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*Impact on Alzheimer's disease and memory mechanisms*
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Treatment with the glutamate modulator riluzole prevents early life stress–induced cognitive deficits and impairments in synaptic plasticity in APPswe/PS1dE9 mice

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Submitted
Abstract

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.

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.

Our results demonstrate that ELS impairs synaptic plasticity, increases plaque load, and impairs reversal learning in 12 months old APPswe/PS1dE9 mice. Notably, reversal 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 from post-weaning onwards.

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.

Keywords: riluzole, Alzheimer’s disease, early life stress, EAAT2, LTP, Barnes maze, glutamate

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**1. Introduction**

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder characterised by progressive cognitive decline\(^1\). In view of current human life expectancy\(^2-^4\), an increase in the numbers of AD patients is expected\(^5\). While familial forms of AD are linked to rare genetic mutations\(^6,^7\), the cause of sporadic AD remains elusive. Various recent lines of evidence suggest that environmental factors play a role in AD risk\(^8-^11\). One of these environmental factors is 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\(^12,^13\), and a higher prevalence and severity of mild cognitive impairment at an older age\(^14,^15\). Likewise, also evidence from rodent studies indicates that early life stress (ELS) triggers an age-related cognitive decline\(^16-^18\). ELS-induced accelerations of cognitive decline are often accompanied by changes in (neuro)biological markers of aging, such as a reduced telomere length\(^19\), reductions in adult hippocampal neurogenesis\(^20-^22\), and enhanced neuro-inflammatory profiles\(^23,^24\). 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-\(^25\) and postnatal stress\(^26,^27\). 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\(^28-^34\). Whereas synaptic NMDA activity is critical for long-term potentiation (LTP) and memory formation, excessive extrasynaptic NMDA activation has been associated with the induction of long-term depression and even excitotoxicity\(^35-^37\). Glutamate uptake by the excitatory amino acid transporter 2 (EAAT2, (GLT-1 or Slc1a2)) is the primary mechanism via which extracellular glutamate regulates physiological glutamatergic neurotransmission in the brain\(^38-^40\). Interestingly, the expression of glutamate transporters, including EAAT2, is decreased after early life stress\(^41\), in aging\(^42,^43\) as well as in AD\(^44,^45\) and has been associated with neurodegeneration\(^44\).

Since (early life) stress can disturb glutamatergic signalling and function, the effects of ELS and AD may thus converge at glutamatergic transmission\(^46,^47\). In the present study we therefore tested in APPswe/PS1dE9 mice, a commonly used mouse model for AD, whether ELS affects mechanisms which 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 increases in...
EAAT2 expression\textsuperscript{48–52}. Riluzole increases synaptic connectivity, strengthens neural connectivity\textsuperscript{53}, and enhance LTP\textsuperscript{54}. Moreover, riluzole prevented age-related cognitive decline in rodents\textsuperscript{55} and AD related changes in gene expression\textsuperscript{52}. Our present results show not only that ELS affects synaptic plasticity and learning and memory processes, in close correlation with EAAT2 expression in the hippocampus, but also that these deficits in LTP and cognitive performance 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, and were approved by the animal welfare committee of the University of Amsterdam. Wild type (WT) and APPswe/PS1dE9 male littermates\textsuperscript{56} of 6 and 12 (± 1) months of age were used. To obtain mice, two 10 weeks old C57BL/6J virgin wild type (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\textsuperscript{27, 57–59}. 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\textsuperscript{22, 27, 57–59}. 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
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

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\textsuperscript{52}) 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\textsubscript{2}/5% CO\textsubscript{2}) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO\textsubscript{3} (25), MgSO\textsubscript{4} (6), KCl (3.5), NaH\textsubscript{2}PO\textsubscript{4} (1.25), CaCl\textsubscript{2} (0.5). Coronal slices (350 \textmu m) 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\textsubscript{4} (1.3), NaH\textsubscript{2}PO\textsubscript{4} (1.25), CaCl\textsubscript{2} (2.5), glucose (10), NaHCO\textsubscript{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\textsuperscript{60–62}. fEPSPs were evoked using a stainless steel bipolar stimulation electrode (60 \textmu m diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded through a glass electrode (2-5 M\Omega 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\textsuperscript{62,63}. 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.
Chapter 6

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 27). 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 (reversal learning). 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. All stainings were performed on parallel series from the same brains within an age group. 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\textsubscript{2}O\textsubscript{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 Brunswig Chemie, Amsterdam, 1:800). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 mL 0.05 M Tris, 0.01% H\textsubscript{2}O\textsubscript{2}).

2.8. Fluorescent immunohistochemistry

For EAAT2 immunohistochemistry, 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\textsuperscript{64}. 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\textsuperscript{65,66}. EAAT2 fluorescence was measured using ImageJ in 50 µm intervals from the cellular layer in the CA1 of the hippocampus\textsuperscript{52}. 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

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 (e.g.27) this procedure reduced body weight gain (Ctrl: 3.6 ± 0.11; ELS: 2.5 ± 0.08; t(55)=8.06, p=0.001), indicative of effective stress exposure. Since effects of ELS are particularly sex-specific22,67, all experiments were further conducted with male mice. From PND 28 onwards, half of the mice received riluzole supplementation to their drinking solution. No difference in water consumption was observed between any of the groups (treatment effect: F(1,49)=0.54, p=0.47).

3.1. Hippocampal synaptic plasticity

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

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:
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

Figure 1. Riluzole treatment rescues ELS-induced impairments in hippocampal LTP in APPswe/PS1dE9 mice after 10 Hz stimulation for 90 seconds. A. LTP in water-treated mice. Both genotype and condition decrease the slope of the fEPSP over the entire 60 minutes after stimulation, resulting in LTD in ELS-APPswe/PS1dE9 mice. B. Riluzole significantly increases the fEPSP, most strongly in APPswe/PS1dE9 mice. C. During the last 10 minutes of recording, riluzole increased the fEPSP significantly in ELS-WT, Ctrl-APPswe/PS1dE9 and ELS-APPswe/PS1dE9 mice. Ctrl-WT-water: N=18; ELS-WT-water: N=13; Ctrl-APPswe/PS1dE9-water: N=10; ELS-APPswe/PS1dE9-water: N=5; Ctrl-WT-riluzole: N=4; ELS-WT-riluzole: N=6; Ctrl-APPswe/PS1dE9-riluzole: N=4; ELS-APPswe/PS1dE9-riluzole: N=10. *: p<0.05.
**Table 1. Basal field potential characteristics for hippocampal CA1 area**

<table>
<thead>
<tr>
<th></th>
<th>$R_{\text{max}}$ (mV/ms)</th>
<th>$I_{\text{h}}$ (µA)</th>
<th>$S$</th>
<th>N (mice (slices))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl – WT</td>
<td>-0.24 ± 0.03</td>
<td>2.27 ± 0.05</td>
<td>-0.22 ± 0.05</td>
<td>10 (27)</td>
</tr>
<tr>
<td>ELS – WT</td>
<td>-0.27 ± 0.03</td>
<td>2.29 ± 0.04</td>
<td>-0.23 ± 0.04</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Ctrl – APPswe/PS1dE9</td>
<td>-0.26 ± 0.04</td>
<td>2.36 ± 0.05</td>
<td>-0.24 ± 0.05</td>
<td>10 (17)</td>
</tr>
<tr>
<td>ELS – APPswe/PS1dE9</td>
<td>-0.16 ± 0.04</td>
<td>2.25 ± 0.10</td>
<td>-0.15 ± 0.04</td>
<td>6 (14)</td>
</tr>
<tr>
<td><strong>Riluzole</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ctrl – WT</td>
<td>-0.36 ± 0.03</td>
<td>2.10 ± 0.05</td>
<td>-0.54 ± 0.15</td>
<td>6 (8)</td>
</tr>
<tr>
<td>ELS – WT</td>
<td>-0.45 ± 0.04</td>
<td>1.87 ± 0.03</td>
<td>-0.54 ± 0.07</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Ctrl – APPswe/PS1dE9</td>
<td>-0.33 ± 0.05</td>
<td>2.14 ± 0.07</td>
<td>-0.58 ± 0.12</td>
<td>5 (7)</td>
</tr>
<tr>
<td>ELS – APPswe/PS1dE9</td>
<td>-0.30 ± 0.05</td>
<td>2.06 ± 0.11</td>
<td>-0.32 ± 0.05</td>
<td>6 (9)</td>
</tr>
<tr>
<td><strong>Main/interaction effects</strong></td>
<td>ns</td>
<td>ns</td>
<td>$T^*$</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M (n). Maximal slope of the fEPSP ($R_{\text{max}}$), half-maximum stimulus intensity ($I_{\text{h}}$), and the slope of the input-output curve (slope factor $S$) in the CA1 area. C: condition effect, G: genotype effect, T: treatment effect.

$F(1,63)=22.62, p<0.001$; 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 ($p<0.001$).

**3.2. Barnes maze**

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. 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 ($F(1,55)=6.23, p=0.02$) (Figure 2A,B). However, neither genotype nor condition affected performance on acquisition learning (genotype effect: $F(1,55)=0.24, p=0.63$; condition effect: $F(1,55)=1.18, p=0.28$). No effects were observed when examining,
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

Figure 2: 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 riluzole treatment. F. The distance travelled to the exit hole during reversal learning was reduced by 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=7; ELS-APPswe/PS1dE9-water: N=9; Ctrl-WT-riluzole: N=7; ELS-WT-riluzole: N=7, Ctrl-APPswe/PS1dE9-riluzole: N=8; ELS-APPswe/PS1dE9-riluzole: N=9. *: p<0.05.
During reversal learning, i.e. 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(1,55)=23.76, p=0.001) (Figure 2D,E). In addition, APPswe/PS1dE9 mice took a longer distance to find the exit hole (F(1,55)=10.36, p=0.002). 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 genotype interaction effect (treatment:
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

\[ F(1,55)=34.46, p=0.001; \text{genotype: } F(1,55)=8.26, p=0.006; \text{condition: } F(1,55)=5.012, p=0.029; \text{genotype } \times \text{treatment: } F(1,55)=7.65, p=0.008 \]  (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.

3.3. 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), reflecting the enhanced EAAT2 expression following riluzole treatment in aged ELS and APPswe/PS1dE9 mice.

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

3.4. Hippocampal plaque load

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

4. Discussion

Previous studies have reported that early life stress can alter reversal learning, synaptic plasticity and amyloid levels in 12 months old APPswe/PS1dE9 mice. In the current study, we investigated whether riluzole, a modulator of glutamate levels, can rescue these effects. We found that ELS-induced impairments in synaptic plasticity, reversal learning and plaque load in APPswe/PS1dE9 mice
can be rescued by prolonged riluzole treatment from post-weaning onward, likely by regulating EAAT2 expression.

Our current model for ELS has previously been shown to induce (age-related) impairments in spatial learning, memory processes (reviewed by\textsuperscript{68,69}) and synaptic plasticity\textsuperscript{70}. In addition, it has been shown that ELS aggravates AD-related neuropathology, including increased soluble Aβ levels, a higher plaque load, and impairs cognitive performance\textsuperscript{24,27,58}. 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\textsuperscript{28,71}. Moreover, ELS-exposure in APPswe/PS1dE9 mice further decreased synaptic plasticity, and even resulted in LTD. We then sought to investigate whether alterations in glutamatergic signalling might attenuate these effects by lifelong treatment with the glutamate modulator riluzole, given right 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, and 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\textsuperscript{52} 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 12 months 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\textsuperscript{72-74}, but future studies are required to investigate the 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\textsuperscript{75,76}, and functional brain abnormalities have been observed in humans decades before the development of other symptoms\textsuperscript{77,78}. We therefore tested whether ELS
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

affected learning and memory in APPswe/PS1dE9 mice. Previously, we have reported that 12 month old ELS-APPswe/PS1dE9 mice are impaired in reversal learning in the Barnes maze\(^2\). In line with these findings, we found at present that ELS exposure in APPswe/PS1dE9 mice did not alter acquisition learning, but impaired reversal learning. While riluzole slightly enhanced acquisition learning, it particularly prevented the deficits on reversal 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\(^42,48,52\), which is relevant for maintaining proper synaptic glutamate levels\(^38\). 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\(^36\), and has been implicated in aging and various neurodegenerative diseases, including AD\(^36,37,43-45,52\). Furthermore, EAAT2 haploinsufficiency aggravates cognitive impairments in an AD mouse model, while EAAT2 overexpression improves cognitive performance\(^79\).

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 Barnes maze performance, 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\(^55\), 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\(^80\). 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\(^81,82\) and presenting as one of the first symptoms of AD\(^77,78\). 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\textsuperscript{83,84}. This is thought to occur through an excessive activation of extra-synaptic NMDA receptors\textsuperscript{83,85}, and a decrease in the expression of synaptic NMDA receptors\textsuperscript{86}. In parallel, neuronal activity, regulated by glutamatergic signalling increases the release of A\textbeta\textsuperscript{3}	extsubscript{1}, possibly resulting in a 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/PS\textsuperscript{1}dE9 mice soluble A\textbeta-40 and A\textbeta-42 levels are increased following ELS\textsuperscript{27}, although plaque load was not affected in this study. EAAT2 overexpression has previously been shown to decrease pathological markers in an AD mouse model\textsuperscript{79}, again supporting the hypothesis that improved regulation of glutamatergic signalling via enhanced EAAT2 uptake could potentially mitigate A\textbeta toxicity and worsen cognitive performance. This may suggest that normalising glutamate levels prevents A\textbeta pathology.

Taken together, the present results indicate that riluzole rescues deficits in reversal learning in 12 month old, ELS-exposed APPswe/PS\textsuperscript{1}dE9 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.

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