

# Effects of riluzole on electrically evoked neurotransmitter release

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**1** The main purpose of the present study was to investigate the effects of the neuroprotective agent riluzole on the electrically evoked release of [<sup>3</sup>H]-glutamate ([<sup>3</sup>H]-Glu) in mouse neocortical slices. The reported selectivity of riluzole for excitatory amino acids was tested in release experiments with further neurotransmitters. Also distinct species, mouse, rat and man were compared.

**2** [<sup>3</sup>H]-Glu was formed endogenously during incubation of slices with [<sup>3</sup>H]-glutamine ([<sup>3</sup>H]-Gln). Released [<sup>3</sup>H]-Glu and tissue [<sup>3</sup>H]-Glu was separated by anion exchange chromatography. Electrically evoked [<sup>3</sup>H]-Glu release was strongly diminished by tetrodotoxin (TTX) and Ca<sup>2+</sup>-withdrawal.

**3** Riluzole (100 µM) depressed the release of [<sup>3</sup>H]-Glu up to 77% (IC<sub>50</sub> = 19.5 µM). Riluzole was also able to inhibit strongly the electrically evoked release of [<sup>3</sup>H]-acetylcholine ([<sup>3</sup>H]-ACh) (at 100 µM by 92%, IC<sub>50</sub> = 3.3 µM, and [<sup>3</sup>H]-dopamine ([<sup>3</sup>H]-DA) (at 32 µM by 72%, IC<sub>50</sub> = 6.8 µM). However, the release of [<sup>3</sup>H]-serotonin ([<sup>3</sup>H]-5-HT) was less diminished (at 100 µM by 53%, IC<sub>50</sub> = 39.8 µM). Riluzole up to 100 µM did not affect [<sup>3</sup>H]-noradrenaline ([<sup>3</sup>H]-NA) release.

**4** Between species, i.e. in mouse, rat and human neocortex, no significant differences between the effects of riluzole could be observed.

**5** The NMDA-receptor blocker MK-801 (1 µM) and the AMPA/Kainate-receptor blocker NBQX (1 µM) did neither affect the electrically evoked [<sup>3</sup>H]-ACh release nor its inhibition by riluzole, indicating that effects of riluzole on transmitter release were neither due to modulation of ionotropic Glu receptors, nor due to indirect inhibition of Glu release through these receptors.

**6** Taken together, riluzole inhibits the release of distinct neurotransmitters differently, but is not selective for the excitatory amino acid Glu.

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**Abbreviations:** ACh, acetylcholine; ALS, amyotrophic lateral sclerosis; CI<sub>95</sub>, 95% confidence intervals; DA, dopamine; EDTA, ethylenediaminetetraacetic acid; Gln, glutamine; Glu, glutamate; HPLC, high performance liquid chromatography; 5-HT, serotonin; I<sub>max</sub>, maximum inhibition; NA, noradrenaline; PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid; pIC<sub>50</sub>, negative logarithm of the concentration at half-maximum inhibition; TTX, tetrodotoxin

## Introduction

Riluzole (2-amino-6-[trifluoromethoxy]-benzothiazole) is known as a neuroprotective agent with anticonvulsive, anxiolytic and anaesthetic properties (Doble, 1996). Clinical trials have shown that riluzole prolongs the life of patients with amyotrophic lateral sclerosis (ALS), a rapidly progressive and fatal disease in which both upper and lower motoneurons degenerate (Lacomblez *et al.*, 1996; Bensimon *et al.*, 1994). As a result of these studies, riluzole has been approved and marketed for the treatment of ALS in many countries. In addition, it proved effective in animal models of Parkinson's disease (Barnéoud *et al.*, 1996), Chorea Huntington (Mary *et al.*, 1995) and cerebral and retinal ischaemia (Malgouris *et al.*, 1989; Pratt *et al.*, 1992; Lagrèze *et al.*, 1999).

According to the literature, these effects of riluzole are due to its ability to diminish excitatory neurotransmission. Riluzole has often been outlined as an antiGlu substance, a Glu release inhibitor and an antagonist of Glu neurotransmission (Benavides *et al.*, 1985; Malgouris *et al.*, 1989; Martin *et al.*, 1993; for review see Doble, 1996). *In vitro*, riluzole inhibited K<sup>+</sup>-evoked release of endogenous Glu and aspartate from hippocampal slices (Martin *et al.*, 1993). *In vivo*, riluzole antagonized the spontaneous release of Glu (Chéramy *et al.*, 1992), as well as the Glu-induced DA release from the rat caudate nucleus (Chéramy *et al.*, 1986). Although riluzole did

not bind to any of the known recognition sites of Glu receptors, it was shown to attenuate cation influx initiated by NMDA and kainate (Debono *et al.*, 1993; Hubert *et al.*, 1994).

Obrenovitch & Urenjak (1998) raised the question, which kind of Glu release may be modulated by riluzole, and whether it inhibits Glu release as a Na<sup>+</sup>-channel blocker selectively or not. Our aims were (1) to determine whether riluzole does also inhibit the electrically evoked release of [<sup>3</sup>H]-Glu; (2) to compare its effects on [<sup>3</sup>H]-Glu release with those on other neurotransmitters: i.e. [<sup>3</sup>H]-ACh, [<sup>3</sup>H]-NA, [<sup>3</sup>H]-DA and [<sup>3</sup>H]-5-HT; and (3) to gain information about the efficacy of riluzole in human neocortex in comparison to mouse and rat brain tissue.

## Methods

### Tissue preparation

Six black mice (25–30 g) and Wistar rats (250–350 g) were decapitated, brains were quickly removed and hemispheres were placed in cold oxygenated physiological buffer. Composition of the buffer was (mM): NaCl 121, KCl 1.8, CaCl<sub>2</sub> 1.3, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, Glucose 11, pH 7.4. When experiments were performed in Ca<sup>2+</sup>-free medium, ethylenediaminetetraacetic acid (EDTA, 100 µM) was added to

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the superfusion buffer. Tissue removal, dissection and slicing were accomplished rapidly and the tissue was placed in cold buffer between steps. All efforts were made to minimize animal suffering, to reduce the number of animals used, according to the obtained licence of the local ethical committee on animal care.

Specimens of human neocortical tissue were obtained from patients undergoing surgical treatment of epilepsy. The procedure was approved by the local Ethical Committee of the University of Freiburg. The tissue was instantly immersed in ice-cold oxygen-saturated buffer and processed immediately. The patients obtained antiepileptic drugs (lamotrigine and carbamazepine) or the psychopharmacological clomipramine which were stopped 1 day before surgery. Mannit and dexamethasone was applied before neurosurgery if signs of elevated intracranial pressure were present. After premedication with flunitrazepam, anaesthesia was performed with thiopental, fentanyl or flunitrazepam. To obtain muscular relaxation pancuronium was used. These drugs given were assumed to be washed out of the slices and not to affect the superfusion experiments.

Slices of mice neocortex (300  $\mu\text{m}$ -thick) were washed three times in 5 ml cold oxygen-saturated buffer and then incubated with either [ $^3\text{H}$ ]-glutamine, [ $^3\text{H}$ ]-choline, [ $^3\text{H}$ ]-NA or [ $^3\text{H}$ ]-5-HT. Slices of mice caudatoputamen (300  $\mu\text{m}$ -thick) washed as described above, were incubated with [ $^3\text{H}$ ]-DA. Rat and human neocortex slices, 350  $\mu\text{m}$ -thick and washed as described, were incubated in [ $^3\text{H}$ ]-choline.

#### [ $^3\text{H}$ ]-Glu release

Slices were incubated in 1 ml buffer containing 2  $\mu\text{M}$  [ $^3\text{H}$ ]-Gln and the Glu uptake blocker L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC, 3  $\mu\text{M}$ ) for 60 min at 37°C. Then they were rinsed and transferred (two slices per chamber) to superfusion chambers (dead volume 100  $\mu\text{l}$ ) and superfused at 22°C at a flow rate of 0.4 ml per minute. After preperfusion for 50 min, 2 ml per 5 min fractions of superfusion fluid were collected and mixed with 13 ml  $\text{H}_2\text{O}$ . [ $^3\text{H}$ ]-Glu release was stimulated twice with a series of rectangular, unipolar pulses at 65 ( $\text{S}_1$ ) and 105 ( $\text{S}_2$ ) min (parameters: 28 mA, 2 ms, 3 Hz, 240 pulses). After superfusion the slices were homogenized by sonification in 1 ml  $\text{H}_2\text{O}$ . This homogenate was centrifuged at 10,000 r.p.m. for 5 min and the supernatant was mixed with 14 ml  $\text{H}_2\text{O}$ . [ $^3\text{H}$ ]-Glu was separated from [ $^3\text{H}$ ]-Gln and other [ $^3\text{H}$ ]-labelled metabolites, mainly [ $^3\text{H}$ ]-GABA, by anion exchange chromatography using Amberlite IRA-67 resin. Chromatography columns (6  $\times$  50 mm) were charged with  $\text{HCO}_3^-$ -loaded Amberlite IRA-67. Columns were washed with 30 ml  $\text{H}_2\text{O}$  and [ $^3\text{H}$ ]-Glu was eluted in three steps each covering 3 ml of 1 M NaCl. Eluates of both slices and superfusion samples were mixed with 10 ml of liquid scintillation cocktail (Ultima Gold XR<sup>®</sup>) and quantified by liquid scintillation counting.

#### Validation of anion exchange chromatography

Separation of Glu by anion exchange chromatography was validated by high performance liquid chromatography (HPLC) and also by use of [ $^3\text{H}$ ]-labelled Glu, Gln and GABA and liquid scintillation counting. HPLC was performed with precolumn derivatization and gradient elution using a modification of the methods of Graser and Godel (Graser *et al.*, 1985; Godel *et al.*, 1984). Glu, Gln and GABA were mixed in a concentration of 20  $\mu\text{M}$  each and separated by anion exchange chromatography as described above. Each fraction

(sample application, rinsing and elution step) was collected and analysed by HPLC. [ $^3\text{H}$ ]-labelled compounds were applied separately and collected fractions were quantified by liquid scintillation counting.

#### [ $^3\text{H}$ ]-ACh release

Slices were incubated for 30 min in buffer containing 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-choline at 37°C. Subsequently they were rinsed, transferred to superfusion chambers (one slice in each) and superfused with buffer at a rate of 0.4 ml min<sup>-1</sup>. Hemicholinium-3 (10  $\mu\text{M}$ ) was added in order to inhibit uptake of [ $^3\text{H}$ ]-choline. After 60 min of preperfusion, the superfusate was collected in 2 ml samples. At 75 min ( $\text{S}_1$ ), 105 min ( $\text{S}_2$ ), 135 min ( $\text{S}_3$ ) and 165 min ( $\text{S}_4$ ) slices were depolarized by electrical pulses (depending on the experimental question, parameters were either 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C or 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C).

It has been shown earlier that the depolarization-evoked [ $^3\text{H}$ ]-overflow from brain slices incubated with [ $^3\text{H}$ ]-choline represents the transmitter [ $^3\text{H}$ ]-ACh (Richardson & Szerb, 1974; Feuerstein *et al.*, 1998).

#### [ $^3\text{H}$ ]-NA release

Slices were incubated for 30 min in buffer containing 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-NA at 37°C and L(+)-ascorbic acid (0.1 g l<sup>-1</sup>). In the superfusion fluid, oxaprotiline (1  $\mu\text{M}$ ) was additionally present in order to inhibit uptake of [ $^3\text{H}$ ]-NA. At 75 min ( $\text{S}_1$ ), 105 min ( $\text{S}_2$ ), 135 min ( $\text{S}_3$ ) and 165 min ( $\text{S}_4$ ) slices were depolarized by electrical pulses (depending on the experimental question, parameters were either 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C; 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C or 60 mA, 2 ms, 100 Hz, 4 pulses, 37°C).

#### [ $^3\text{H}$ ]-DA release

Slices were incubated for 30 min in buffer containing 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-DA at 37°C and L(+)-ascorbic acid (0.1 g l<sup>-1</sup>). In the superfusion fluid, nomifensine (3.2  $\mu\text{M}$ ) was additionally present in order to inhibit uptake of [ $^3\text{H}$ ]-DA. At 75 min ( $\text{S}_1$ ), 105 min ( $\text{S}_2$ ) and 135 min ( $\text{S}_3$ ) slices were depolarized by electrical pulses (parameters: 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C).

#### [ $^3\text{H}$ ]-5-HT release

Slices were incubated for 60 min in buffer containing 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-5-HT at 37°C and L(+)-ascorbic acid (0.1 g l<sup>-1</sup>). In the superfusion fluid, fluvoxamine (1  $\mu\text{M}$ ) was additionally present in order to inhibit uptake of [ $^3\text{H}$ ]-5-HT. After 60 min of preperfusion, the superfusate was collected in 2 ml samples. At 75 min ( $\text{S}_1$ ), 105 min ( $\text{S}_2$ ) and 135 min ( $\text{S}_3$ ) slices were depolarized by electrical pulses (parameters: 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C).

In all experiments, except those with [ $^3\text{H}$ ]-Glu, the slices were removed from the chambers after superfusion and solubilized in 0.5 ml of Soluene<sup>®</sup>. The solubilized slices were mixed with 10 ml, the superfusate fractions with 3 ml, of liquid scintillation cocktail (Ultima Gold<sup>®</sup>), and tritium content was determined by liquid scintillation counting.

#### Calculation

The outflow of tritiated compounds was expressed as fractional rate, i.e. the radioactivity of the superfusates was

divided by the radioactivity in the tissue at the beginning of the corresponding collection period. Effects of drugs or  $\text{Ca}^{2+}$ -withdrawal on basal [ $^3\text{H}$ ]-outflow were evaluated by the ratio of the fractional rate before the second, third and fourth stimulation ( $b_2$ ,  $b_3$ ,  $b_4$ ) and that before the first stimulation ( $b_1$ ). The  $b_x/b_1$  ratios were then compared between treatments and controls using the 95% confidence intervals ( $\text{CI}_{95}$ ) of the  $b_x/b_1$  ratios (see legend to Table 3). The stimulation-evoked overflow of tritium of the first ( $S_1$ ) and the following ( $S_x$ ) stimulations are calculated by subtracting the basal radioactivity from the total radioactivity in the stimulation fractions (basal outflow was assumed to decline linearly). The difference was expressed as percentage of the radioactivity in the slice at the beginning of the respective stimulation period. Drugs to be tested were given 15 min before  $S_x$ ; their effects were evaluated by comparing the ratios  $S_x/S_1$ . Control experiments were always run in parallel to drug experiments. The  $S_x/S_1$  ratios were normalized by dividing each individual ratio by the mean ratio of the corresponding control stimulation. All results were expressed as means with  $\text{CI}_{95}$ .

Concentration-inhibition curves of riluzole for each transmitter (except [ $^3\text{H}$ ]-NA) were evaluated using the logistic function,  $S_x/S_1 = I_{\max} * 10^{\text{Lril}} / (10^{-\text{pIC}_{50}} + 10^{\text{Lril}})$  with  $\text{Lril}$  = logarithm of the applied concentration of riluzole (independent variable) and the individual  $S_x/S_1$  ratios (dependent variable), yielding the parameter estimates  $I_{\max}$  = maximum inhibition of [ $^3\text{H}$ ]-transmitter release due to riluzole and  $\text{pIC}_{50}$  = negative logarithm of the riluzole concentration at half-maximum inhibition (Feuerstein & Limberger, 1999).

## Materials

Soluene<sup>®</sup> and Ultima Gold<sup>®</sup> (XR) were obtained from Packard, Frankfurt, Germany. [ $^3\text{H}$ ]-glutamine L-[3,4- $^3\text{H}$ (N)] (49.9 Ci  $\text{mmol}^{-1}$ ) was purchased from NEN, Du Pont, Bad Homburg, Germany; [methyl- $^3\text{H}$ ]-choline chloride (81 Ci  $\text{mmol}^{-1}$ ); [7,8- $^3\text{H}$ ]-dopamine (47.0 Ci  $\text{mmol}^{-1}$ ); [ $^3\text{H}$ ]-noradrenaline (55 Ci  $\text{mmol}^{-1}$ ) from Amersham Buchler, Braunschweig, Germany; hydroxytryptamine binoxalate 5-[1,2- $^3\text{H}$ ] and TTX from Bio Trend, Köln, Germany (27.5 Ci  $\text{mmol}^{-1}$ ); (+)-oxaprotilin HCl from Novartis Pharma, Nürnberg, Germany; fluvoxamine maleate from Solvay, Hannover, Germany; EDTA, nomifensine and hemicholinium from Sigma, Deisenhofen, Germany and PDC, MK 801 maleate and NBQX from

Tocris, Cookson LTD, Bristol, U.K. Riluzole was a gift from Rhône-Poulenc-Rorer, Köln, Germany.

## Results

The major focus of the present study was the effect of riluzole on electrically evoked [ $^3\text{H}$ ]-Glu release in mouse neocortex. As a prerequisite of this, [ $^3\text{H}$ ]-Glu was successfully separated from other [ $^3\text{H}$ ]-compounds by anion exchange chromatography, which was validated HPLC and also by use of [ $^3\text{H}$ ]-labelled Glu, Gln and GABA (Table 1). For comparison with glutamate experiments, other neurotransmitters and other species, rat and human neocortex, were used.

### Effect of TTX, $\text{Ca}^{2+}$ -withdrawal and riluzole on [ $^3\text{H}$ ]-Glu release

In mouse neocortical slices, electrical field stimulation (2 ms, 28 mA, 240 pulses, 3 Hz, 22°C) evoked [ $^3\text{H}$ ]-Glu efflux beyond basal levels (Figure 1). The mean of  $S_1$  was 0.65% of tissue [ $^3\text{H}$ ]-Glu,  $\text{CI}_{95}$  (0.58%, 0.72%). Resulting  $S_2/S_1$  ratios of control experiments amounted to 0.74,  $\text{CI}_{95}$  (0.64, 0.85), to be compared with ratios obtained under drug treatment. In order to prove the physiological relevance of the electrical stimulation paradigm used, the modulation of [ $^3\text{H}$ ]-Glu release by the  $\text{Na}^+$ -channel blocker TTX at 1  $\mu\text{M}$ , and by withdrawal of  $\text{Ca}^{2+}$  was investigated. TTX and  $\text{Ca}^{2+}$  withdrawal strongly reduced the electrically evoked release (Figure 2a). The basal [ $^3\text{H}$ ]-Glu efflux was neither affected by TTX nor by riluzole at the concentrations used. Withdrawal of  $\text{Ca}^{2+}$ , however, led to a slight increase of basal [ $^3\text{H}$ ]-Glu efflux (Table 3).

[ $^3\text{H}$ ]-Glu release was diminished by riluzole in a concentration-dependent fashion as shown in Figure 2b. Nonlinear regression analysis using the logistic function given in Methods yielded a  $\text{pIC}_{50}$  value of 4.71,  $\text{CI}_{95}$  (4.25, 5.18), and an  $I_{\max}$  estimate of 93.4%,  $\text{CI}_{95}$  (68.5%, 136.1%).

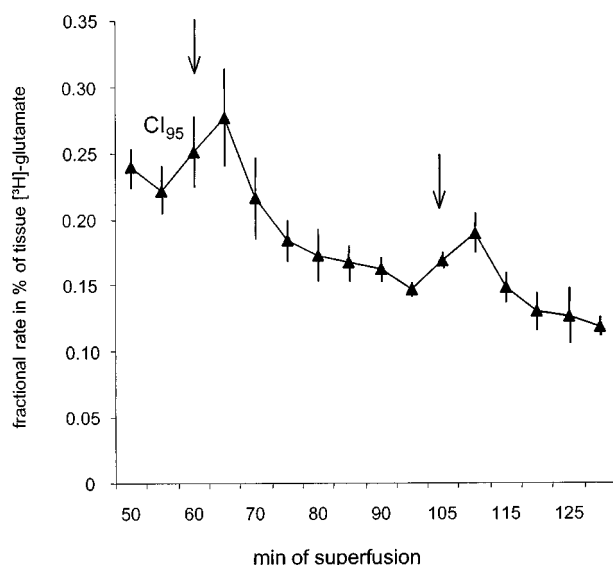
### Effect of TTX, $\text{Ca}^{2+}$ -withdrawal and riluzole on [ $^3\text{H}$ ]-ACh release, [ $^3\text{H}$ ]-NA, [ $^3\text{H}$ ]-5-HT, [ $^3\text{H}$ ]-DA in mouse neocortex and caudatoputamen

To compare effects of riluzole in different transmitter systems, we needed to use the same experimental parameters

**Table 1** Validation of the anion exchange chromatography

HPLC	Sample application (Glu, Gln, GABA + 2 ml buffer, 14 ml $\text{H}_2\text{O}$ )	Rinsing (30 ml $\text{H}_2\text{O}$ )	Elution (3 × 3 ml 1M NaCl)
Glu	0.58%, $\text{CI}_{95}$ (0.35%, 0.83%)	0%, $\text{CI}_{95}$ (0%, 0%)	99.41%, $\text{CI}_{95}$ (97.32%, 101.50%)
Gln	79.82%, $\text{CI}_{95}$ (74.96%, 84.70%)	20.17%, $\text{CI}_{95}$ (17.97%, 22.38%)	0%, $\text{CI}_{95}$ (0%, 0%)
GABA	87.04%, $\text{CI}_{95}$ (81.52%, 92.58%)	12.60%, $\text{CI}_{95}$ (11.80%, 13.41%)	0.34%, $\text{CI}_{95}$ (-0.33%, 1.03%)
<i>[<math>^3\text{H}</math>]-determination</i>			
[ $^3\text{H}$ ]-Glu	3.95%, $\text{CI}_{95}$ (3.68%, 4.22%)	0.55%, $\text{CI}_{95}$ (0.51%, 0.59%)	90.95%, $\text{CI}_{95}$ (90.43%, 91.47%)
[ $^3\text{H}$ ]-Gln	61.61%, $\text{CI}_{95}$ (61.13%, 62.09%)	23.1%, $\text{CI}_{95}$ (20.21%, 25.73%)	14.22%, $\text{CI}_{95}$ (13.61%, 14.83%)
[ $^3\text{H}$ ]-GABA	85.39%, $\text{CI}_{95}$ (84.54%, 86.24%)	12.58%, $\text{CI}_{95}$ (12.21%, 12.95%)	1.14%, $\text{CI}_{95}$ (1.04%, 1.24%)

Glu, Gln and GABA were mixed at concentrations of 20  $\mu\text{M}$  each and separated by anion exchange chromatography (for details, see Methods). Each fraction, i.e. the sample application, the rinsing and the elution step, was collected. After that, all fractions were analysed by HPLC. [ $^3\text{H}$ ]-Glu, [ $^3\text{H}$ ]-Gln and [ $^3\text{H}$ ]-GABA were applied separately and collected fractions were quantified by liquid scintillation counting. Results are given in per cent of recovered Glu, Gln and GABA with corresponding  $\text{CI}_{95}$ .



**Figure 1** The electrically evoked [ $^3\text{H}$ ]-Glu release as fractional rates (see Methods) after [ $^3\text{H}$ ]-Gln incubation of mouse neocortex slices. Each value is the mean value of nine controls with corresponding  $\text{CI}_{95}$ s. Series of electrical pulses (parameters: 28 mA, 2 ms, 3 Hz, 240 pulses, at  $22^\circ\text{C}$ ) were applied at 60 and 105 min. The Glu uptake inhibitor PDC ( $3\text{ }\mu\text{M}$ ) was present throughout the experiment.

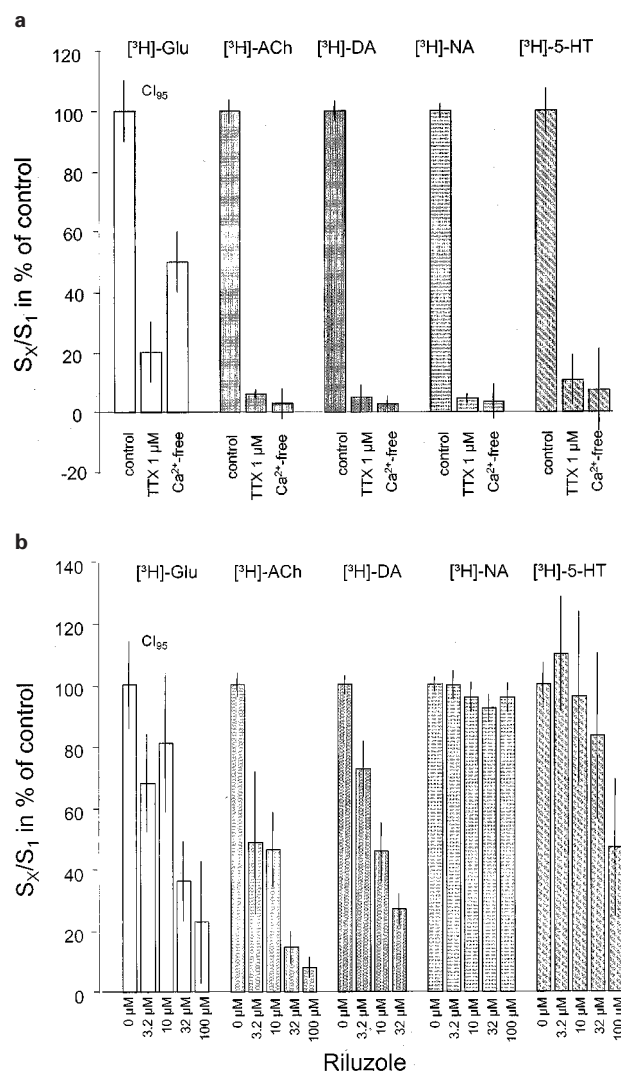
for each investigated transmitter. Therefore, the experimental conditions of release were based on the parameters used for [ $^3\text{H}$ ]-Glu experiments. Electrical stimulation using these parameters (2 ms, 28 mA, 240 pulses, 3 Hz,  $22^\circ\text{C}$ ) increased [ $^3\text{H}$ ]-efflux from mouse neocortical slices prelabeled with [ $^3\text{H}$ ]-choline.  $\text{S}_1$  was 1.54% of tissue tritium,  $\text{CI}_{95}$  (1.41%, 1.66%); in controls the  $\text{S}_2/\text{S}_1$  ratio was 1.05,  $\text{CI}_{95}$  (0.87, 1.24),  $\text{S}_3/\text{S}_1$  was 1.33,  $\text{CI}_{95}$  (1.18, 1.47), and  $\text{S}_4/\text{S}_1$  was 1.43,  $\text{CI}_{95}$  (1.23, 1.62).

Under the same parameters the mean  $\text{S}_1$  value of electrically evoked [ $^3\text{H}$ ]-NA was 7.94% of tissue tritium,  $\text{CI}_{95}$  (7.27%, 8.60%); in controls the  $\text{S}_2/\text{S}_1$  ratio was 0.95,  $\text{CI}_{95}$  (0.92, 0.99),  $\text{S}_3/\text{S}_1$  was 0.93,  $\text{CI}_{95}$  (0.88, 0.98), and  $\text{S}_4/\text{S}_1$  was 0.90,  $\text{CI}_{95}$  (0.85, 0.95).

Under the same parameters the mean  $\text{S}_1$  value of electrically evoked [ $^3\text{H}$ ]-5-HT was 2.09% of tissue tritium,  $\text{CI}_{95}$  (1.75%, 2.44%); in controls  $\text{S}_2/\text{S}_1$  was 0.70,  $\text{CI}_{95}$  (0.60, 0.79) and  $\text{S}_3/\text{S}_1$  was 0.68,  $\text{CI}_{95}$  (0.57, 0.80).

Under the same parameters the mean  $\text{S}_1$  value of electrically evoked [ $^3\text{H}$ ]-DA was 3.14% of tissue tritium,  $\text{CI}_{95}$  (2.72%, 3.58%); in controls the  $\text{S}_2/\text{S}_1$  ratio 0.76,  $\text{CI}_{95}$  (0.72, 0.81) and  $\text{S}_3/\text{S}_1$  was 0.64,  $\text{CI}_{95}$  (0.60, 0.69).

TTX and  $\text{Ca}^{2+}$  withdrawal abolished the electrically evoked release of [ $^3\text{H}$ ]-ACh, [ $^3\text{H}$ ]-NA, [ $^3\text{H}$ ]-5-HT, and [ $^3\text{H}$ ]-DA (Figure 2a). Figure 2b shows that riluzole concentration-dependently diminished the release of [ $^3\text{H}$ ]-ACh and [ $^3\text{H}$ ]-DA, but was less potent in reducing [ $^3\text{H}$ ]-5-HT release. Accordingly, the  $\text{pIC}_{50}$  estimates by nonlinear regression analysis of the inhibition of [ $^3\text{H}$ ]-ACh, [ $^3\text{H}$ ]-DA, and [ $^3\text{H}$ ]-5-HT release were, 5.48,  $\text{CI}_{95}$  (5.26, 5.72), 5.17,  $\text{CI}_{95}$  (5.00, 5.34), and 4.40,  $\text{CI}_{95}$  (4.03, 4.74), respectively. For the concentration-response relationship of [ $^3\text{H}$ ]-ACh and [ $^3\text{H}$ ]-DA the  $I_{\text{max}}$  value was estimated to 93.1%,  $\text{CI}_{95}$  (84.4%, 102.3%), and 89.2%,  $\text{CI}_{95}$  (79.6%, 101.2%), respectively, which was very similar to the maximum inhibition seen with [ $^3\text{H}$ ]-Glu release. For the inhibition of [ $^3\text{H}$ ]-5-HT release, however,  $I_{\text{max}}$  could not be estimated from the data points, but was assumed to be 50% from Figure 2b in order to roughly assess the corresponding  $\text{pIC}_{50}$  value mentioned above. [ $^3\text{H}$ ]-NA release was not changed by riluzole.



**Figure 2** (a) Effects of TTX ( $1\text{ }\mu\text{M}$ ) and  $\text{Ca}^{2+}$ -free medium on electrically evoked neurotransmitter release of mouse neocortex. Stimulation was induced by the following parameters: 28 mA, 2 ms, 3 Hz, 240 pulses, at  $22^\circ\text{C}$ . Columns represent mean  $\text{S}_x/\text{S}_1$  values in per cent of corresponding controls with  $\text{CI}_{95}$ s. TTX or  $\text{Ca}^{2+}$  withdrawal was applied from 15 min before the second stimulation onwards ( $n=7-10$  per column). The  $\text{S}_1$  as well as the control  $\text{S}_x/\text{S}_1$  values are given in the text. (b) Effects of riluzole on electrically evoked neurotransmitter release in mouse neocortex. Stimulation was induced by the following parameters: 28 mA, 2 ms, 3 Hz, 240 pulses, at  $22^\circ\text{C}$ . Columns represent mean  $\text{S}_x/\text{S}_1$  values in per cent of corresponding controls ( $0\text{ }\mu\text{M}$  riluzole) with  $\text{CI}_{95}$ s. Riluzole was given (at increasing concentrations during  $\text{S}_2$ ,  $\text{S}_3$  and  $\text{S}_4$  in the case of [ $^3\text{H}$ ]-ACh, [ $^3\text{H}$ ]-DA, [ $^3\text{H}$ ]-NA and [ $^3\text{H}$ ]-5-HT) from 15 min before the second stimulation onwards ( $n=6-20$  per column). Since at least three controls were running in each superfusion experiment the number of controls accumulated was up to 20 per column whereas the number of drug experiments was only 6–10 per column. The  $\text{S}_1$  as well as the control  $\text{S}_x/\text{S}_1$  values are given in the text.

#### [ $^3\text{H}$ ]-NA release using other experimental conditions

Since riluzole, even at the highest concentration, did not affect [ $^3\text{H}$ ]-NA release under the conditions used for [ $^3\text{H}$ ]-Glu release, other parameters were tested. Electrical field stimulation with 60 mA, 2 ms, 90 pulses, 3 Hz, at a superfusion temperature of  $37^\circ\text{C}$  (parameters usually used for NA experiments) led to  $\text{S}_1=7.98\%$  of tissue tritium,  $\text{CI}_{95}$  (7.48%, 8.47%). In controls, the  $\text{S}_2/\text{S}_1$  ratio of controls was 0.92,  $\text{CI}_{95}$  (0.84, 1.01), and the  $\text{S}_3/\text{S}_1$  ratio was 0.90,  $\text{CI}_{95}$  (0.79, 1.01). We also examined pseudo-one pulse stimulation parameters for autoinhibition-free release (Singer, 1988; 60 mA, 2 ms, 4 pulses, 100 Hz,

37°C), resulting in  $S_1$ -values of 2.18% of tissue tritium,  $CI_{95}$  (1.96%, 2.40%). In the controls the  $S_2/S_1$  ratio was 0.96,  $CI_{95}$  (0.88, 1.04), and the  $S_3/S_1$  ratio was 1.00,  $CI_{95}$  (0.92, 1.07). The effects of riluzole under these conditions are shown in Table 2. It remained ineffective under autoinhibition-free release conditions, but showed significant inhibitory effects at 32 and 100  $\mu\text{M}$  when autoinhibition was operative at 3 Hz stimulation frequency. However, the magnitude of these effects of riluzole were less than those observed with all other transmitters.

#### Comparison of riluzole effects on [ $^3\text{H}$ ]-ACh release in mouse, rat and human neocortex tissue

Investigating the question, whether the efficacy of riluzole was similar within different species we found the  $S_1$ -values to be in mouse 2.22% of tissue tritium,  $CI_{95}$  (2.11%, 2.32%), in rat 1.42%,  $CI_{95}$  (1.28%, 1.56%), and in human neocortex 1.03%,  $CI_{95}$  (0.87%, 1.19%). In controls, the  $S_2/S_1$  ratio was 1.03,  $CI_{95}$  (0.98, 1.07), and the  $S_3/S_1$  ratio was 1.08,  $CI_{95}$  (1.02, 1.14) in mouse tissue, in rat tissue the  $S_2/S_1$  ratio was 0.93,  $CI_{95}$  (0.89, 0.97), and the  $S_3/S_1$  was 0.84,  $CI_{95}$  (0.79, 0.89) and in human tissue the  $S_2/S_1$  ratio was 1.04,  $CI_{95}$  (0.90, 1.18) and  $S_3/S_1$  was 1.02,  $CI_{95}$  (0.92, 1.13). The concentration-dependent inhibition of [ $^3\text{H}$ ]-ACh release by riluzole in human neocortical slices was not significantly different from that seen in mouse and rat (Figure 3). Note that the maximum inhibition by riluzole in the present experimental setup was significantly less than that observed with [ $^3\text{H}$ ]-ACh release under the conditions of [ $^3\text{H}$ ]-Glu release (Figure 2b).

#### Effects of PDC, NBQX + MK 801, and riluzol + NBQX + MK 801 on [ $^3\text{H}$ ]-ACh release

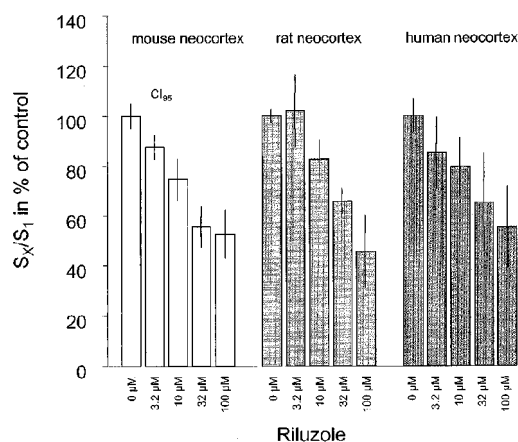
To investigate whether Glu itself might influence [ $^3\text{H}$ ]-ACh release, we increased extracellular Glu with PDC (100  $\mu\text{M}$ ). [ $^3\text{H}$ ]-ACh release was unchanged in the presence of PDC (Figure 4). To exclude the effects of riluzole on [ $^3\text{H}$ ]-ACh release through ionotropic Glu receptors these receptors were blocked by MK 801 and NBQX (1  $\mu\text{M}$  each). The inhibition due to riluzole was not changed by MK 801 and NBQX (Figure 4).

## Discussion

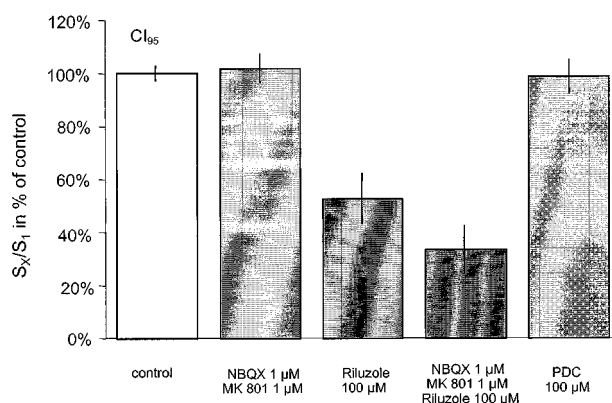
The present study examined the effects of riluzole on electrically evoked neurotransmitter release in mouse, rat and human brain tissue. We chose an electrical stimulation paradigm because electrical pulses, according to their TTX sensitivity, simulate action potentials to induce  $\text{Ca}^{2+}$ -dependent, i.e. exocytotic, transmitter release.

According to Waldmeier *et al.* (1993), [ $^3\text{H}$ ]-Glu release experiments were performed at 22°C, to reduce both the

spontaneous activity of neurons and the reuptake of released Glu. Reuptake was additionally suppressed by PDC (3  $\mu\text{M}$ ). Thus, the signal to noise ratio (evoked to basal transmitter efflux) was improved. Our model of [ $^3\text{H}$ ]-Glu release from slices preloaded with [ $^3\text{H}$ ]-Gln mainly reflects release from the neuronal Glu pool, since Gln, the major precursor of the



**Figure 3** Effects of riluzole on electrically evoked [ $^3\text{H}$ ]-ACh release. Stimulation was induced by the following parameters: 60 mA, 2 ms, 3 Hz, 90 pulses, at 37°C. Columns represent mean  $S_x/S_1$  values in per cent of corresponding controls (0  $\mu\text{M}$  riluzole) with  $CI_{95}$ s. Riluzole was given (at increasing concentrations during  $S_2$  and  $S_3$ ) from 15 min before the second stimulation onwards ( $n=6-9$  per column). The  $S_1$  as well as the control  $S_x/S_1$  values are given in the text.



**Figure 4** Effects of MK 801 + NBQX, riluzole, MK 801 + NBQX + riluzole and PDC on electrically evoked [ $^3\text{H}$ ]-ACh release of mouse neocortex. Stimulation was induced by the following parameters: 60 mA, 2 ms, 3 Hz, 90 pulses, at 37°C. Columns represent mean  $S_x/S_1$  values in per cent of corresponding controls with  $CI_{95}$ s. Drugs were given from 15 min before the second stimulation onwards ( $n=6-20$  per column, see comment in the legend of Figure 2b). The corresponding  $S_1$  as well as the control  $S_x/S_1$ -values are given in the text.

**Table 2** Effects of riluzole on electrically evoked [ $^3\text{H}$ ]-NA release in the absence and presence of autoinhibition in mouse neocortex

Stimulation parameters	Control	Riluzole (3.2 $\mu\text{M}$ )	Riluzole (10 $\mu\text{M}$ )	Riluzole (32 $\mu\text{M}$ )	Riluzole (100 $\mu\text{M}$ )
60 mA, 2 ms, 4 pulses, 100 Hz, 37°C	0.00%, $CI_{95}$ (-6.16%, 6.16%)	-2.74%, $CI_{95}$ (-14.33%, 8.84%)	-3.95%, $CI_{95}$ (-32.79%, 24.90%)	7.06%, $CI_{95}$ (-0.66%, 14.77%)	1.75%, $CI_{95}$ (-25.10%, 28.61%)
60 mA, 2 ms, 90 pulses, 3 Hz, 37°C	0.00%, $CI_{95}$ (-4.61%, 4.61%)	0.63%, $CI_{95}$ (-2.55%, 3.81%)	9.01%, $CI_{95}$ (-1.30%, 19.32%)	25.62%, $CI_{95}$ (22.01%, 29.23%)	33.25%, $CI_{95}$ (25.01%, 41.58%)

Data represent inhibition by riluzole in per cent of corresponding controls with  $CI_{95}$ s. Riluzole was given from 15 min before the second stimulation onwards at increasing concentrations ( $n=6-20$  per column). The corresponding  $S_1$  as well as the control  $S_x/S_1$  values are given in the text.

**Table 3** Effects of riluzole, TTX and  $\text{Ca}^{2+}$ -withdrawal on the basal, unstimulated [ $^3\text{H}$ ]-transmitter outflow

Transmitter	Control	Riluzole (3.2 $\mu\text{M}$ )	Riluzole (10 $\mu\text{M}$ )	Riluzole (32 $\mu\text{M}$ )	Riluzole (100 $\mu\text{M}$ )	TTX (1 $\mu\text{M}$ )	$\text{Ca}^{2+}$ -free
[ $^3\text{H}$ ]-Glu 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C, mouse	1.00, Cl <sub>95</sub> (0.97, 1.03)	1.03, Cl <sub>95</sub> (0.97, 1.1)	1.02, Cl <sub>95</sub> (0.98, 1.06)	0.95, Cl <sub>95</sub> (0.91, 1.00)	1.02, Cl <sub>95</sub> (1.00, 1.06)	1.03, Cl <sub>95</sub> (1.00, 1.06)	1.20, Cl <sub>95</sub> (1.07, 1.34)
[ $^3\text{H}$ ]-ACh 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C, mouse	1.00, Cl <sub>95</sub> (0.97, 1.03)	0.96, Cl <sub>95</sub> (0.90, 1.01)	0.98, Cl <sub>95</sub> (0.93, 1.02)	0.89, Cl <sub>95</sub> (0.81, 0.96)	0.95, Cl <sub>95</sub> (0.91, 0.99)	0.90, Cl <sub>95</sub> (0.86, 0.94)	1.07, Cl <sub>95</sub> (0.99, 1.15)
[ $^3\text{H}$ ]-ACh 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C, mouse	1.00, Cl <sub>95</sub> (0.95, 1.05)	1.01, Cl <sub>95</sub> (0.88, 1.14)	1.05, Cl <sub>95</sub> (1.02, 1.09)	1.02, Cl <sub>95</sub> (1.00, 1.04)	1.11, Cl <sub>95</sub> (1.06, 1.16)		
[ $^3\text{H}$ ]-ACh 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C, rat	1.00, Cl <sub>95</sub> (0.96, 1.04)	1.03, Cl <sub>95</sub> (0.97, 1.09)	1.02, Cl <sub>95</sub> (0.92, 1.12)	0.99, Cl <sub>95</sub> (0.95, 1.02)	0.95, Cl <sub>95</sub> (0.86, 1.05)		
[ $^3\text{H}$ ]-ACh 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C, human	1.00, Cl <sub>95</sub> (0.97, 1.03)	1.00, Cl <sub>95</sub> (0.95, 1.04)	1.04, Cl <sub>95</sub> (0.96, 1.12)	0.95, Cl <sub>95</sub> (0.90, 1.00)	0.94, Cl <sub>95</sub> (0.88, 1.00)		
[ $^3\text{H}$ ]-DA 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C, mouse	1.00, Cl <sub>95</sub> (0.94, 1.06)	0.88, Cl <sub>95</sub> (0.80, 0.96)	0.87, Cl <sub>95</sub> (0.79, 0.96)	0.72, Cl <sub>95</sub> (0.64, 0.80)	1.08, Cl <sub>95</sub> (0.99, 1.17)	0.77, Cl <sub>95</sub> (0.74, 0.81)	0.92, Cl <sub>95</sub> (0.72, 1.12)
[ $^3\text{H}$ ]-5-HT 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C, mouse	1.00, Cl <sub>95</sub> (0.93, 1.07)	0.92, Cl <sub>95</sub> (0.85, 1.00)	0.83, Cl <sub>95</sub> (0.66, 0.99)	0.83, Cl <sub>95</sub> (0.71, 0.94)	0.74, Cl <sub>95</sub> (0.65, 0.82)	0.98, Cl <sub>95</sub> (0.92, 1.04)	1.18, Cl <sub>95</sub> (1.09, 1.27)
[ $^3\text{H}$ ]-NA 28 mA, 2 ms, 3 Hz, 240 pulses, 22°C, mouse	1.00, Cl <sub>95</sub> (0.94, 1.06)	1.04, Cl <sub>95</sub> (0.66, 1.42)	0.91, Cl <sub>95</sub> (0.74, 1.08)	0.86, Cl <sub>95</sub> (0.70, 1.03)	0.82, Cl <sub>95</sub> (0.70, 0.94)	0.71, Cl <sub>95</sub> (0.60, 0.83)	0.75, Cl <sub>95</sub> (0.56, 0.94)
[ $^3\text{H}$ ]-NA 60 mA, 2 ms, 100 Hz, 4 pulses, 37°C, mouse	1.00, Cl <sub>95</sub> (0.98, 1.02)	0.89, Cl <sub>95</sub> (0.87, 0.92)	0.91, Cl <sub>95</sub> (0.71, 1.12)	0.59, Cl <sub>95</sub> (0.51, 0.67)	0.76, Cl <sub>95</sub> (0.69, 0.84)		
[ $^3\text{H}$ ]-NA 60 mA, 2 ms, 3 Hz, 90 pulses, 37°C, mouse	1.00, Cl <sub>95</sub> (0.93, 1.07)	1.06, Cl <sub>95</sub> (0.82, 1.29)	0.83, Cl <sub>95</sub> (0.67, 0.98)	0.89, Cl <sub>95</sub> (0.49, 1.28)	0.66, Cl <sub>95</sub> (0.62, 0.69)		

Results ( $b_x/b_1$ ) were given in per cent of corresponding control value with the Cl<sub>95</sub>. Apart from a tendency to reduce the basal [ $^3\text{H}$ ]-outflow in experiments with monoaminergic transmitters, no consistent effect of riluzole on the  $b_x/b_1$  ratio can be detected. TTX seems to reduce the  $b_x/b_1$  ratio in [ $^3\text{H}$ ]-DA and [ $^3\text{H}$ ]-NA experiments, whereas  $\text{Ca}^{2+}$ -withdrawal enhanced this ratio in the case of [ $^3\text{H}$ ]-5-HT and [ $^3\text{H}$ ]-Glu. These slight effects on basal [ $^3\text{H}$ ]-outflow are not assumed to have an impact on our results.

neurotransmitter Glu, is metabolized predominantly in neurons (Bradford *et al.*, 1978; Reubi, 1980). Because of the [ $^3\text{H}$ ]-Gln incubation, [ $^3\text{H}$ ]-Glu had to be separated by anion-exchange chromatography from other [ $^3\text{H}$ ]-compounds including [ $^3\text{H}$ ]-Gln that were excreted in the superfusion medium. At variance to the present approach, information about the source of Glu is vague if endogenous Glu is determined, e.g. in microdialysis studies (Timmerman & Westerink, 1997).

Because the  $\text{Na}^+$  channel blocker TTX as well as  $\text{Ca}^{2+}$ -free medium inhibited the electrically evoked [ $^3\text{H}$ ]-Glu release, we assume that mainly action potential-induced, exocytotic Glu release occurred in our experiments. With this model, we were able to investigate the effects of substances on release of neuronal Glu.

The first question to answer was whether or not riluzole is able to suppress electrically evoked [ $^3\text{H}$ ]-Glu release. As expected from other studies, using potassium depolarization, which demonstrated riluzole to inhibit Glu release (e.g. Martin *et al.*, 1993) this drug did also suppress electrically evoked [ $^3\text{H}$ ]-Glu release in mouse neocortical slices in a concentration-dependent manner and even more efficaciously. Obrenovitch & Urenjak (1998) presumed for experiments using potassium depolarization that release of Glu might be due to a reversal of the Glu transporter and/or due to osmotic pressure (see also Szatkowski *et al.*, 1990). We assume that in our hands electrically evoked [ $^3\text{H}$ ]-Glu release is of neuronal origin and, to a large extent, exocytotically released. Thus, we demonstrated that riluzole can efficiently suppress this electrically stimulated release of Glu.

In earlier articles (Doble, 1996; Martin *et al.*, 1993) riluzole was suggested to be a selective Glu release inhibitor. Therefore,

we investigated riluzole effects in five different transmitter systems to test this proposed selectivity. We chose the same parameter constellation in all experiments, namely that for [ $^3\text{H}$ ]-Glu release, to be sure that distinct riluzole effects were not due to differences in parameters, but to the transmitter systems themselves. The physiological relevance of these rather unusual release conditions, including temperature, was assured by experiments with TTX and  $\text{Ca}^{2+}$ -free medium for all transmitter systems. Riluzole inhibited [ $^3\text{H}$ ]-Glu, [ $^3\text{H}$ ]-ACh and [ $^3\text{H}$ ]-DA release to a similar extent and with similar potency, in contrast to its minor inhibition of the release of [ $^3\text{H}$ ]-5-HT. The inhibition of [ $^3\text{H}$ ]-ACh by riluzole was also minor when stimulation conditions with less electrical pulses were applied, as in the case of the comparison of mouse, rat and human neocortical tissue. This may be explained by the smaller number of pulses and, correspondingly, the smaller use-dependent action of riluzole, as described by MacIver *et al.* (1996).

Concerning the lack of effects of riluzole on [ $^3\text{H}$ ]-NA release, we further tested other parameter constellations (4 pulses, 60 mA, 100 Hz and 90 pulses, 60 mA, 3 Hz). We found only a weak inhibitory effect of riluzole on the release of [ $^3\text{H}$ ]-NA evoked by 90 pulses at 3 Hz. The fact that high concentrations of riluzole significantly depressed the release of this transmitter solely under the conditions of autoinhibition may also be explained by use-dependent effects of riluzole since autoinhibition conditions had 90 pulses whereas 'autoinhibition-free' conditions only had 4 pulses. When 240 pulses were applied with a current of only 28 mA, however, no effect of riluzole on the release of [ $^3\text{H}$ ]-NA was observed, at variance to the strong effects of this drug on the release of [ $^3\text{H}$ ]-ACh, [ $^3\text{H}$ ]-Glu and [ $^3\text{H}$ ]-DA under this constellation of



parameters. This comparison indicates that the effects of riluzole on different transmitter systems cannot be similarly explained, e.g. by a similar action on  $\text{Na}^+$  channels.

In comparison with other studies our  $\text{IC}_{50}$  value of the inhibition by riluzole of  $[\text{H}^3]\text{-Glu}$  release ( $19.5 \mu\text{M}$ ) compares with  $> 50 \mu\text{M}$  of Lingamaneni & Hemmings (1999) who evoked Glu release by  $30 \text{ mM K}^+$ . Martin *et al.* (1993) found an  $\text{IC}_{50}$  value of about  $30 \mu\text{M}$  for evoked Glu release evoked with  $50 \text{ mM K}^+$ . In addition the  $I_{\text{max}}$  value of our study was larger. These differences may be due to the fact that in our hands  $[\text{H}^3]\text{-Glu}$  was mainly of neuronal origin which may be subject to a more pronounced modulation. For ACh release, evoked by electrical pulses of only 1 ms duration, an  $\text{IC}_{50}$  value of about  $25 \mu\text{M}$  (Benavides *et al.*, 1985) is at variance to our value of only  $3.3 \mu\text{M}$ . This discrepancy may be explained by the different stimulation conditions applied, including that pulses with 2 ms duration may allow the generation of more action potentials, and the proposed development of use-dependence (MacIver *et al.*, 1996) than pulses of 1 ms. In the study of Boireau *et al.* (1998) the ouabain-induced  $[\text{H}^3]\text{-DA}$  release was half-maximally inhibited by  $0.9 \mu\text{M}$  riluzole which is less than the  $\text{IC}_{50}$  found in the present experiments ( $6.8 \mu\text{M}$ ).  $\text{IC}_{50}$  values between 1.6 and  $9.5 \mu\text{M}$  have been found by Keita *et al.* (1997) using different stimulating drugs to evoke  $[\text{H}^3]\text{-DA}$  release. Veratridine-induced catecholamine release from adrenal chromaffin cells was inhibited by riluzole with an  $\text{IC}_{50}$  of about  $5 \mu\text{M}$  in the study of Yokoo *et al.* (1998). Since in our hands riluzole was hardly effective on  $[\text{H}^3]\text{-NA}$  release we cannot provide an  $\text{IC}_{50}$  for comparison. As far as we know, *in vitro* studies investigating the effect of riluzole on 5-HT release do not exist. We found a rather low potency of riluzole ( $\text{IC}_{50}$  of  $39.8 \mu\text{M}$ ) with respect to this neurotransmitter.

Riluzole inhibits several ion channels that are assumed to be essential for the release of all neurotransmitter (i.e. Benoit & Escande, 1991; Hubert, 1994; Huang *et al.*, 1997; Zona *et al.*, 1998). As outlined above, the mechanism of riluzole to block presynaptic  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels, however, hardly explains the missing inhibition seen with the transmitter NA, and the weaker effects on 5-HT release we found. Two other studies (MacIver *et al.*, 1996; Martin *et al.*, 1993) also compared the effects of riluzole on different transmitter systems, this time on Glu and GABA, and concluded that Glu neurotransmission is preferentially diminished.

Concerning our results, we propose that inhibition of neurotransmitter release by riluzole may not occur through those ion channels which are nearly equal in most nerve terminals, but is rather mediated through receptors, which might differ between neurotransmitter systems. This hypothesis of receptor-mediated effects of riluzole is supported by earlier findings (Doble *et al.*, 1992; Huang *et al.*, 1997) demonstrating that G-proteins are involved in riluzole effects. However, the receptors influenced by riluzole to depress transmitter release are not known. A specific modulation of glutamatergic transmission rather exists postsynaptically, as shown by Keita *et al.* (1997) and Hubert *et al.* (1994), but not

presynaptically with respect to transmitter release.

We excluded that inhibition of  $[\text{H}^3]\text{-ACh}$  release by riluzole is mediated through blockade of NMDA heteroreceptors on axon terminals or through blockade of other ionotropic Glu receptors. This can be concluded from the finding that the effects of riluzole were maintained in the presence of MK 801 and NBQX, antagonists at NMDA and AMPA/kainate receptors, respectively. Thus, riluzole does obviously not affect ionotropic Glu receptors to inhibit  $[\text{H}^3]\text{-ACh}$  release. The release of this transmitter in mouse neocortex was also not modulated by the elevation of extracellular Glu through PDC. Taken together, the terminal release of  $[\text{H}^3]\text{-ACh}$  in mouse neocortex seems not affected by the neurotransmitter Glu which is also underlined by the lack of Glu to evoke  $[\text{H}^3]\text{-ACh}$  release from nerve terminals (Lupp *et al.*, 1992).

Riluzole is used in the treatment of ALS, a disease leading to selective degeneration of the first and second motoneuron (Hugon, 1996; Lacomblez *et al.*, 1996). Glu excitotoxicity is considered as a major pathogenetic factor in ALS (Leigh & Meldrum, 1996) which prompted us to focus on this neurotransmitter in the present study. The marked neuroprotective effects of riluzole in several animal studies of the last years, *in vivo* and *in vitro* (see Estevez *et al.*, 1995; Rothstein & Kuncl, 1995; Pratt *et al.*, 1992) seem at variance to the rather short prolongation of life-expectancy and the small improvement in motor function gained by ALS patients receiving riluzole. To test whether riluzole has only minor effects in humans and since, to our knowledge, riluzole has not been tested in human tissue *in vitro* before, we compared the effects of riluzole in several species, including humans. We found that riluzole also affects the release of  $[\text{H}^3]\text{-ACh}$  in human neocortex, but we did not find any relevant difference between the species mouse, rat and human. This indicates that riluzole does affect the cholinergic neurotransmission in patients, and probably also other neurotransmitter systems in man. Among the reasons, which could explain the weak effects of riluzole in ALS, is the fact that it may be given too late to protect motoneurons. In addition, its inhibition of  $[\text{H}^3]\text{-ACh}$  release in human tissue suggests that it may also inhibit the cholinergic neurotransmission at muscular endplates, thus possibly diminishing its beneficial effect on motor function.

In conclusion, the neuroprotective agent and major ALS medication, riluzole, is an inhibitor of electrically evoked Glu release. It acts differently on the release of several neurotransmitters, but not selectively on excitatory amino acids. The modulation of ACh release by riluzole is not mediated by glutamate receptors. In human tissue riluzole seems to have the same effects as in animal tissue.

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