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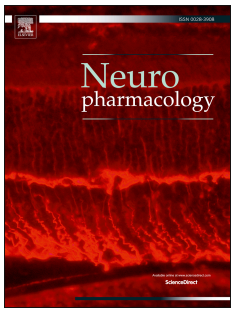

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Sylvie L. Lesuis, Paul Kaplick, Paul J. Lucassen, Harm J. Krugers

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Sylvie L. Lesuis, Paul Kaplick, Paul J. Lucassen, Harm J. Krugers

Brain Plasticity Group, SILS-CNS, University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, The Netherlands

Corresponding author: S.L. Lesuis, Sylvie@lesuis.nl, Brain Plasticity Group, SILS-CNS, University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, The Netherlands.

Contact information:

Sylvie L. Lesuis: Sylvie@lesuis.nl

Paul Kaplick: Paul.Kaplick@gmail.com

Paul J. Lucassen: P.J.Lucassen@uva.nl

Harm J. Krugers: H.Krugers@uva.nl

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Abstract

Background: Environmental factors like stress affect age-related cognitive deficits and promote Alzheimer's disease (AD)-related pathology in mice. Excess glutamate has been proposed as a possible mediator underlying these effects in the hippocampus, a vulnerable brain region implicated in learning and memory.

Methods: Here, we examined a) whether stress applied during a sensitive developmental period early in life affects later synaptic plasticity, learning and memory and plaque load in the APPswe/PS1dE9 mouse model for Alzheimer's disease and b) whether these effects could be rescued using long-term treatment with the glutamate modulator riluzole.

Results: Our results demonstrate that ELS impairs synaptic plasticity in 6-month-old mice and increases plaque load in 12-month-old APPswe/PS1dE9 mice, while impairing flexible spatial learning in the Barnes maze. Notably, spatial learning correlated well with hippocampal expression of the transporter EAAT2, which is important for extracellular glutamate uptake. The changes in LTP, plaque load and cognition after ELS were all prevented by riluzole treatment that started from post-weaning.

Conclusion: These results suggest that normalising glutamate signalling may be a viable therapeutic strategy for treating vulnerable individuals at risk of developing stress-aggravated AD, particularly in relation to adverse early life experiences.

Highlights

- In APP/PS1 mice, early life stress impairs LTP and flexible spatial learning.
- Early life stress increases plaque load in APPswe/PS1dE9 mice.
- EAAT2 correlates positively with flexible spatial learning.
- Riluzole treatment prevented ELS changes in LTP, flexible spatial learning and plaque load.

- Thus, normalising glutamate signalling rescues ELS-induced deficits in AD mice.

1. Introduction

Alzheimer's disease (AD) is a frequent age-related neurodegenerative disorder characterised by progressive cognitive decline (Selkoe and Schenk, 2003) that is, in view of current human life expectancy (Jagust, 2013; Prince et al., 2013; Small et al., 2002), even expected to increase in the future (Brookmeyer et al., 2007). While familial forms of AD are linked to rare genetic mutations (Querfurth and LaFerla, 2010; Scheltens et al., 2016), the cause of sporadic AD remains elusive. Various recent lines of evidence suggest that environmental factors play a role in AD risk (Baumgart et al., 2015; Herbert and Lucassen, 2016; Matthews et al., 2016; Xu et al., 2015). One of these environmental factors may be exposure to stress, particularly when experienced during the sensitive period of early life. For instance, Individuals with a history of childhood adversity have a higher probability to develop later diseases (Ferraro et al., 2016; Schafer and Ferraro, 2012), and a higher prevalence and severity of mild cognitive impairment at an older age (Kang et al., 2017; Wang et al., 2016). Likewise, evidence from rodent studies indicates that early life stress (ELS) triggers age-related cognitive decline (Oitzl et al., 2000; Solas et al., 2010; Vallée et al., 1999). Such ELS-induced accelerations of cognitive decline are often accompanied by (neuro)biological changes of aging, such as a reduced telomere length (Price et al., 2013), reductions in adult hippocampal neurogenesis (Bath et al., 2016; Lucassen et al., 2015; Naninck et al., 2015), and enhanced neuro-inflammatory profiles (Hoeijmakers et al., 2016; Johnson and Kaffman, 2018). In line with the hypothesis that ELS may affect the course of AD related changes, ELS has been shown to worsen cognitive decline in various genetic mouse models for AD both following pre- (Sierksma et al., 2013) and postnatal stress (Hui et al., 2017; Lesuis et al., 2018). Yet how early life adversity aggravates aging and AD is unknown.

Studies in transgenic animal models for AD have implicated glutamatergic N-methyl-D-aspartate (NMDA) receptors in AD and reveal that glutamatergic synapses are particularly affected

(Haass and Selkoe, 2007; Kamenetz et al., 2003; Kessels et al., 2013; Rowan et al., 2003; Townsend et al., 2006; Turner et al., 2003; Walsh et al., 2002). Whereas synaptic NMDA activity is critical for long-term potentiation (LTP) and memory formation, excessive extra-synaptic NMDA activation has been associated with the induction of long-term depression and even excitotoxicity (Hardingham, 2006; Hardingham and Bading, 2010; Rusakov and Kullmann, 1998). Glutamate uptake by the excitatory amino acid transporter 2 (EAAT2, (also known as GLT-1 or Slc1a2)) is the primary mechanism via which extracellular glutamate regulates physiological glutamatergic neurotransmission in the brain (Furuta et al., 1997; Huang and Bergles, 2004; Tzingounis and Wadiche, 2007). Interestingly, the expression of glutamate transporters, including EAAT2, is decreased after early life stress (Odeon et al., 2015), in aging (Brothers et al., 2013; Potier et al., 2010) as well as in AD (Jacob et al., 2007; Masliah et al., 1996) and has been associated with neurodegeneration (Masliah et al., 1996).

Since (early life) stress can disturb glutamatergic signalling and function, the effects of ELS and AD may thus converge at glutamatergic transmission (O'Connor et al. 2013; Musazzi et al. 2011). In the present study we therefore tested in APPswe/PS1dE9 mice whether ELS affects mechanisms which are critical for the uptake of glutamate from synapses (i.e. EAAT2), synaptic plasticity, and whether these effects can be modulated by the glutamate modulator riluzole. This drug alters glutamatergic neurotransmission by decreasing presynaptic glutamate release, and by facilitating glial glutamate uptake via increased EAAT2 expression (Azbill et al., 2000; Frizzo et al., 2004; Fumagalli et al., 2008; Pereira et al., 2016; Pittenger et al., 2008). Riluzole increases synaptic connectivity, strengthens neural connectivity (Larkum and Nevian, 2008), and enhances LTP (De Roo et al., 2008). Moreover, riluzole prevents age-related cognitive decline in rodents (Pereira et al., 2014) and AD related changes in gene expression (Pereira et al., 2017). Our present results show not only that ELS affects synaptic plasticity and spatial memory in APPswe/PS1dE9 mice, in close correlation with EAAT2 expression in the hippocampus, but also that these deficits in LTP and spatial memory in 12-month-old AD mice were completely prevented by prolonged riluzole treatment.

2. Materials and Methods

2.1. Mice and breeding.

All experimental procedures were conducted under Dutch national law and European Union directives on animal experiments (2010/63/EU), and were approved by the animal welfare committee of the University of Amsterdam. Wild type (WT) and APPswe/PS1dE9 male littermates (Jankowsky et al., 2001) of 6 and 12 (± 1) months of age were used. To obtain mice, two 10 weeks old C57BL/6J virgin WT females (Harlan Laboratories B.V., Venray, The Netherlands) and one heterozygous male APPswe/PS1dE9 mouse were housed together for one week to allow mating. Pregnant females were housed individually in a standard cage covered with a filter top and monitored daily for the birth of pups (Arp et al., 2016; Lesuis et al., 2018, 2016; Rice et al., 2008). When a litter was born before 10.00 a.m., the previous day was considered the day of birth (postnatal day 0; PND 0), after which the early life stress paradigm was initiated from PND 2-9. At PND 21, mice were weaned and ear biopsies were collected for identification and genotyping. Mice were housed with 2-6 same sex littermates per cage. All experimental mice were left undisturbed (except for cage cleaning once a week) until the start of the experimental procedures at 6 and 12 months of age. Number of mice used: 6 months old: 56 mice; 12 months old: 57 mice.

2.2. Early life stress.

At postnatal day (PND) 2, litters were culled to 6 pups per litter, and dams and their litters were randomly assigned to the early life stress (ELS) or control condition until PND 9, after which all mice were treated equally, as described before (Arp et al., 2016; Lesuis et al., 2018, 2016; Naninck et al., 2015; Rice et al., 2008). Briefly, control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Tecnilab-BMI,

Someren, the Netherlands)). ELS dams were provided with a strongly reduced amount of sawdust bedding and half the nesting material (1/2 piece of nesting material), and a fine-gauge stainless steel mesh was placed 1 cm above the cage floor.

2.3. Riluzole treatment.

Riluzole (Selleckchem, The Netherlands) was added to the drinking water from weaning (PND 28) onwards, and provide fresh every 3-4 days. Bottles were shielded from light to prevent light exposure. A dosage of 4.0 mg/kg per day per animal (adapted from (Pereira et al., 2016)) was dissolved in tap water and stirred until the water was completely transparent.

2.4. Field potential recordings.

Field potential recordings were conducted in 6-month-old male animals. At PND 180 ± 14 mice were sacrificed between 9 and 10 a.m. through quick decapitation. Immediately after decapitation, the brain was rapidly removed, and collected in ice-cold oxygenated (95% O_2/5% CO_2) solution containing (in mM): Cholinechloride (120), glucose (10), $NaHCO_3$ (25), $MgSO_4$ (6), KCl (3.5), NaH_2PO_4 (1.25), $CaCl_2$ (0.5). Coronal slices (350 µm) were cut using a microtome (Leica VT1000S). For recovery, slices were incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), $MgSO_4$ (1.3), NaH_2PO_4 (1.25), $CaCl_2$ (2.5), glucose (10), $NaHCO_3$ (25), after which the sections were maintained at room temperature (22 °C). Sections containing the dorsal hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF. Field excitatory synaptic potentials (fEPSPs) were recorded as described previously (Bagot et al., 2009; Pu et al., 2007; Wiegert et al., 2006). fEPSPs were evoked using a stainless steel bipolar stimulation electrode (60 µm diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded

through a glass electrode (2-5 MΩ impedance, filled with aCSF) positioned in the CA1 stratum radiatum. A stimulus-response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored by stimulating at 0.033 Hz for 10 minutes. When recordings were stable, afferent fibres were stimulated at 10 Hz for 90 seconds (Mayford et al., 1996; Wiegert et al., 2006). We used this paradigm since it elicits synaptic plasticity at the threshold for LTP and LTD, and is therefore will-suited to examine subtle and potentially bi-directional changes in synaptic plasticity (Derks et al., 2016; Mayford et al., 1996; Wiegert et al., 2006). Next, the degree of potentiation was determined by recording fEPSPs every 30 seconds for 1h. Synaptic transmission was measured by determining the slope of the fEPSP. The average baseline value was normalised to 100% and all values of the experiment were normalised to this baseline average.

2.5. Barnes maze.

Mice (12 months) were transferred to a reversed light/dark cycle (lights on 8 p.m., lights off 8 a.m.) one month before behavioural testing commenced and were single-housed in the behaviour room for one more week before testing. Three days prior to testing, mice were handled for five minutes per day. Testing was conducted during the dark, active phase of the mice between 12 and 6 p.m. During testing, recording was done with a video camera connected to a computer with Ethovision software version 14 (Noldus, The Netherlands). Twelve-month-old APPswe/PS1dE9 and WT male mice were tested for spatial memory in the spatial Barnes maze task. A classic set up was used (110 cm diameter, 12 exit holes) in which mice were trained for one (day 1 and 2) or two (day 3 and 4) sessions a day (adapted from (Lesuis et al., 2018)). During training, mice were placed in the centre of the maze twice (inter-trial interval of 30 minutes) and were allowed to navigate to the exit hole leading to the home cage (acquisition learning). Behavioural flexibility was tested by relocating the

exit hole to another location on the maze (180 degrees) for two sessions per day on two consecutive days. Cages containing used bedding material were placed at equal distances under the maze to avoid guidance by odour cues, the board was rotated after each trial, and the maze was cleaned with 25 % EtOH to dissipate odour cues. The location of the exit hole was always fixed relative to the distal extra-maze cues in the room. The distance the mice travelled until the exit hole was reached was analysed.

2.6. Tissue preparation.

One week after behavioural testing, mice were sacrificed by quick decapitation, between 8.00 and 9.00 p.m. (beginning of the inactive phase). The brains were removed, and the left hemisphere was immersion-fixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4) for 48 h and then stored in 0.01% sodium-azide in 0.1 M PB at 4 °C until further processing. Paraformaldehyde-fixed tissue was overnight cryoprotected in 30% sucrose/0.1 M PB. Frozen hemispheres were cut in 40 µm thick coronal sections in six parallel series using a sliding microtome and stored in antifreeze solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05 M PBS) at -20 °C until immunohistochemical staining.

2.7. DAB immunohistochemistry.

Immunocytochemistry was used to visualise amyloid plaques. Prior to staining, sections were mounted on glass (Superfrost Plus slides, Menzal, Braunschweig, Germany) and antigen retrieval was performed by heating the sections in 0.1 M citrate buffer (pH 6) in a microwave (Samsung M6235) to a temperature of ±95 °C for 15 min. Sections were incubated with 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity, and were next incubated for 30 min in blocking buffer (1% BSA, 0.3% Triton X-100 in 0.05 M TBS). Primary antibody 6E10 (1:1500, BioLegend) was incubated for two hours at room temperature and overnight at 4 °C. Sections were incubated with biotinylated

secondary antibody (1:200, sheep anti-mouse, GE Healthcare) for 2h at room temperature followed by a 90 min incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amsterdam, 1:800). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 mL 0.05 M Tris, 0.01% H_2O_2).

2.8. Fluorescent immunohistochemistry.

A random subset of brains (N=4-5 mice/group) was used for EAAT2 immunohistochemistry. All stainings were performed on parallel series from the same brains within an age group. Sections were incubated with blocking mix containing goat anti-mouse Fab fragments (1:200) in 0.1 M PBS. Primary mouse anti-EAAT2 (1:250, Cell Signalling) was incubated for 1h at RT followed by incubation at 4 °C overnight. Sections were incubated in the secondary antibodies (1:200 sheep anti-mouse) for 2h, and mounted and coverslipped with Vectashield.

2.9. Imaging and quantification.

Quantification was performed on coronal sections of the left hemisphere on 8–10 sections per animal of matched anatomical levels along the rostro-caudal axis (Lesuis et al., 2017). Using a Nikon DS-Ri2 microscope, representative images of 20x magnification were systematically captured. For images from DAB staining, ImageJ software was used to binarise the pictures to 8-bit black-and-white pictures, and a fixed intensity threshold was applied defining the DAB staining. Measurements were performed for the percentage area covered by DAB staining (Christensen et al., 2009; Marlatt et al., 2013). EAAT2 fluorescence was measured using ImageJ in 50 µm intervals from the cellular layer in the CA1 of the hippocampus (Pereira et al., 2016). All images were quantified by an experimenter blinded to the experimental procedures and animals.

2.10. Statistical analysis.

Data were analysed using SPSS 22.0 (IBM software). Data are expressed as mean ± standard error of the mean (S.E.M.). Data were considered statistically significant when $p<0.05$. Outliers were determined using a Grubb's test, which identifies a maximum of one value to be excluded from the analysis. Repeated measures ANOVA was performed to assess Barnes maze learning curves over the different trials, and to assess synaptic plasticity. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. To enhance the readability of the graphs, the repeated measures data for the LTP and Barnes maze have been split up in separate graphs (Figure 1A, B and Figure 2A-D), although statistical analysis was performed on all data combined. To compare between groups accounting for the main and interaction effects of genotype (WT vs. APPswe/PS1dE9), condition (Ctrl vs. ELS), and treatment (water vs. Riluzole), a 2x2x2 ANOVA was performed, with planned contrasts as post hoc tests to correct for the relevant comparisons conducted. Pearson's correlation test was conducted to determine correlations.

3. Results

3.1. Early life stress model

APPswe/PS1dE9 and WT littermates were housed with limited nesting and bedding materials from PND 2 to 9 in order to induce ELS. In line with previous reports (Lesuis et al., 2018; Naninck et al., 2015) this procedure reduced body weight gain (Ctrl: 3.6 ± 0.11 gram; ELS: 2.5 ± 0.08 gram; $t(55)=8.06$, $p=0.001$), indicative of effective stress exposure. Since effects of ELS are particularly sex-specific (Loi et al., 2017; Naninck et al., 2015), all experiments were further conducted with male mice. From PND 28 onwards, half of the mice received riluzole supplementation to their drinking solution. Water consumption was measured at 3 different time points throughout the experiment

241 (Table 1). No differences in consumption of water with or without riluzole were observed (see Table
242 1).

243 Table 1. Consumption of water with and without riluzole at different time points throughout the
244 experiment.

	PND 35	6 months	11 months
Water	4.1 ± 1.0 (*20*)	4.7 ± 1.0 (*20*)	5.2 ± 1.0 (*21*)
Water + Riluzole	4.2 ± 1.0 (*16*)	4.7 ± 1.0 (*16*)	5.5 ± 0.9 (*16*)
	Ns	Ns	Ns

245 Water consumption is expressed as average ml/mouse/day. Data expressed as mean ± S.E.M
246 (*number of mice*).

247 **3.2. Hippocampal synaptic plasticity**

248 To investigate whether ELS and/or an APPswe/PS1dE9 background affected synaptic plasticity, we
249 measured hippocampal long-term potentiation (LTP) at 6 months of age, and tested whether effects
250 could be rescued by riluzole treatment. We found no differences of condition, genotype or
251 treatment on maximum slope or the half-maximum stimulation intensity, as determined from the
252 input-output curve (Table 2). There was a main effect of treatment ($F(1,97)=30.84$, $p<0.001$) on the
253 slope factor.

254 Table 2. Basal field potential characteristics for hippocampal CA1 area

		Max Slope (mV/ms)	Half Max Intensity (µA)	Slope Factor S	N (mice (slices))
water	**Ctrl – WT**	-0.24 ± 0.03	2.27 ± 0.05	-0.22 ± 0.05	10 (27)
	ELS – WT	-0.27 ± 0.03	2.29 ± 0.04	-0.23 ± 0.04	8 (21)
	Ctrl – APPswe/PS1dE9	-0.26 ± 0.04	2.36 ± 0.05	-0.24 ± 0.05	10 (17)
	ELS – APPswe/PS1dE9	-0.16 ± 0.04	2.25 ± 0.10	-0.15 ± 0.04	6 (14)

riluzole	**Ctrl – WT**	-0.36 ± 0.03	2.10 ± 0.05	-0.54 ± 0.15	6 (8)
	ELS – WT	-0.45 ± 0.04	1.87 ± 0.03	-0.54 ± 0.07	5 (6)
	Ctrl – APPswe/PS1dE9	-0.33 ± 0.05	2.14 ± 0.07	-0.58 ± 0.12	5 (7)
	ELS – APPswe/PS1dE9	-0.30 ± 0.05	2.06 ± 0.11	-0.32 ± 0.05	6 (9)
Main/interaction effects		ns	ns	T*	

Data expressed as mean ± S.E.M. Maximal slope of the fEPSP (*Max slope*), half-maximum stimulus intensity (*Half Max Intensity*), and the slope of the input-output curve (*Slope Factor S*) in the CA1 area. C: condition effect, G: genotype effect, T: treatment effect.

In water treated mice, both condition and genotype reduced LTP (condition: $F(1,40)=4.47$, $p=0.04$; genotype: $F(1,40)=7.86$, $p=0.008$) (Figure 1A). When combining all data, riluzole treatment increased LTP in all groups (main treatment effect: $F(1,63)=61.62$, $p<0.001$) (Figure 1A,B). However, these effects were most pronounced in APPswe/PS1dE9 mice (genotype*treatment: $F(1,63)=22.62$, $p<0.001$; post hoc difference between: Ctrl-APPswe/PS1dE9 water vs. riluzole: $p<0.001$; ELS-APPswe/PS1dE9 water vs. riluzole $p<0.001$), while there was also an interaction between condition and treatment ($F(1,63)=4.40$, $p=0.04$) (Figure 1A,B). The average of the signal during the last 10 minutes was analysed separately (Figure 1C). Here, too, riluzole treatment significantly increased synaptic potentiation ($F(1,63)=62.41$, $p<0.001$), most strongly in APPswe/PS1dE9 mice ($F(1,63)=15.34$, $p<0.001$). Post hoc testing revealed a significant effect of riluzole treatment in ELS-WT mice ($p=0.01$), Ctrl-APPswe/PS1dE9 mice ($p<0.001$), and ELS-APPswe/PS1dE9 mice ($p<0.001$).

3.3. Barnes maze training

We next investigated whether ELS-induced changes in synaptic plasticity also affect spatial memory performance in WT and APPswe/PSdE9 mice, and whether such effects could be prevented by

riluzole in 12-month-old mice (Lesuis et al., 2018). For acquisition learning, there was a mild but significant effect of treatment, in which riluzole resulted in a shorter distance to locate the exit hole ($F(1,58)=6.91$, $p=0.01$) (Figure 2A,B). However, neither genotype nor condition affected performance on acquisition learning (genotype effect: $F(1,58)=0.27$, $p=0.61$; condition effect: $F(1,58)=1.31$, $p=0.26$). No effects were observed when examining, the last trial of acquisition learning, indicating that after 6 training sessions, all groups learned to find the location of the exit hole to a similar degree (Figure 2C).

When the exit hole was relocated to a new location, riluzole again improved performance, resulting in a shorter distance travelled to the exit hole ($F(1,58)=24.90$, $p<0.001$) (Figure 2D,E). In addition, APPswe/PS1dE9 mice took a longer distance to find the exit hole ($F(1,58)=9.97$, $p=0.003$). Analysis of the last trial, as an indication of how well mice had learned to locate the exit hole, revealed an effect of treatment, genotype and condition, as well as a condition x treatment interaction effect (treatment: $F(1,58)=39.03$, $p<0.001$; genotype: $F(1,58)=5.95$, $p=0.018$; condition: $F(1,58)=8.56$, $p=0.005$; condition x treatment: $F(1,58)=7.68$, $p=0.003$) (Figure 2F). Post hoc testing revealed that in APPswe/PS1dE9 mice, ELS resulted in a longer distance to the exit hole than Ctrl animals. Riluzole treatment also resulted in a shorter travelling distance to the exit hole in both groups.

3.4. EAAT2 expression

Immunocytochemical labelling revealed that EAAT2 was reduced in the distal portion of the CA1 area with age ($F(1,34)=81.38$, $p=0.001$) (Figure 3A). We further found that EAAT2 expression in aged riluzole treated animals was enhanced when compared to untreated young and aged mice (treatment effect: $F(1,34)=250.22$, $p=0.001$). Moreover, in water-treated animals, genotype reduced EAAT2 expression at all ages ($F(1,34)=5.6$, $p=0.025$). We found an interaction effect between condition x treatment ($F(1,34)=14.42$, $p=0.001$) and genotype x treatment ($F(1,34)=8.76$, $p=0.006$),

299 reflecting the enhanced EAAT2 expression following riluzole treatment in aged ELS and
300 APPswe/PS1dE9 mice.

301 Importantly, EAAT2 expression correlated significantly with cognitive performance of the
302 last learning trial of the Barnes maze in aged mice (r=-0.75, n=32, p=0.001) (Figure 3B), which
303 suggests a potential mechanism by which riluzole may rescue cognitive performance.

304

305 **3.5. Hippocampal plaque load**

306 Finally, we investigated plaque load, an important pathological hallmark of AD, and we found a
307 significant interaction effect between condition and treatment in the hippocampal CA1 area
308 ($F(1,37)=7.52$, $p=0.009$). ELS-APPswe/PS1dE9 mice treated with water displayed an increased plaque
309 load, which was absent in APPswe/PS1dE9 animals treated with riluzole treatment ($p<0.05$) (Figure
310 3C). Plaque load did not correlate with cognitive decline ($r=0.09$, $n=32$, $p=0.59$) (Figure 3D).

311

312 **4. Discussion**

313 Previous studies have reported that early life stress can alter flexible spatial learning, synaptic
314 plasticity and amyloid levels in 12-month-old APPswe/PS1dE9 mice (Lesuis et al., 2018). In the
315 current study, we investigated whether riluzole, a modulator of glutamate levels (Brothers et al.,
316 2013; Pittenger et al., 2008) can rescue these effects. We found that ELS-induced impairments in
317 synaptic plasticity, flexible spatial learning and plaque load in APPswe/PS1dE9 mice can be rescued
318 by prolonged riluzole treatment from post-weaning onward, likely by regulating EAAT2 expression.

319 Our current model for ELS has previously been shown to induce (age-related) impairments in
320 spatial learning, memory processes (reviewed by (Walker et al., 2017; Yam et al., 2017)) and synaptic
321 plasticity (Brunson et al., 2005). In addition, it has been shown that ELS aggravates AD-related
322 neuropathology, including increased soluble Aβ levels, increased plaque load, and impaired cognitive

performance (Hoeijmakers et al., 2016; Lesuis et al., 2018, 2016). In agreement, we found that ELS impaired synaptic plasticity in WT mice. In addition, LTP was impaired in APPswe/PS1dE9 mice which is in line with earlier studies showing impairments in synaptic plasticity in (transgenic) mouse models of AD (Jacobsen et al., 2006; Rowan et al., 2003). Moreover, ELS-exposure in APPswe/PS1dE9 mice further decreased synaptic plasticity, and even resulted in LTD-like changes. We then investigated whether alterations in glutamatergic signalling might attenuate these effects by long-term treatment with the glutamate modulator riluzole, administered immediately after weaning. While riluzole did not affect LTP in Ctrl-reared wild type mice, it increased LTP in all other experimental groups, suggesting that the impairments resulting from both ELS and an APPswe/PS1dE9 background are indeed mediated by disturbances in glutamatergic signalling. Interestingly, riluzole treatment was most effective in APPswe/PS1dE9 mice. This effect was most pronounced in the first 10 minutes after stimulation, which could point to a different recovery of the presynaptic glutamate release between WT and APPswe/PS1dE9 mice after the 90 seconds of high frequency stimulation (which may have resulted in a depletion of synaptic vesicles). These effects of riluzole may be related to one of the many pathways associated to synaptic plasticity that are differentially regulated by AD (Pereira et al., 2016) and the exact nature of this interaction requires further investigation. Clearly, riluzole was able to prevent ELS and APPswe/PS1dE9-induced alterations in synaptic plasticity in 6-month-old mice.

We have previously reported that ELS resulted in aberrantly *increased* LTP in older APPswe/PS1dE9 mice, which was paralleled by less specific memory formation on a fear conditioning task (Lesuis et al., submitted). Although these animals were recorded at different ages (6 vs. 12 months old), the opposing phenotypes are remarkable. Importantly, both excessively enhanced and decreased levels of LTP have been implicated in cognitive deficits (Hancock et al., 1991; Migaud et al., 1998; Willshaw and Dayan, 1990), but future studies are required to investigate the possible age-dependent effects and the exact nature of ELS-induced effects on synaptic plasticity in APPswe/PS1dE9 mice.

LTP is an important cellular model for learning and memory (Kessels and Malinow, 2009; Malinow and Malenka, 2002), and functional brain abnormalities have been observed in humans decades before the development of other symptoms (Reiman et al., 2004; Sperling et al., 2009). We therefore tested whether ELS affected learning and memory in APPswe/PS1dE9 mice. Previously, we have reported that 12-month-old ELS-APPswe/PS1dE9 mice are impaired in flexible spatial learning in the Barnes maze (Lesuis et al., 2018). In line with these findings, we found at present that ELS exposure in APPswe/PS1dE9 mice did not alter acquisition learning, but impaired flexible spatial learning. While riluzole slightly enhanced acquisition learning, it particularly prevented the deficits on flexible spatial learning. Interestingly, riluzole treatment improved performance in both transgenic groups, as well as in the ELS-WT mice. Together, these observations indicate that in cognitively impaired animals, be it after ELS or due to an APPswe/PS1dE9 background, riluzole improves cognitive performance.

A possible mechanism via which the effect of riluzole may rescue both these impairments, could be through regulating EAAT2 expression (Brothers et al., 2013; Pereira et al., 2016; Pittenger et al., 2008), which is relevant for maintaining proper synaptic glutamate levels (Tzingounis and Wadiche, 2007). EAAT2 regulates reuptake of glutamate outside the synaptic cleft, preventing excess glutamate from binding to extra-synaptic NMDA receptors, reducing synaptic efficiency and inducing LTD and excitotoxicity (Hardingham and Bading, 2010), and has been implicated in aging and various neurodegenerative diseases, including AD (Hardingham and Bading, 2010; Jacob et al., 2007; Masliah et al., 1996; Pereira et al., 2016; Potier et al., 2010; Rusakov and Kullmann, 1998). Furthermore, EAAT2 haploinsufficiency aggravates cognitive impairments in an AD mouse model, while EAAT2 overexpression improves cognitive performance (Takahashi et al., 2015).

In line with this, we observed that EAAT2 immunoreactivity was significantly reduced with aging, while both ELS and an APPswe/PS1dE9 background further lowered EAAT2, which was strongest in APPswe/PS1dE9 mice exposed to ELS. Riluzole treatment strongly increased EAAT2

levels in the CA1 area of the hippocampus in all groups, irrespective of their genetic background or early life experience. Interestingly, EAAT2 expression correlated significantly with flexible spatial learning , indicating that EAAT2 is indeed relevant for memory formation. Increased immunoreactivity for EAAT2 was observed in the same region as where we observed decreases in synaptic plasticity in ELS-APPswe/PS1dE9 mice. In addition, others have previously observed increased spine clustering in the same area in riluzole-treated rats, which also correlated with cognitive performance (Pereira et al., 2014), suggesting a potential mechanism by which riluzole can increase cognitive performance. However, in addition to regulating glutamate levels, the drug has additional pharmacological effects such as inhibiting Na^+ channels (Bellingham, 2011). A possible contribution of these mechanisms to the present results cannot be ruled out.

Synaptic dysfunction is an important mechanism implicated in AD-related cognitive deficits (Selkoe 2002; DeKosky and Scheff 1990) and presenting as one of the first symptoms of AD (Sperling et al. 2009; Reiman et al. 2004). Amyloid-β (Aβ), one of the hallmarks of AD neuropathology, is closely related to glutamatergic dysregulation, since Aβ oligomers disrupt glutamate uptake, reduce synaptic transmission, facilitate LTD and inhibit LTP (Li et al. 2009; Cheng et al. 2009). This is thought to occur through an excessive activation of extra-synaptic NMDA receptors (Li et al., 2011, 2009), and a decrease in the expression of synaptic NMDA receptors (Snyder et al., 2005). In parallel, neuronal activity, regulated by glutamatergic signalling increases the release of Aβ (Kamenetz et al., 2003), possibly resulting in vicious cycle of neurotoxicity. In the current study, we find that plaque load was increased following ELS, an effect that was rescued by riluzole treatment. Likewise, we have previously shown that in APPswe/PS1dE9 mice soluble Aβ-40 and Aβ-42 levels are increased following ELS (Lesuis et al., 2018), although plaque load was not affected in this study. EAAT2 overexpression has previously been shown to decrease pathological markers in an AD mouse model (Takahashi et al., 2015), again supporting the hypothesis that improved regulation of glutamatergic signalling via enhanced EAAT2 uptake could potentially mitigate Aβ toxicity and worsen cognitive performance. This may suggest that normalising glutamate levels prevents Aβ pathology.

5. Conclusions

The present results indicate that riluzole rescues deficits in flexible spatial learning in 12-month-old ELS-exposed APPswe/PS1dE9 mice. The effects of riluzole are possibly mediated by alterations in synaptic plasticity that emerge already from a young age onwards (at least 6 months) since LTP deficits were completely rescued by riluzole supplementation. Future studies are required to investigate in more detail the critical time windows in which riluzole can prevent the ELS-induced impairments. Ultimately, reducing glutamatergic signalling could represent future therapeutic strategy for treating vulnerable individuals at risk of developing stress-aggravated AD, particularly in relation to adverse early life experiences.

6. List of abbreviations

aCSF: artificial cerebrospinal fluid

AD: Alzheimer's disease

Aβ: amyloid-β

Ctrl: control

EAAT2: excitatory amino acid transporter 2

ELS: early life stress

fEPSP: Field excitatory synaptic potential

LTP: long-term potentiation

NMDA: N-methyl-D-aspartate

PND: postnatal day

WT: wild-type

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The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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693

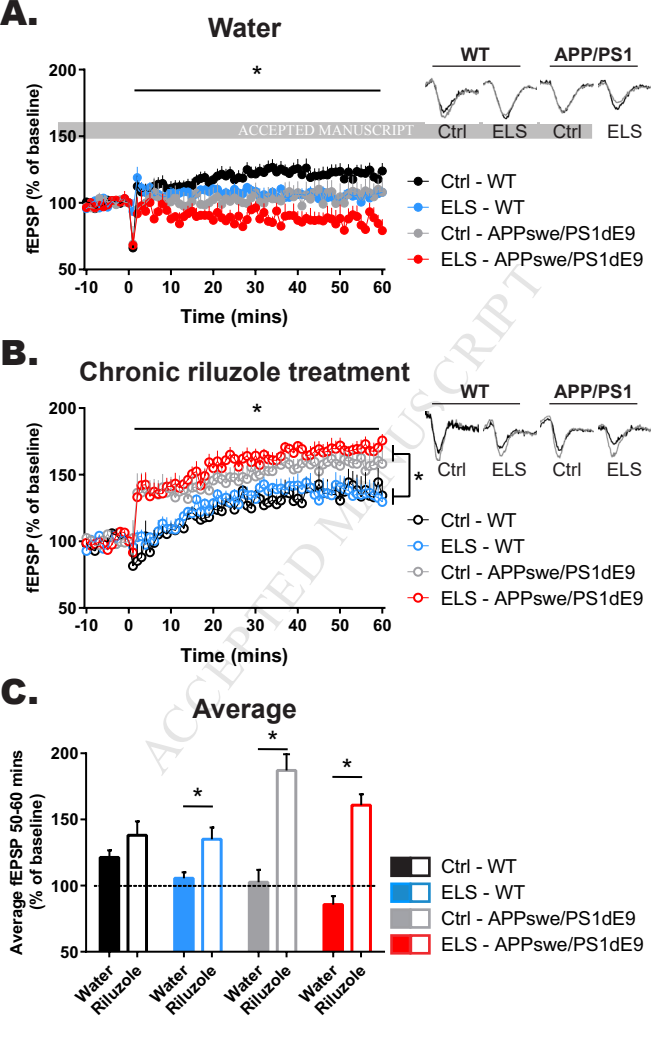
694 **Figure legends**

695 **Figure 1: Chronic riluzole treatment rescues ELS-induced impairments in hippocampal LTP in**
696 **APPswe/PS1dE9 mice after 10 Hz stimulation for 90 seconds.** (A) LTP in water-treated mice. Both
697 genotype and condition decrease the slope of the fEPSP over the entire 60 minutes after stimulation,
698 resulting in LTD in ELS-APPswe/PS1dE9 mice. Right panel: typical example of a fEPSP at baseline
699 (black), and 50 minutes after stimulation (grey). (B) Chronic riluzole treatment significantly increases
700 LTP, most strongly in APPswe/PS1dE9 mice. (C) During the last 10 minutes of recording, chronic
701 riluzole treatment increased LTP significantly in ELS-WT, Ctrl-APPswe/PS1dE9 and ELS-
702 APPswe/PS1dE9 mice. Ctrl-WT-water: N=18; ELS-WT-water: N=13; Ctrl-APPswe/PS1dE9-water:
703 N=10; ELS-APPswe/PS1dE9-water: N=5; Ctrl-WT-riluzole: N=4; ELS-WT-riluzole: N=6; Ctrl-
704 APPswe/PS1dE9-riluzole: N=4; ELS-APPswe/PS1dE9-riluzole: N=10. *: $p<0.05$.

705

Figure 2: Chronic riluzole-treated aged APPswe/PS1dE9 mice were protected against ELS-induced deficits in Barnes maze performance. (A,B) The distance travelled before the mice located the exit hole was comparable between all groups (water-treated mice: full line; riluzole-treated mice: dashed line). (C) The distance travelled during the last trial of acquisition learning was also comparable between all groups. (D) When the exit hole was relocated to a novel location, in WT mice, long-lasting riluzole treatment (dashed line) resulted in a slight improvement in the distance travelled to the exit hole, compared to water-treated mice (full line). (E) Water-treated APPswe/PS1dE9 mice took longer to locate the exit hole compared to WT mice, especially when exposed to ELS. The distance travelled was improved in all groups after chronic riluzole treatment. (F) The distance travelled to the exit hole when the exit hole was relocated to a new location was reduced by long-term riluzole treatment in all groups, except for Ctrl-WT mice. Ctrl-WT-water: N=7; ELS-WT-water: N=9; Ctrl-APPswe/PS1dE9-water: N=9; ELS-APPswe/PS1dE9-water: N=9; Ctrl-WT-riluzole: N=7; ELS-WT-riluzole: N=8; Ctrl-APPswe/PS1dE9-riluzole: N=8; ELS-APPswe/PS1dE9-riluzole: N=9. *: $p<0.05$.

Figure 3. Chronic riluzole increases EAAT2 expression. (A) Quantification of fluorescent intensity of CA1 hippocampal sections labelled for EAAT2. Chronic Riluzole administration significantly increased labelling in the region 150-200 µm from the pyramidal cell bodies in aged mice. N=4/group. * indicates a significant difference from the Ctrl-WT group of the respective age or treatment group. (B) Distance travelled during the last trial of the Barnes maze correlated with the expression of EAAT2 in the CA1 area. (C) Plaque load analysis revealed a larger area of the CA1 covered with plaques in ELS compared to Ctrl APPswe/PS1dE9 mice. This was again normalised by chronic riluzole treatment. N=8-9/group. (D) No correlation was observed between plaque load and distance travelled in the last trial of the Barnes maze.


A.
Water
WT
APP/PS1
Ctrl
ELS
Ctrl
ELS
ACCEPTED MANUSCRIPT
fEPSP (% of baseline)
Time (mins)
Ctrl - WT
ELS - WT
Ctrl - APPswe/PS1dE9
ELS - APPswe/PS1dE9
B.
Chronic riluzole treatment
WT
APP/PS1
Ctrl
ELS
Ctrl
ELS
fEPSP (% of baseline)
Time (mins)
Ctrl - WT
ELS - WT
Ctrl - APPswe/PS1dE9
ELS - APPswe/PS1dE9
C.
Average
Average fEPSP 50-60 mins (% of baseline)
Water
Riluzole
Water
Riluzole
Water
Riluzole
Water
Riluzole
Ctrl - WT
ELS - WT
Ctrl - APPswe/PS1dE9
ELS - APPswe/PS1dE9