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# Increased concentrations of both NMDA receptor co-agonists D-serine and glycine in global ischemia: a potential novel treatment target for perinatal asphyxia

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**Abstract** Worldwide, perinatal asphyxia is an important cause of morbidity and mortality among term-born children. Overactivation of the *N*-methyl-D-aspartate receptor (NMDAr) plays a central role in the pathogenesis of cerebral hypoxia–ischemia, but the role of both endogenous NMDAr co-agonists D-serine and glycine remains largely elusive. We investigated D-serine and glycine concentration changes in rat glioma cells, subjected to oxygen and glucose deprivation (OGD) and CSF from piglets exposed to hypoxia–ischemia by occlusion of both carotid arteries and hypoxia. We illustrated these findings with analyses of cerebrospinal fluid (CSF) from human newborns affected by perinatal asphyxia. Extracellular concentrations of glycine and D-serine were markedly increased in rat glioma cells exposed to OGD, presumably through increased synthesis from L-serine. Upon reperfusion glycine concentrations normalized and D-serine concentrations were significantly

lowered. The *in vivo* studies corroborated the finding of initially elevated and then normalizing concentrations of glycine and decreased D-serine concentrations upon reperfusion. These significant increases of both endogenous NMDAr co-agonists in combination with elevated glutamate concentrations, as induced by global cerebral ischemia, are bound to lead to massive NMDAr activation, excitotoxicity and neuronal damage. Influencing these NMDAr co-agonist concentrations provides an interesting treatment target for this common, devastating and currently poorly treatable condition.

**Keywords** D-Serine · Glutamate · NMDA receptor · Perinatal asphyxia · Ischemia

## Introduction

Perinatal asphyxia is the consequence of impaired blood supply between mother and fetus, leading to insufficient delivery of oxygen, glucose and other blood-borne fuels to fetal organs, including the brain. The short-term clinical consequences include persistent low Apgar scores, multi-organ failure and neurological sequelae. In the long-term, this might result in cerebral palsy, mental retardation, visual and acoustic impairment and epilepsy (Robertson et al. 1989; Shankaran et al. 1991). In spite of improvements in perinatal care, the incidence and outcome of perinatal asphyxia remain relatively unchanged. Of the yearly estimated 4 million neonatal deaths worldwide, approximately 1 million is caused by perinatal asphyxia (Lawn et al. 2005). Of infants with moderate encephalopathy, 10% die, and 30% of those who survive, have disabilities. Among infants with severe encephalopathy, 60% die, and many, if not all survivors are handicapped

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(Robertson et al. 1989; Shankaran et al. 1991). Treatment is currently aimed at supportive intensive care, recently combined with hypothermia (Selway 2010).

It has been firmly established that excessive excitation of the *N*-methyl-D-aspartate receptor (NMDAR) plays a central role in the pathogenesis of hypoxic-ischemic damage, especially in the developing brain (Sattler and Tymianski 2001). The NMDAR requires simultaneous binding by glutamate and a co-agonist for activation. It had long been assumed that glycine was the endogenous co-agonist. However, recent studies have shown that D-serine is the main endogenous co-agonist for most NMDAR subtypes in most brain areas (Mothet et al. 2000). Since NMDARs appear not to be saturated during physiological conditions (Bergeron et al. 1998), elevations in glycine or D-serine concentrations may lead to increased toxicity during pathological conditions, such as hypoxia–ischemia. NMDAR activation and D-serine concentrations are strongly developmentally regulated (Fuchs et al. 2006), implying a specific role in central nervous system (CNS) development. Alterations in D-serine concentrations and NMDAR activation during this intense period of embryonic and early postnatal CNS development, induced for instance by perinatal asphyxia, may therefore be particularly deleterious. The synthesizing (Wolosker et al. 1999) and metabolizing (Molla et al. 2006) enzymes of D-serine and glycine have been characterized (Fig. 1). By pharmacological manipulation, this might yield new potential candidates for pharmacological intervention after perinatal asphyxia.

Despite this exciting hypothesis, most research has focused on glutamate, and the contribution of D-serine and

glycine to the pathogenesis of perinatal asphyxia remains largely elusive. Therefore, we investigated D-serine and glycine concentration changes in different models for global ischemia.

### Cell cultures

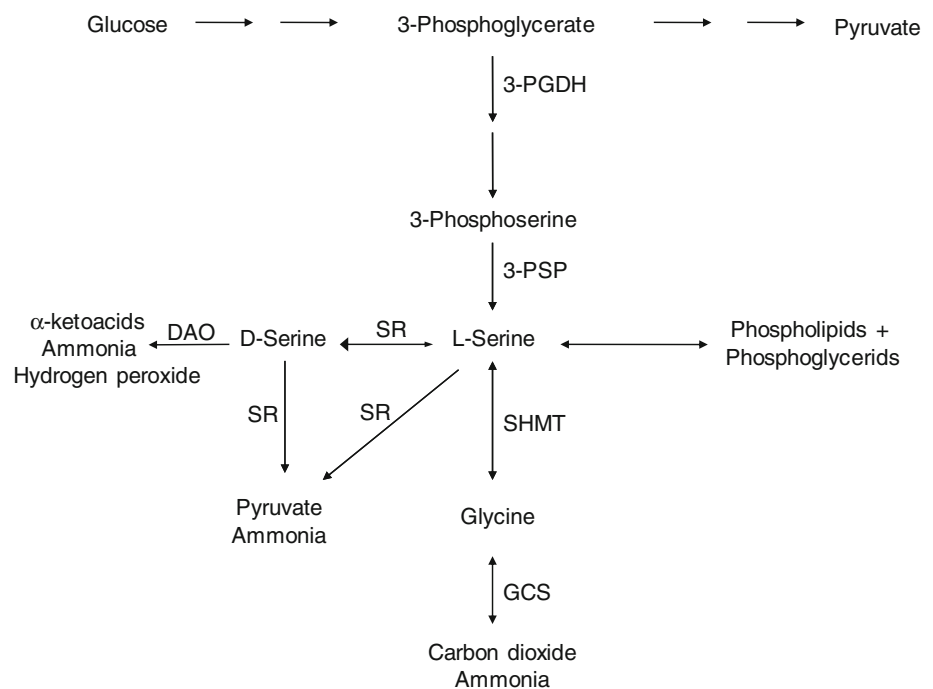
We subjected rat glioma cells to oxygen–glucose deprivation (OGD), a commonly used in vitro model for global ischemia, although the duration and depth of oxygen and glucose deprivation vary between studies (Jones et al. 2004). Although neurons are far more susceptible to ischemic brain damage than neighboring astrocytes, glioma cells were studied, because of emerging evidence that astrocytes contribute substantially to neuronal fate in cerebral hypoxia–ischemia (Rossi et al. 2007). By studying astrocytes in isolation, the direct effects of OGD on astrocytes could be visualized without the effects of stressed neuronal cells complicating interpretation.

### Piglets

Analysis of CSF from piglets exposed to hypoxia–ischemia by occlusion of both common carotid arteries, combined with hypoxia. Piglet brains correspond well with those of term-born neonates (Dobbing and Sands 1979), and this animal model is commonly used to study global ischemia (Fritz et al. 1999; Peeters-Scholte et al. 2002).

We illustrated these findings with cerebrospinal fluid (CSF) analyses from a small number of newborns with and without perinatal asphyxia. Despite being a global marker

**Fig. 1** Pathways involved in L-serine and D-serine biosynthesis, catabolism and exogenous sources. *3-PGDH* 3-phosphoglycerate dehydrogenase, *3-PSP* 3-phosphoserine phosphatase, *SHMT* serine hydroxymethyltransferase, *GCS* glycine cleavage system, *SR* serine racemase, *DAO* D-amino acid oxidase



for concentration differences in brain regions, CSF is the most accessible CNS marker in these critically ill newborns.

In all models, we found consistent time-dependent changes in D-serine and glycine concentrations upon hypoxia–ischemia and reperfusion. Together with the known increases in extracellular glutamate concentrations (Benveniste et al. 1984; Gucuyener et al. 1999; Puka-Sundvall et al. 1996; Ueda et al. 1992) after hypoxia–ischemia, the increased concentrations of both NMDAr co-agonists ought to lead to massive NMDAr activation, resulting in excitotoxicity and neuronal damage.

## Materials and methods

### Cell cultures

#### Materials

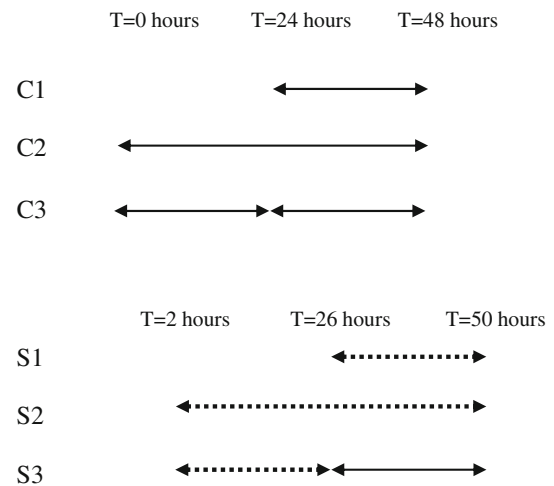
Rat glioma C6 cells were cultured in F-12 Kaighn's medium supplemented with 10% fetal calf serum and 100 µg/ml penicillin/streptomycin (all from Gibco Life Technologies, Invitrogen, Breda, The Netherlands). To create normoglycemic and hypoglycemic conditions, media identical in inorganic salt, amino acid and vitamin composition and only different in glucose content (RPMI 1640 BE-12 702F: 2,000 mg/l and BE-12 752F: 0 mg/l, Bio Whittaker, Breda, The Netherlands), supplemented with 10% fetal calf serum and 100 µg/ml penicillin/streptomycin (Gibco Life Technologies, Invitrogen, Breda, The Netherlands) were used. Hypoxia was applied by culturing samples in an in VIVO<sub>2</sub> Hypoxia workstation 1000 (Biotrace International, Mid Glamorgan, UK), equipped with a Ruskinn gas mixer module V<sub>2</sub> (Biotrace International, Mid Glamorgan, UK). Oxygen level was set at 1%, CO<sub>2</sub> at 5% and H<sub>2</sub> at 0%.

#### Experimental design

The experiment was started by washing 6 × 6 well plates (C2–C3 and S2–S3 started on day 1; C1 and S1 started 24 h (h) later, Fig. 2) of near confluent glioma C6 cells twice with phosphate buffered saline and applying the experimental conditions (6 different samples per condition). A sample of the external medium was collected at the end of each condition and stored at –20°C until analysis. This experiment (6 conditions;  $n = 6$  for each condition) was repeated on four different occasions.

#### Quantitative amino acid determination

D-Serine, L-serine and glycine concentrations were measured by stable isotope dilution gas and liquid



**Fig. 2** Experimental design of the Glioma C6 studies. *Straight line with arrows* indicate normoxia and normoglycemia (21% O<sub>2</sub>; RPMI 12-702 medium). *Dotted line with arrows* oxygen and glucose deprivation (1% O<sub>2</sub>; RPMI 12-752 medium) C1–C3 control 1–3; S1–S3 sample 1–3

chromatographic–mass spectrometric methods as described before (Fuchs et al. 2008).

#### Piglets

Seven newborn Dutch store piglets with a postnatal age range from 1 to 3 days were exposed to hypoxia–ischemia by occlusion of both common carotid arteries and reduction of the fraction of inspired oxygen for 1 h as described previously (Peeters-Scholte et al. 2002). CSF was withdrawn at 3 h ( $n = 2$ ) or 24 h ( $n = 5$ ) after the start of hypoxia–ischemia. CSF was also withdrawn in three control piglets, which underwent the same surgical procedure but were not exposed to hypoxia–ischemia. Experimental protocols were approved by the Animal Research Committee of the Utrecht University, The Netherlands.

#### Statistical analyses

Amino acid concentration differences between the external medium of glioma C6 cells and “fresh medium” (from the bottle, supplemented with fetal calf serum and penicillin/streptomycin) were compared between experimental and control conditions using the unpaired Student's *t* test. Similarly, amino acid concentrations in CSF from piglets 24 h after hypoxia–ischemia were compared with control CSF, using the unpaired Student's *t* test. The level of significance was set at  $p < 0.05$ . Since there was CSF of only two piglets 3 h after hypoxia–ischemia, these were not included in the statistical analyses but presented to show the trend of concentration changes.

## Results

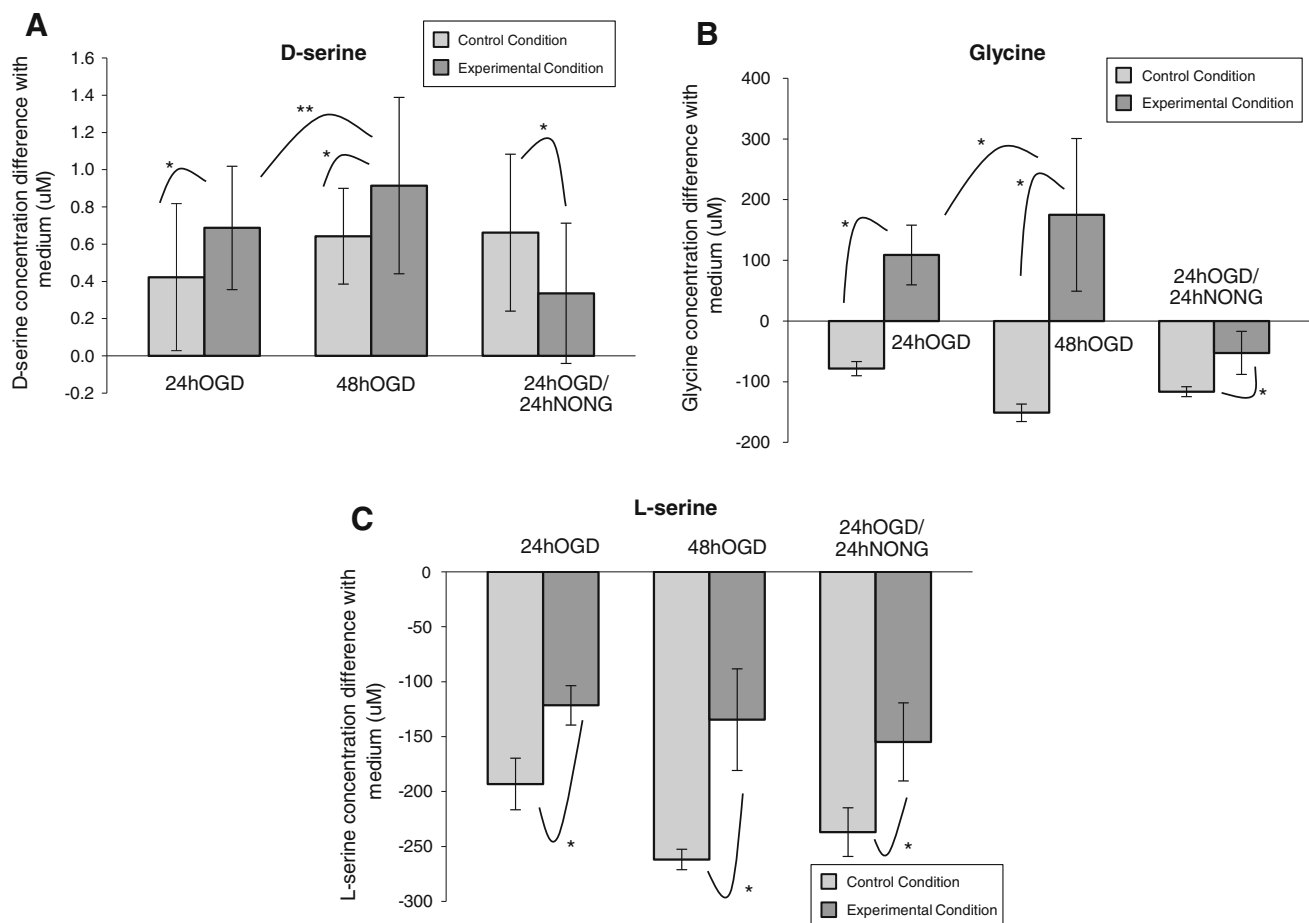
Rat glioma C6 cells were incubated according to our experimental protocol (see “Materials and methods”). In control conditions, D-serine concentrations in external medium increased after 24 h to  $+0.42 \mu\text{M}$  (Fig. 3) and after 48 h to  $+0.64 \mu\text{M}$  ( $p < 0.05$  when comparing 24 h with 48 h), potentially through the release from vesicular storage (Hur et al. 2010; Martineau et al. 2008; Mothet et al. 2005). Glycine and L-serine concentrations decreased during 24–48 h of normal conditions, suggesting uptake by glioma cells.

Application of OGD for 24 or 48 h resulted in increases in D-serine concentrations from  $+0.42 \mu\text{M}$  (control condition) to  $+0.69 \mu\text{M}$  at 24 h and from  $+0.64 \mu\text{M}$  (control condition) to  $+0.91 \mu\text{M}$  at 48 h ( $p < 0.05$  in both cases). After OGD, glycine concentrations increased from  $-78 \mu\text{M}$

(control condition) to  $+109 \mu\text{M}$  at 24 h and from  $-151 \mu\text{M}$  (control condition) to  $+175 \mu\text{M}$  at 48 h ( $p \ll 0.01$  in both cases;  $p < 0.05$  when comparing 24–48 h OGD).

Interestingly, when simulating reperfusion by applying normoxia/normoglycemia (NO/NG) after 24-h OGD, D-serine concentrations were significantly lowered when compared to control conditions [ $+0.66 \mu\text{M}$  (control condition) vs.  $+0.34 \mu\text{M}$  (24 h OGD + 24 h NO/NG),  $p < 0.01$ ]. Concentrations of glycine normalized, by decreasing to a lesser extent than during control conditions [ $-116 \mu\text{M}$  (control condition) vs.  $-52 \mu\text{M}$  (24 h OGD + 24 h NO/NG)].

Concentrations of L-serine, the common precursor of D-serine and glycine, and other amino acids (including aspartate, another excitatory amino acid, data not shown) decreased after OGD and in the reperfusion experiment, but lesser than during control conditions, suggesting that



**Fig. 3** Medium concentration differences of the NMDA<sub>R</sub> agonists D-serine, glycine and their common precursor L-serine after oxygen and glucose deprivation (OGD). The figures represent the mean  $\pm$  standard deviation of the concentration differences of D-serine (a), glycine (b) and L-serine (c) between fresh medium (directly taken from the bottle, supplemented with fetal calf serum and penicillin/

streptomycin, as was used in the experiments) and the external medium after 24 h OGD, 48 h OGD, and 24 h OGD followed by 24 h normoxia/normoglycemia (NO/NG). These graphs represent the mean of six different samples and six different controls during four different experiments. \*Significant change with a  $p$  value  $< 0.05$  and \*\* $0.05 < p < 0.1$

the rise in D-serine and glycine is caused by specific synthesis and not mere efflux due to, for instance, increased cell wall permeability.

In order to gain insight into the mechanism responsible for the changes in D-serine and glycine concentrations, a fifth condition was added (similar to S1 and C1, Fig. 2) by addition of 3 mg of the labeled stable isotope  $^{13}\text{C}_3^{15}\text{N}$  L-serine (Cambridge Isotope Laboratories, Inc., Andover, USA) to 100 ml medium (to a concentration of 285  $\mu\text{M}$ ), to which NO/NG, OGD, normoxia/hypoglycemia and hypoxia/normoglycemia was applied during 48 h, because the effects were most evident after 48 h (Fig. 3). By labeling L-serine, the common endogenous precursor of D-serine and glycine, we investigated D-serine and glycine synthesis from L-serine by quantifying the resulting labeled D-serine and glycine (Table 1). In this experiment, D-serine concentrations remained unaffected by 48 h OGD when compared to NO/NG (0.04  $\mu\text{M}$  and 0.03  $\mu\text{M}$ , respectively, non-significant), but these concentrations were very low, nearing the limit of detection of the analytical method. Glycine concentrations increased significantly and markedly after 48 h OGD (57.0  $\mu\text{M}$ ), when compared to control conditions (34.3  $\mu\text{M}$ ,  $p < 0.01$ ). Synthesis of labeled glycine after 48 h of control conditions is interesting when considering the decrease in total glycine concentrations in medium after 48 h (Fig. 3), implying simultaneous synthesis and use of glycine under normal conditions. Under NO/NG conditions, D-serine synthesis accounted for 0.02% and glycine synthesis for 18% of L-serine use. D-Serine synthesis from L-serine was not significantly increased by hypoxia, hypoglycemia or the combination ( $0.05 < p < 0.1$  for OGD), but again, concentrations of labeled D-serine were very low for quantification with our analytical method. Glycine synthesis from L-serine almost doubled to 28% ( $p < 0.01$ ) upon hypoxia, decreased to 4.6% ( $p < 0.01$ ) upon hypoglycemia and almost tripled to 51.5% ( $p < 0.01$ ) upon OGD.

## Piglets

Three hours after starting hypoxia–ischemia, piglets showed an almost eightfold increase in glycine concentrations in CSF when compared to control piglets (Fig. 4). Glycine concentrations remained markedly increased (fourfold  $p < 0.05$ ) in CSF 24 h after starting hypoxia–ischemia when compared to control piglets, but lesser than 3 h after hypoxia–ischemia (twofold reduction). D-Serine concentrations seemed to rise marginally 3 h after hypoxia–ischemia (but  $n = 2$ ), and were significantly reduced at 24 h when compared with control piglets (40% reduction,  $p < 0.05$ ). L-Serine concentrations appeared to increase in CSF 3 h after hypoxia–ischemia (50%, but again  $n = 2$ ) and were similar to control values 24 h after hypoxia–ischemia.

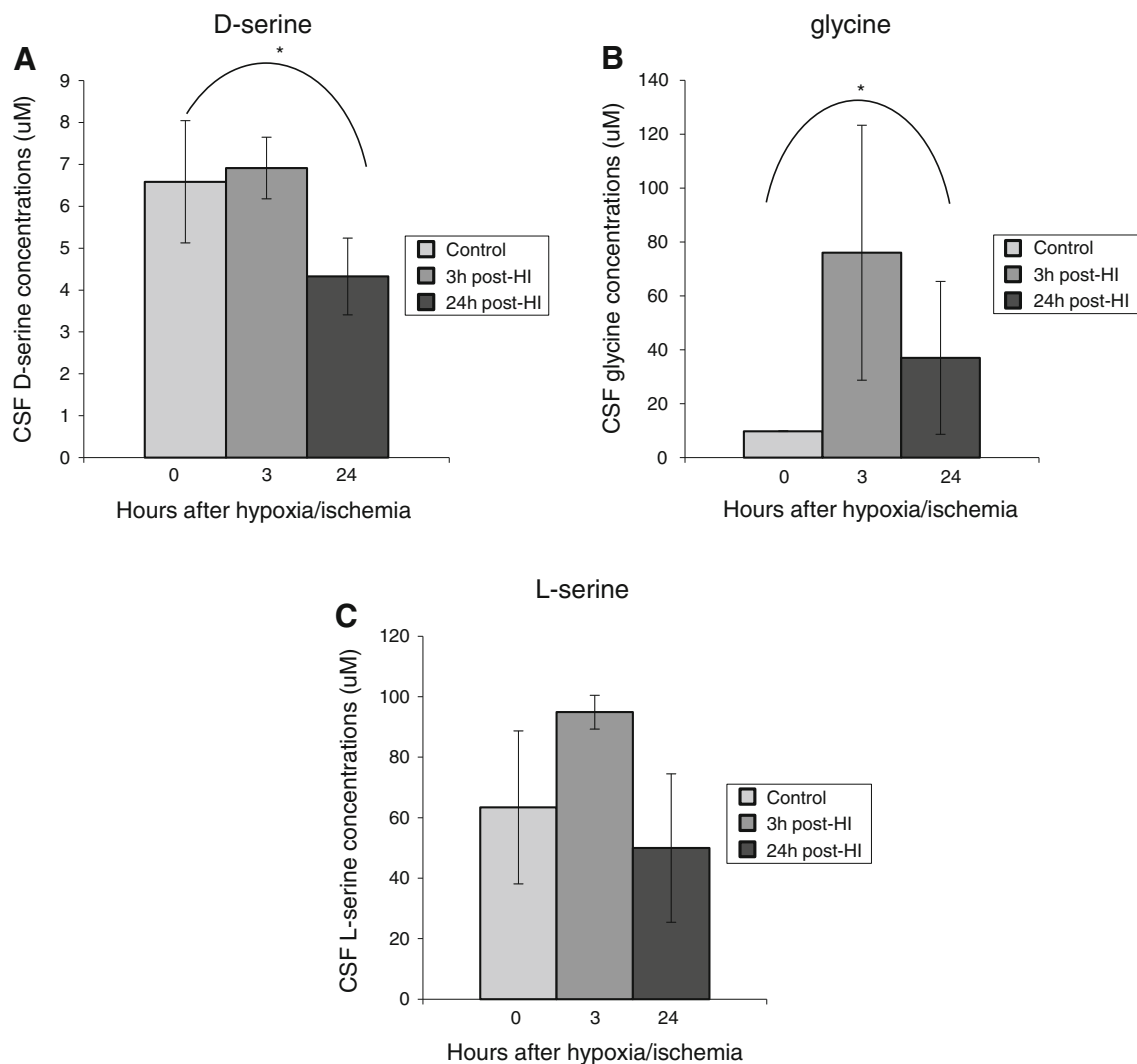
## Discussion

Since perinatal asphyxia is an important contributor to neonatal mortality and morbidity with poor curative treatment options, this study was aimed to elucidate mechanisms of NMDAR-associated excitotoxicity in hypoxia–ischemia in an attempt to yield novel treatment strategies. The necessity for NMDARs to be activated not only by glutamate, but also by glycine or D-serine may be regarded as a safety mechanism to prevent excitotoxicity. Our results imply that this safety mechanism fails in global cerebral ischemia, since we found that concentrations of both glycine and D-serine significantly increased during hypoxic–ischemic conditions. Furthermore, our studies provide further evidence that glia are relevant signaling partners and not merely supporting cells for neurons, as was long believed (Billard 2008; Halassa et al. 2007; Oliet and Mothet 2009; Panatier et al. 2006). In fact, glia do not only protect neurones by glutamate uptake, but our results show

**Table 1** Changes in D-serine and glycine synthesis from L-serine after hypoxia, hypoglycemia and OGD

	$^{13}\text{C}_3$ L-serine use ( $\mu\text{M}$ )	$^{13}\text{C}_3$ D-serine synthesis ( $\mu\text{M}$ )	$^{13}\text{C}_2$ glycine synthesis ( $\mu\text{M}$ )	$^{13}\text{C}_3$ D-serine synthesis/ $^{13}\text{C}_3$ L-serine use (%)	$^{13}\text{C}_2$ glycine synthesis/ $^{13}\text{C}_3$ L-serine use (%)
NO/NG	191.3 (SD 3.8)	0.03 (SD 0.04)	34.3 (SD 1.8)	0.02 (SD 0.02)	18.0 (SD 1.0)
HO	162.8 (SD 6.7)	0.12 (SD 0.07)	45.8 (SD 4.4)	0.08 (SD 0.05, $p = 0.05$ )	28.3 (SD 4.0, $p < 0.01$ )
HG	231.7 (SD 4.6)	0.12 (SD 0.07)	10.7 (SD 1.3)	0.05 (SD 0.03, $p = 0.09$ )	4.6 (SD 0.6, $p < 0.01$ )
OGD	111.7 (SD 10.8)	0.04 (SD 0.06)	57.0 (SD 3.6)	0.04 (SD 0.06, $p = 0.51$ )	51.5 (SD 7.4, $p < 0.01$ )

D-Serine and glycine synthesis from L-serine was studied by analysis of resulting  $^{13}\text{C}_3$  D-serine and  $^{13}\text{C}_2$  glycine concentrations in the medium upon addition of  $^{13}\text{C}_3$  L-serine. The table depicts medium concentration changes in  $^{13}\text{C}_3$  L-serine (added to a medium concentration of 285  $\mu\text{M}$ ),  $^{13}\text{C}_3$  D-serine and  $^{13}\text{C}_2$  glycine after rat glioma C6 cells were exposed to 48 h of NO/NG, hypoxia (HO), hypoglycemia (HG) or OGD ( $n = 6$  for each condition). The last two columns represent the percentage of  $^{13}\text{C}_3$  D-serine and  $^{13}\text{C}_2$  glycine synthesis, respectively, of total  $^{13}\text{C}_3$  L-serine use and the  $p$  value (Student's  $t$  test) after comparison with the NO/NG condition ( $N = 6$  for all conditions)



**Fig. 4** D-Serine, glycine and L-serine concentrations in CSF from control piglets and 3 and 24 h after application of cerebral hypoxia–ischemia CSF concentrations of D-serine (a), glycine (b), and L-serine (c) as determined by GC–MS in control piglets ( $n = 3$ ), 3 h after start

of hypoxia–ischemia (HI) ( $n = 2$ ) and 24 h after start of hypoxia–ischemia ( $n = 5$ ). Changes between control piglets and 24 h after start of hypoxia–ischemia were statistically evaluated and \*significant change with  $p < 0.05$

that glia also contribute to neurotoxicity by increasing extracellular D-serine and glycine concentrations.

Both D-serine and glycine concentrations appear to rise through increased synthesis from L-serine. A central role for D-serine synthesis from L-serine by SR during OGD is evidenced by SR knock-out mice, displaying 90% reduced D-serine concentrations and decreased neurotoxicity and dramatically diminished infarct volume after middle cerebral artery occlusion (Mustafa et al. 2010). For glycine, we showed in our isotope studies that glycine synthesis from L-serine almost tripled from 18% under control conditions to 51.5% after OGD. Since use of labeled L-serine decreased after OGD, this might represent a specific shift of L-serine use towards glycine synthesis. However, the concentration differences might also in part be explained by ischemia-induced changes in re-uptake pumps, as have

been described for glutamate (Huang et al. 1993). This was not specifically assessed in our study and would be interesting to investigate in future experiments.

The relative contribution of D-serine and glycine to NMDAR-induced neuronal death following ischemia remains unclear. Addition of D-amino acid oxidase (DAO), the enzyme that degrades D-serine and not glycine, led to diminished cell death upon application of NMDA or OGD (Katsuki et al. 2004). Similarly, NMDA-elicited neurotoxicity in rat hippocampal slices was virtually abolished by complete removal of D-serine (Shleper et al. 2005). The effects of endogenous glycine could be observed only after simultaneous removal of endogenous D-serine and blockage of the glycine transporter GlyT1. Thus, although levels of glycine were tenfold higher than D-serine in this study, endogenous D-serine might be the dominant co-agonist for

**Table 2** Patient characteristics, amino acid concentrations and their ratios in CSF from neonates with and without perinatal asphyxia

Term (weeks + days)	Weight (g)	Gender	Age (days)	Clinical picture	D-Serine ( $\mu\text{M}$ )	L-Serine ( $\mu\text{M}$ )	Glycine ( $\mu\text{M}$ )	D-Serine/total serine (%)	Glycine/total L-serine (%)	D-Serine/ glycine
<b>Patients</b>										
1 39 + 6	3,875	F	1	Perinatal asphyxia (AS 3/6/6, pH 7.21, BE -15.4), convulsions, death (upon withholding supportive treatment because of poor prognosis). No signs of infection in blood, urine or CSF	7.13	34.37	14.66	17.18	29.90	0.49
2 38 + 5	2,840	M	2	Perinatal asphyxia (AS 7/8, pH 6.95, BE -16), no convulsions, no microorganism in blood, urine or CSF, CRP max 139 mg/l. No adequate psychomotor development, some attention problems	11.61	34.91	14.95	24.96	29.98	0.78
<b>Controls</b>										
1 42 + 2	4,800	F	1	AS 9/9, pH 7.20, BE -6. Suspected perinatal infection (temperature 38.1°C), no signs of infection in blood, urine or CSF, CRP max 23 mg/l	12.2	48.10	8.53	20.23	15.06	1.43
2 41 + 5	3,725	M	2	AS 9/10, pH 7.39, BE 2. Suspected perinatal infection (temperature > 38.0°C), no signs of infection in urine or CSF. Blood culture yielded <i>Corynebacterium striatum</i> (probably contamination), CRP max 35 mg/l	11.90	47.94	10.32	19.89	17.71	1.15

Characteristics of neonates with and without perinatal asphyxia and their CSF amino acid concentrations determined by LC-MS and their ratios. Use of the samples for our study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands. *F*: female, *M*: male. Age at the moment of the lumbar puncture, performed to rule out meningitis. This is routinely performed in newborns with fever, because symptoms are nonspecific at this age. CSF Samples were kept at 4°C for 1 week, before being stored at -80°C until analysis. AS Apgar score, pH, BE base excess represent the acidity of cord blood. CRP C reactive protein, a commonly clinically used infection parameter



NMDA-elicited neurotoxicity (Shleper et al. 2005). This important role of D-serine in neurotoxicity following ischemia is confirmed by the dramatically diminished neurotoxicity and infarct volume in SR knock-out mice (Mustafa et al. 2010). Moreover, simultaneous elevation of both NMDAr co-agonists as seen in our experiments, in combination with the known increases in extracellular glutamate concentrations (Benveniste et al. 1984; Gucuyener et al. 1999; Puka-Sundvall et al. 1996; Ueda et al. 1992) must lead to NMDAr-associated excitotoxicity.

During the reperfusion phase, we observed increased but normalizing glycine concentrations *in vitro* and *in vivo*. This concurs with the few reports describing increased glycine concentrations in mammalian CSF or microdialysates after global ischemia (Baker et al. 1991; Phillis and Walter 1989; Roldan et al. 1999). Interestingly, we observed significantly reduced D-serine concentrations during the reperfusion phase, both *in vitro* and *in vivo*. This concurs with the finding of delayed transient decreases of SR (mRNA and protein) and D-serine in the ipsilateral temporoparietal mouse cortex after permanent focal ischemia (Wang and Zhu 2004). In accordance with the model of a safety mechanism, this reduction in D-serine concentrations during reperfusion might represent an attempt to compensate for increased NMDAr activity. Potentially, the elevated glycine concentrations contribute to this phenomenon, since glycine inhibits SR activity at these concentrations (Strisovsky et al. 2005).

This pattern of concentration changes induced by hypoxia–ischemia and reperfusion was consistent with the analyses performed in CSF withdrawn from human newborns with and without perinatal asphyxia 24–48 h after birth, thus representing the reperfusion phase (Table 2). Asphyxiated neonates displayed higher glycine concentrations and percentage glycine of the sum of L-serine and glycine (an indication for the amount of glycine synthesis from L-serine) and in the most serious case of asphyxia also lower D-serine concentrations and percentage D-serine of the sum of D- and L-serine (an indication for the quantity of D-serine synthesis from L-serine) than controls. The finding of alterations in the CSF, which represents just a global marker for changes in the CNS, emphasizes the magnitude of concentration changes of the NMDAr co-agonists in specific brain areas.

In conclusion, our results encourage the development of effective and safe drugs preventing NMDAr overactivation after global ischemia. Therapeutic agents for hypoxia–ischemia, including competitive and non-competitive NMDAr inhibitors, as well as NR1 antagonists have been largely disappointing (Madden 2002; Warach et al. 2006), partly due to significant side effects (Groenendaal et al. 2002; Levene et al. 1995). New strategies, such as manipulating co-agonist concentrations by influencing enzyme

kinetics might form a more subtle alternative. The protective effect of hypothermia, a currently promising method to decrease neuronal damage in asphyxiated infants with severe encephalopathy, might at least in part rely on reducing glutamate (Baker et al. 1991; Illievich et al. 1994) and glycine (Baker et al. 1991; Simpson et al. 1991) concentrations, as observed in rabbit hippocampus microdialysates and in superfusates from rat cerebral cortical slices. Although D-serine concentrations have not yet been investigated in hypothermia, these results show that the reduction of the concentrations of the NMDAr (co)-agonists might be beneficial in global ischemia. Targeting the glycine and particularly the D-serine synthetic pathway may provide an attractive novel treatment option for asphyxiated neonates.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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