ORIGINAL ARTICLE

Differential expression of glycine receptor subunit messenger RNA in the rat following spinal cord injury

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Study design: Spinal cord injury (SCI) results in alterations in the regulation of many genes that may influence neuronal death and the subsequent loss of motor function and neuropathic pain. The subtype expression mRNA levels of glycine receptors (GlyRs) after SCI are unknown.

Methods: Using real-time reverse transcription PCR, this study analyzed changes in the mRNA abundance of the four major GlyR subunits ($\alpha 1$ and β) at 6, 24 h and 3, 7 and 10 days after SCI in adult male rats. SCI was induced at the T9 level by transection.

Results: Our results show a complicated temporal and spatial pattern of alteration in GlyR mRNA expression levels after SCI. Temporal and spatial variations with different degrees and direction (up or downregulation) of expression alteration were observed. The greatest variation was seen in GlyR $\alpha 1$, whereas GlyR $\alpha 2$ was downregulated in all regions following SCI.

Conclusion: This study shows that alteration in GlyR expression starts as early as 6 h after SCI. The most significant points of this research are temporal elevation of GlyR $\alpha 1$ and continuous reduction of GlyR $\alpha 2$. Alterations in GlyR expression within the spinal cord may have a key role in the development of pathological pain. Therefore, control of GlyR expression could represent a novel therapeutic avenue for the development of new painkiller agents in SCI.

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Introduction

Glycine receptors (GlyRs) are pentameric proteins belonging to the Cys-loop family of ligand-gated ion channels.¹ In the rat, GlyRs are constructed from a family of four different subunits, $\alpha 1$ – $\alpha 3$ and β subunits,¹ assembled either as homomeric receptors containing a single α -subunit or as heteromeric receptors that contain α - and β -subunits. GlyRs are considered to consist mainly of $\alpha 1\beta$ heteromers in the mature spinal cord and brainstem, whereas in the fetal/neonatal nervous system, homomeric $\alpha 2$ subunit GlyRs are suggested to predominate.²

Thousands of genes are essential for the proper functioning of the central nervous system. Spinal cord injury (SCI) results in an alteration in the expression of many genes, with major functional consequences such as paraplegia, which results from an interruption of the flow of information passing through the long spinal tracts.^{3–6} Analysis of alterations in gene expression is essential to identify the precise molecular pathways altered as a function of time and to develop a practical therapy for SCI, especially within the

first few days after SCI. This could involve the development of strategies for favorable manipulation of beneficial pathways and inhibition of toxic pathways. Earlier studies identified several SCI-induced gene and protein expression alterations.

GlyRs may assume different roles during the course of the physiological response to SCI. Recent discoveries revealed the involvement of GlyRs in the development of spinal pain sensitization.⁷ Although the GlyR $\alpha 3$ subunit has recently emerged as a key factor in inflammatory pain pathways in the spinal cord dorsal horn,⁸ the full extent of location and cell types expressing different GlyR subunits is not yet known. Therefore, a more quantitative approach is necessary to reveal the roles of GlyRs and the changes in their expression profiles in SCI repair. Quantitative approaches make it possible to compare the activity of GlyRs at different times following injury.

In this study, we used a quantitative real-time PCR technique to analyze the changes in GlyRs mRNA expression in the rat spinal cord following experimentally induced injury to the spinal cord. We analyzed the changes in GlyRs mRNA abundance at 6 h and 1, 3, 7 and 10 days after traumatic injury to rat spinal cord. Our results demonstrate that SCI causes a significant change in the expression of these genes.

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Materials and methods

Animals

A total of 54 1-year-old adult male Wistar rats weighing 180–240 g at the beginning of the experiment were used (45 as SCI models and 9 as control). They were housed in groups (1–6) under a 12:12-h light/dark cycle with *ad libitum* access to standard rat chow and water. All experiments were approved by the Animal Ethics Committee for the University of Isfahan.

SCI

Animals were anesthetized with halothane (induction 4%, maintenance 2%, in an oxygen and nitrous oxide 50:50 mixture) and a T9 laminectomy was performed exposing a circle of dura and the spinal cord was injured by bisection. The incisions were closed and rats were returned to their cages after recovering from anesthesia. After SCI, rats were housed in groups of three in a cage to reduce stress from isolation and kept at 22–25 °C on highly absorbent bedding. Their bladders were manually expressed twice daily until a reflex bladder was established, usually at 10 days after injury.

Tissue preparation

At 0 (control group), 6 h, 24 h, 3, 7 and 10 days after surgery, whole spinal cords were rapidly removed from the anesthetized rat ($n=9$ for each time point) and placed into ice-cold saline. The dura was removed and around 2.5 cm piece of cord centered at the T9 injury site was cut into three segments: 8 mm tissue centered on the injury epicenter, 8 mm tissue rostral and 8 mm tissue caudal to the epicenter. Control samples were prepared in a similarly from the equivalent regions of uninjured rats. The tissue segments were stored at -70°C .

RNA extraction and RT

Total cellular RNA was isolated from frozen tissues using the RNX plus solution (CinnaGen, Iran) according to the manufacturer's instructions. The purity and integrity of the extracted RNA was evaluated by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. Complementary DNA synthesis reactions were performed using 1 μg DNase (CinnaGen, Iran)-treated total RNA from each sample and AccuPower RT Premix (Bioneer, Republic Korea) with random hexamer priming in a 20 μl reaction.

Quantitative real-time PCR

Real-time PCR was performed in the Chromo4 Detection System (Bio-Rad, Hercules, CA, USA). Briefly, 25 ng of cDNA and gene-specific primers were added to SYBR Green PCR Master Mix (IQ SYBR Green I Dye, Bio-Rad) and subjected to PCR amplification (cycle 1 at 95 °C for 5 min, and 40 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s). All PCRs were run in duplicate. The amplified transcripts were quantified using the comparative C_T method (<http://www.pebiiodocs.com/pebiiodocs/04303859.pdf>).

The primers used for real-time PCR were

$\alpha 1$: 5'-GCACCAAGCACTACAACAC-3'/5'-AGGACAGGATGACGATAAGC-3';

$\alpha 2$: 5'-GAGACAGCAGTGGAACGATTC-3'/5'-TCCGCAGCAACTTGTATCAG-3';

$\alpha 3$: 5'-GCCTTCTGATTGTCTTCTGTC-3'/5'-CTCTGCGTGTGTCATCGTAAG-3';

β : 5'-AAGGCACTGGTTACTACAC-3'/5'-CTGAGCACGGAGAAGATG-3'.

Expression levels were normalized to that of the constitutive 18S ribosomal RNA with primers:

18S: 5'-GTAACCCGTTGAACCCCATTC-3'/5'-CCATCCAATCGGTAGTAGCG-3'.

Relative expression data were quantified using $2^{-\Delta\Delta C_T}$ method, where C_T is the cycle threshold. All target genes were normalized to the 18S housekeeping gene.

Statistical analysis

To determine significance, all data were subjected to statistical analysis using a computerized statistics program (GraphPad Prism). One-way ANOVA was used, as indicated in the figure legends, followed by a *post hoc* test (Tukey) of differences between specific time points. A level of $P<0.05$ was considered significant.

Results

Total RNA quality

RNA isolated from both control and injured rats had A260/A280 ratios that ranged from 1.70 to 2.05. Agarose gel electrophoresis showed three bands at 28S, 18S and 5S in each sample, indicating an absence of degradation during preparation (Figure 1).

Temporal and spatial alterations in GlyR $\alpha 1$ expression

GlyR genes showed significant alterations in mRNA expression after SCI. Figure 2 shows the temporal changes in mRNA expression of GlyR $\alpha 1$, GlyR $\alpha 2$, GlyR $\alpha 3$ and GlyR β in the rostral, epicenter and caudal samples after SCI. In each case, data were expressed as a ratio to that expressed in the same region of control spinal cord.

One-way ANOVA indicated significant effects of both time and location after SCI and of the interaction between these factors. In tissue rostral to the injury site, GlyR $\alpha 1$ mRNA was increased by 35.5 ± 12.6 -, 6.1 ± 3.1 -, 12.9 ± 4.2 - and 5.7 ± 2.4 -fold at 6 h, 1, 3 and 7 days, respectively, then returned to near control level by 10 days (2.0 ± 1.1). Only alteration at 6 h after injury was significant ($P<0.001$) (Figure 2 panel a). In the epicenter tissue, GlyR $\alpha 1$ mRNA began to increase 6 h (5.9 ± 1.0 -fold) after SCI, and then continued to increase to 9.1 ± 2.5 -fold at 24 h, 19.9 ± 3.9 -fold at 3 days and reduced to 9.1 ± 2.8 -fold at 7 days after injury, and thereafter returned to near control level by 10 days (2.7 ± 0.5). Enhancements at days 1, 3 and 7 were significant ($P<0.001$) (Figure 2 panel b). The effect of SCI on GlyR $\alpha 1$ expression was less in caudal tissue with no more than a 12.7 ± 1.2 -fold increase in the 7 days after injury (Figure 2 panel c). Thus, the pattern

Figure 1 Agarose gel electrophoresis of RNA. Three bands at 28S, 18S and 5S in each sample were observed.

of GlyR α 1 mRNA increase resembled a wave traveling rostrally-caudally.

Temporal and spatial alterations in GlyR α 2 expression

A very different pattern was seen for the expression of GlyR α 2 after SCI (Figure 2 panels d–f). GlyR α 2 mRNA was significantly ($P < 0.001$) decreased by 94% at 6 h in rostral tissue, and decreased by 95% in epicenter tissue. In caudal tissue, it was decreased by 99%. GlyR α 2 decreased at 6 h after SCI and no recovery was observed until day 10.

Temporal and spatial alterations in GlyR α 3 expression

In contrast, GlyR α 3 mRNA (Figure 2 panels g–i) was higher than normal in all regions at 3 days, but showed a complex pattern of changes at earlier and later time points. It was rapidly and profoundly decreased at the epicenter at 6 and 24 h after SCI and then increased to above normal levels by 3 days. In the rostral tissue, there was a profound decrease at day 1, followed by an increase at days 3 and 7. The greatest alterations were seen in the tissue caudal to the epicenter, in which GlyR α 3 mRNA increased to more than two times the normal level at 6 and 24 h and 3 days after SCI. At 7 and 10 days, epicenter and caudal to the epicenter tissue regions had mRNA about the normal levels (Figure 2 panels h and i).

At day 10 in the rostral region, the mRNA expression level of GlyR α 3 was significantly ($P < 0.05$) less than the normal level. Thus, the pattern of GlyR α 3 mRNA increase resembled a wave traveling caudally–rostrally, opposite in direction to that of GlyR α 1 mRNA.

Temporal and spatial alterations in GlyR β expression

GlyR β mRNA levels (Figure 2l) remained near normal in tissue caudal to the epicenter at 6 h, 1 and 3 days after surgery. However, significant reductions ($P < 0.001$) were observed at days 7 and 10. In the rostral tissue (Figure 2j), there was a significant increase, by 3.6 ± 0.5 -fold at 6 h and 24 h after SCI, which was largely increased by day 7 (6.1 ± 0.3 -fold). The epicenter tissue exhibited increases of 1.5 ± 0.2 -fold at 6 h and 3.6 ± 0.8 -fold at 24 h (Figure 2k). On day 3, it returned to 1.6 ± 0.1 -fold of normal levels. At days 7 and 10, mRNA levels again increased to 2.2 ± 0.3 - and 3.4 ± 0.7 -fold, respectively.

SCI had little effect on GlyR β expression in the caudal tissue at 6 h, 24 h or day 1, but by 7 and 10 days it showed a strong decrease in GlyR mRNA compared with control.

Discussion

We analyzed the expression of mRNA for the four GlyR subunits after SCI. GlyRs mediate most of the inhibitory neurotransmission in the mammalian spinal cord and participate in plastic changes in the efficacy of synaptic transmission, as well as excitotoxic neuronal cell death that occurs in a variety of acute and chronic neurological disorders.^{9,10}

To our knowledge, this is the first time that a complex temporal and spatial pattern of altered mRNA expression levels after SCI for GlyR genes has been shown. Real-time reverse transcription PCR allowed us to obtain quantitative data at different times over the first 10 days after injury and in different regions with respect to the injury epicenter. Each of the GlyR subunits appeared unique in its temporal–spatial pattern of alterations after SCI.

Song *et al.* (2001) reported a reduction in expression levels of vesicular GABA transporter, γ -aminobutyric acid_A receptor subunits ($\alpha 5$ and $\gamma 2$) and GlyR following SCI.¹¹ Vesicular GABA transporter maintains constant levels of inhibitory neurotransmitters γ -aminobutyric acid and glycine in the synaptic vesicles.¹² Therefore, this downregulation may adversely influence the inhibitory synaptic transmission. However, possible changes in expression profiles of most ionic receptors after SCI have not been explored completely yet. This study showed an alteration in expression of GlyR transcripts after SCI, indicating an interruption of neuronal transmission.

In this study, the greatest alterations in mRNA expression after SCI were seen in GlyR α 1 (Figures 2a–c), which was upregulated in all regions and at most time points examined during the first week after SCI. Expression increased more in tissue from the rostral, where it was 35-fold the normal level at 6 h after injury and remained 10-fold the normal level at 1, 3 and 7 days. The increase was less dramatic in the tissue

Figure 2 (a–l), Temporal and spatial changes in GlyRs mRNA expression in rostral, epicenter and caudal tissue after spinal cord injury as compared with the values in control. Circles represent the mean of mRNA expression at each time point in rostral tissue; squares represent the epicenter; and triangles show values for caudal tissue. Compared with control levels, alteration in GlyRs mRNA was observed after injury. Error bars show mean $\pm$ s.d. For each experiment, $n = 4$ for each subunit, * $P < 0.05$, ** $P < 0.01$.

epicenter and caudal to the epicenter, where it peaked at 20- and 12-fold, respectively. GlyR α 2 was downregulated at all time points and regions. Variations in the alteration of GlyR α 3 and GlyR β genes were observed.

Gene expression alteration is different in the acute and chronic phase of SCI. Previous studies showed that the glycine level of the spinal cord hit the highest point, minutes after SCI in rats.^{13–15} Elevated glycine levels could mediate acute spinal shock after SCI.¹⁶ This may be because of an

increase in glycinergic neuronal activity after SCI. Our results also showed a significant elevation in GlyR α 1 expression 6 h after SCI. In the chronic phase after SCI, various spinal reflexes become active. In this phase, the glycine level in the lumbosacral cord showed a reversible decrease at 2–8 weeks in rats with SCI.¹⁷ These findings propose that in the chronic phase of SCI, a decrease in glycinergic neuronal activity in the spinal cord occurred. Reduction in GlyR α 1 at day 10 (chronic phase) in our study is in agreement with this

finding. On the other hand, the injection of increasing amounts of $\alpha 1$ -subunit cDNA into *Xenopus* oocytes results in a progressive increase in both maximum current (I_{max}) and glycine sensitivity.^{18,19} This may explain why GlyR $\alpha 1$ overexpressed a few days after SCI.

The overall increase in GlyR $\alpha 1$ mRNA levels 7 days after SCI was consistent with known loss of oligodendrocytes at and near the injury epicenter²⁰ and with decreased mRNA detected at 24 h after SCI in a gene array study²¹ using 1 cm of spinal cord tissue centered on the injury epicenter. Spatial GlyR $\alpha 1$ alteration may also define a special role for this glycine receptor subunit after SCI. All together, these data show that GlyR $\alpha 1$ may have a physiological and pathophysiological role in the spinal cord.

GlyRs may be localized to presynaptic terminals.²² They are also likely to be expressed by interneurons and could be expressed on glial cells.²³ Recent results showed high proliferation of oligodendrocyte precursor cells in tissue rostral and caudal to the epicenter.^{24,25} Therefore, alteration in GlyRs could be due to damage to each of these sources of GlyR in the spinal cord.

In summary, our results show a multifaceted temporal and spatial pattern of altered GlyRs mRNA expression after SCI. A number of these show a surprising spatial variation with different degrees and even direction (upregulation or down-regulation) of alterations in tissue at different locations with respect to the epicenter. Using the reliable and extremely sensitive technique of quantitative real-time PCR for regional tissue analyses will increase our understanding of the complex changes in gene expression that occur after SCI and how these may be manipulated to increase recovery after traumatic injury. Furthermore, classifying the subtype of GlyRs expressed after SCI in spinal cord will facilitate future research into the role of these receptors using an increasingly available subtype-specific pharmacology.

Conflict of interest

The authors declare no conflict of interest.

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References

- Lynch JW. Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev* 2004; **84**: 1051–1095.
- Betz H, Langosch D, Hoch W, Prior P, Pribilla I, Kuhse J *et al*. Structure and expression of inhibitory glycine receptors. *Adv Exp Med Biol* 1991; **287**: 421–429.
- Osterholm JL. The pathophysiological response to spinal cord injury. The current status of related research. *J Neurosurg* 1974; **40**: 5–33.
- Senter HJ, Venes JL. Altered blood flow and secondary injury in experimental spinal cord trauma. *J Neurosurg* 1978; **49**: 569–578.
- Wrathall JR, Teng YD, Choiniere D, Mundt DJ. Evidence that local non-NMDA receptors contribute to functional deficits in contusive spinal cord injury. *Brain Res Rev* 1992; **586**: 140–143.
- Young W. Secondary injury mechanisms in acute spinal cord injury. *J Emerg Med* 1993; **11**: 13–22.
- Zeilhofer HU. The glycinergic control of spinal pain processing. *Cell Mol Life Sci* 2005; **62**: 2027–2035.
- Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H *et al*. GlyR 3: An essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* 2004; **304**: 884–887.
- Mayer ML, Westbrook GL. The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog Neurobiol* 1987; **28**: 197–276.
- Dingledine R, Boland LM, Chamberlin NL, Kawasaki K, Kleckner NW, Traynelis SF *et al*. Amino acid receptors and uptake systems in the mammalian central nervous system. *Crit Rev Neurobiol* 1988; **4**: 1–96.
- Song G, Cechvala C, Resnick DK, Dempsey RJ, Raghavendra Rao VL. GeneChip analysis after acute spinal cord injury in rat. *J Neurochem* 2001; **79**: 804–815.
- Chaudhry FA, Reimer RJ, Belloccchio EE, Danbolt NC, Osen KK, Edwards RH *et al*. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci* 1998; **18**: 9733–9750.
- Smith CP, Somogyi GT, Bird ET, Chancellor MB, Boone TB. Neurogenic bladder model for spinal cord injury: spinal cord microdialysis and chronic urodynamics. *Brain Res Brain Res Protoc* 2002; **9**: 57–64.
- Sugaya K, Ogawa Y, Hatano T, Nishijima S, Nishizawa O. Micturition in thoracic spinal cord injured cats with autografting of the adrenal medulla to the sacral spinal cord. *J Urol* 2001; **166**: 2525–2529.
- Nishijima S, Sugaya K, Miyazato M, Shimabukuro S, Morozumi M, Ogawa Y. Activation of the rostral pontine reticular formation increases the spinal glycine level and inhibits bladder contraction in rats. *J Urol* 2005; **173**: 1812–1816.
- Simpson Jr RK, Robertson CS, Goodman JC. Glycine: an important potential component of spinal shock. *Neurochem Res* 1993; **18**: 887–892.
- Nishijima S, Sugaya K, Morozumi M, Hatano T, Ogawa Y. Changes of amino acid levels in the central nervous system and serum after spinal cord injury. *Neurology and Neurodynamics* 2001; **20**: 1–12.
- De Saint Jan D, David-Watine B, Korn H, Bregestovski P. Activation of human $\alpha 1$ and $\alpha 2$ homomeric glycine receptors by taurine and GABA. *J Physiol* 2001; **535** (Pt 3): 741–755.
- Taleb O, Betz H. Expression of the human glycine receptor $\alpha 1$ subunit in *Xenopus* oocytes: apparent affinities of agonists increase at high receptor density. *EMBO J* 1994; **13**: 1318–1324.
- Grossman SD, Rosenberg LJ, Wrathall JR. Temporal-spatial pattern of acute neuronal and glial loss after spinal cord contusion. *Exp Neurol* 2001; **168**: 273–282.
- Di Giovanni S, Knoblich SM, Brandoli C, Aden SA, Hoffman EP, Faden AI. Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. *Ann Neurol* 2003; **53**: 454–468.
- Turecek R, Trussell L. Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. *Nature* 2001; **411**: 587–590.
- Lee SC, Zhong YM, Yang XL. Expression of glycine receptor and transporter on bullfrog retinal Müller cells. *Neurosci Lett* 2005; **387**: 75–79.
- McTigue DM, Wei P, Stokes BT. Proliferation of NG2- positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J Neurosci* 2001; **21**: 3392–3400.
- Zai LJ, Wrathall JR. Cell proliferation and replacement following contusive spinal cord injury. *Glia* 2005; **50**: 247–257.