

# Effect of Strychnine, a Glycine Inhibitor, on the Programmed Cell Death of Motoneurons during the Chick Development

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In this study, we report that the treatment of strychnine (STR), an inhibitor of glycine receptor, induced premature onset of programmed cell death (PCD) of developing chick motoneurons (MNs). Treatment of STR on E4 chick embryo increased the apoptosis of MN on E5 when MN PCD does not occur normally. On the other hand, treatment of STR from E3 or E5 for 24 hours did not significantly influence the extent of MN PCD, indicating that the STR effect is developmental stage-specific. However, the expression of glycine receptor isoform was low on E3-4, and other glycine receptor antagonists did not exhibit PCD-promoting activity, suggesting that the STR action on PCD is not related to the glycine receptor activation. Identification of the target molecule for STR action may provide novel mechanism how the onset of developmental PCD is regulated.

**Key words:** strychnine, glycine receptor, programmed cell death, chick embryo, development

## INTRODUCTION

Programmed cell death (PCD) during the nervous system development is believed to be a key mechanism for establishment of the numerically optimal neurons-target connections [1, 2]. Within a discrete period of the embryonic development (E5~E8), for instance, approximately 50% motoneurons (MNs) undergo PCD in the chick embryo. PCD of MNs initiates with provisional synaptic connections with target muscles, and neurotrophic hypothesis explains the principle of developmental PCD: There was a competition among MN to acquire sufficient survival factors, and the limited amounts of available target-derived neurotrophic signals determine the extent of PCD [3-5]. In consistent with this

hypothesis, surgical removal or addition of target muscles in chick embryos accordingly resulted in the augmentation or reduction of the PCD, respectively [2, 6]. However, neurotrophic hypothesis does not explain how PCD initiates. Before their target muscle innervation, young MNs do not undergo PCD although they never be able to obtain target-derived signals. Considering that surgical removal of target does not modify the onset timing of PCD, it appears that the onset of PCD may be independent to the target-derived signals. Some paracrine/autocrine signals or cell-autonomous modifications may be involved in the onset of MN PCD, but molecular mechanisms and responsible extracellular factors are largely unknown.

Neuronal activity during the embryonic development plays significant role in the innervations and maturation of synaptic circuits, via modulation of the growth cone guidance and formation of synapses [7-9]. During the early development, inhibitory neurotransmitters such as glycine and GABA act as excitatory signals and trigger neuronal depolarization [10-12].

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In addition, several excitatory or inhibitory neurotransmissions directly or indirectly affect the PCD of MNs [13-15]. In the course of study examining the role of these inhibitory neurotransmission on the PCD of MNs, we found that the treatment of strychnine, a glycine receptor antagonist, advanced the onset of PCD in chick embryos. This new observation may trigger new insight how the onset of the MN PCD is modulated during the development.

## MATERIALS AND METHODS

### *Animals and treatments*

Fertilized chicken eggs were obtained from Pulmuone Co. (Korea). Eggs were incubated in humidified incubator at 38°C. Stage of chick embryo was identified according to the Hamilton-Hamburger's criteria [3]. Strychnine (300 µg in 100 µl Saline, Sigma S8753, St. Louis, MO), Ha966 (300 µg in 100 µl Saline, Tocris 0281, Ellisville, Missouri) or L-701324 (300 µg in 100 µl Saline, Tocris 0907, Ellisville, Missouri) were applied twice with 12 hours interval on E3, E4 or E5, and sacrificed 12 hours after last treatment.

### *Histology*

Immunohistochemical analyses were performed as previously reported [16]. Briefly, trunk tissues were isolated from embryos and immersion-fixed with 4% paraformaldehyde overnight. Tissues were then transferred in 30% sucrose, sectioned (7 µm) and attached on a gelatin-coated slide glass. After blocking the sections with PBS containing 3% BSA and 0.1% Triton-X100, activated caspase-3 antibody (1:500; Cell signaling Technology, Beverly, MA) was applied overnight. After several washes with PBS, Alexa488-conjugated donkey anti-rabbit antibody was applied for 30 min. Subsequently, sections were washed, counterstained with Hechst33342, mounted and observed with a confocal microscope (Zeiss LSM510, Goettingen, Germany).

### *RT-PCR*

Total RNAs (1 µg) purified from lumbar spinal cords of chick embryo were reverse-transcribed with reverse transcriptase, oligo (dT) primer and RNasin (Promega). An aliquot of the synthesized cDNA was subjected to PCR amplification with specific primers for the target genes. Primer sets for Glycine receptor alpha-1 (5'-AGA GCC CAT TCC TCC CTC CC-3' as 5'-primer and 5'-GGC AGA TCG TGC TGC TGC TT-3' as 3'-primer), Glycine receptor alpha-2 (5'-CCA GCC AGA GTT GCA CTG GG -3' as 5'-primer and 5'-AGG AAG GCG AGT GGG AAT GC-3' as 3'-primer), Glycine receptor alpha-3 (5'-CAG ACA GCG CAA GAT CCC GT-3' as 5'-primer and 5'-TGA GAT CCA TTG GGC AGG

ACA-3' as 3'-primer), Glycine receptor alpha-4 (5'-GGA GCG GCA GAT GGG CTA TT-3' as 5'-primer and 5'-CAG CCC GTA GCC TCG GAA GT-3' as 3'-primer), Glycine receptor beta (5'-CAC AGC GCT GCA AGA TGC AA-3' as 5'-primer and 5'-TGC CTT GGC AAT CTT GGC TT-3' as 3'-primer) and GAPDH (5'-GCT CCC TCA GCT GAT GCC CCC-3' as 5'-primer and 5'-AGG GAT GAC TTT CCC CAC AGC C-3' as 3'-primer) were used. To obtain semi-quantitative data, unsaturated range of PCR amplification cycles were determined, as 30 (Glycine receptor isoforms) or 25 (GAPDH) cycles. Amplified PCR products were resolved on 2% agarose gel, stained with ethidium bromide, and signal densities were measured by densitometer.

### *Statistical analysis*

Data are expressed as mean±standard error of the mean of independent experiments. Comparisons were made using one-way analysis of variance followed by Sheffé's multiple comparisons test and t-test. Statistical tests were carried out using sigmaplot 10.0. A value of p less than 0.05 was considered statistically significant.

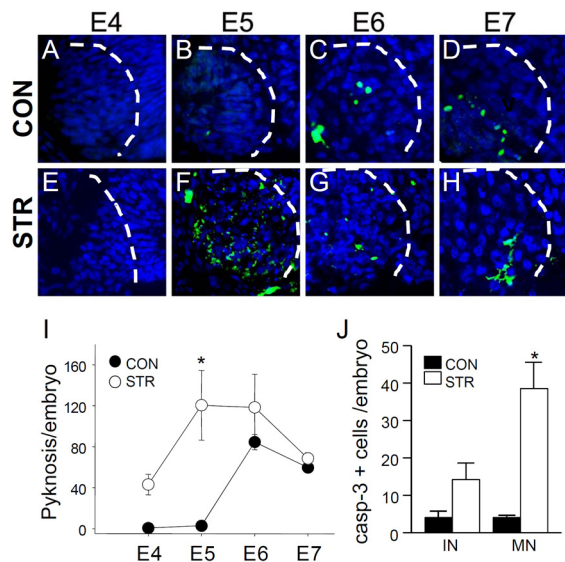
## RESULTS

### *Premature onset of MN PCD by strychnin treatment on E4 chick embryo*

Strychnin (300 µg in 100 µl PBS/embryo) was applied twice a day with 12-hours interval onto the different stage of chick embryo from E3-E6, and the embryos were harvested 24 hours after drug treatment (Fig. 1). In control embryos, evidence of apoptotic neuronal death such as nuclear condensation (pyknosis) and cleavage of caspase-3 was first seen on E5, and increased on E6-E7. On the other hand, apoptosis of MNs dramatically increased on E5 when STR was applied on E4. However, STR treatment on E3, E5 or E6 did not affect MN PCD (Fig. 1A~I). Whereas PCD was augmented by STR treatment on MN population, however, PCD of interneurons (IN) was not significantly modified (Fig. 1J), suggesting that STR preferentially affected PCD of MNs.

### *Expression levels of glycine receptor subtypes in developing chick spinal cord*

To evaluate the possible mechanisms of STR function on developing spinal cord, we evaluated the expression of glycine receptor (GlyR) subunits by semi-quantitative RT-PCR (Fig. 2). Functional glycine receptors are formed by a multimer of alpha and beta subunits, and 4 alpha subunit isoforms and one beta subunit were identified in chicks [17-19]. The expression of GlyR-α1 was only marginal in the embryonic chick spinal cord,



**Fig. 1.** Effect of strychnin (STR) treatments on the programmed cell death (PCD) of motoneurons (MNs) in developing chick embryos. Saline control (CON, A~D) or STR (E~H) was treated 24 hours before sacrifice, and the PCD of MNs was monitored by activated caspase-3 immunolabeling (green) on E4 (A, E), E5 (B, F), E6 (C, G) or E7 (D, H). White dotted lines indicate the border of lateral motor column (LMC) where spinal MNs localize. Nuclei were counter-stained with Hoechst33342 (blue). I: Quantification of dying cells (pyknosis) in each time point. Values are mean±SEM. \**p*<0.05. N= 3. J: Quantification of activated caspase-3 labeled dying MNs and interneurons (IN) on E5 embryo. \**p*<0.05, in one-way ANOVA and post-hoc t-test evaluation. N=3.

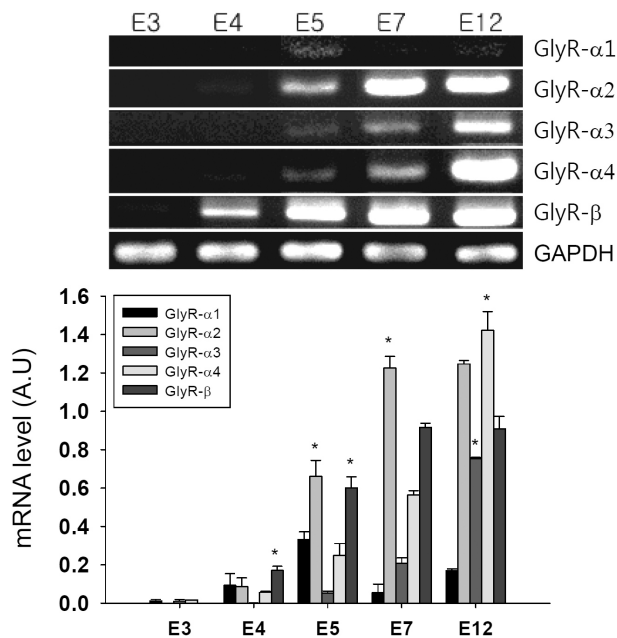
whereas other alpha subunits ( $\alpha 2\sim 4$ ) were detected at significant levels from E5 and their expressions were progressively increased. GlyR- $\beta$  was first identified at high level on E4, and its expression level was also progressively increased. These results suggest that functional glycine receptor may be formed from E4-E5, but their expression levels were substantially increased by a maturation of the spinal cord.

**Effect of other glycine receptor inhibitors on the MN PCD**

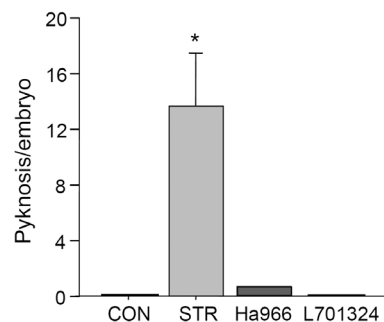
Because STR affected the MN PCD only when glycine receptor expression was very low, we asked whether STR indeed act via glycine receptor. To address this issue, we tested two additional glycine receptor antagonists, Ha966 and L701324. These drugs bind to different target sites of glycine receptors, and inhibit receptor activation competitively or non-competitively [20, 21]. However, different from STR, both inhibitors did not influence the PCD of MNs on E5 (Fig. 3), indicating that STR effect is not mediated by glycine receptor.

**DISCUSSION**

In this study, we identified that strychnin promoted premature



**Fig. 2.** Developmental changes in the expression of glycine receptor subunits. Upper panels show the typical images of EtBr-stained amplified cDNA gel runnings after semiquantitative RT-PCR. Quantification of band intensities by densitometer was shown in graph (lower panel). Band intensities were normalized by GAPDH level. Values are expressed as mean±SEM. \**p*<0.05, N=3.



**Fig. 3.** Effects of glycine receptor antagonists on the onset of MN PCD. The number of pyknotic (apoptotic) nuclei was quantified from 3~4 independent embryos. Values are expressed as mean±SEM. \**p*<0.05, in one-way ANOVA and post-hoc t-test evaluation.

onset of the PCD in developing chick MN. Although it is well known that the extent of MN PCD is mainly controlled by the amounts of available neurotrophic signals from the target muscles, little is known about the mechanism how MN PCD initiates. Surgical elimination of target muscle did not alter the time course of PCD, although it greatly increased the number of dying MNs [22, 23]. In this respect, it appeared that target-derived signals are not major factors controlling the time course of MN PCD. On the contrary, Wang and Scott have demonstrated that the transplantation of older limb bud (E5) to young embryo (E4)

resulted in the premature innervation of young MNs into the older target muscles, and the early onset of MN PCD [24]. This result indicates that innervation may signal to MNs to initiate PCD, suggesting the contribution of target in the PCD onset. Therefore, more comprehensive analyses are required.

In this respect, it is interesting to note that the effect of STR on the MN PCD is highly selective on the onset of PCD, but not on the extent of PCD. This result suggests that the mechanisms controlling the timing of PCD and extent of PCD are dissociable. While we found that approximately 30% of embryos die after STR treatment (data not shown), we do not believe that MN death is caused by general toxicity. We did not include dead embryos in the analysis, ruling out the post-mortem degeneration of MNs. Furthermore, we found that the effect of STR was selective on MN population, and STR only marginally affected the survival of interneurons. Different from MNs, interneurons do not undergo PCD during the development in the chick [25, 26]. Therefore, this selective vulnerability suggests that STR effect was specific to the population undergoing PCD. We also found the augmented PCD in the dorsal root ganglion sensory neurons (data not shown), in consistent with our hypothesis. Finally, we found high level of embryonic toxicity (>30%) after treatment of 1 mg concentration of picrotoxin which is a selective GABA receptor antagonist. However, we failed to monitor significant changes in the MN PCD in picrotoxin treated embryos (data not shown). Collectively, these results demonstrate that the effects of STR on the MN PCD are not caused by general toxicity.

Although STR selectively influences the MN PCD, two independent lines of our current observations favor the idea that this is not mediated by GlyR. First, we observed that the expressions of GlyR subunits were absent or very low in embryos before PCD period. It is reported that the expression of glycine receptors are increasing during the nervous system maturation, and there was a transition of embryonic/neonatal form to adult form of glycine receptors [27, 28]. In rat embryonic spinal cord, expression of GlyRa2 is predominant, whereas  $\alpha 1$  and  $\alpha 3$  expressions were more abundant in adults, suggesting that GlyRa2 is typical isoforms determining the characteristics of embryonic form of GlyRs [29]. In this study, we found that expression of GlyRa2 isoforms was predominant in the embryonic chick spinal cords similarly to the rat. Substantial level of GlyRa2 mRNA was first identified on E5. Considering that STR treatment on E4 selectively influence the MN PCD, this expression patterns did not correlated with stage-specific action of STR, although we cannot rule out the possibility that small amounts of GlyR expression may mediate STR action. Another line of evidence more clearly demonstrated the receptor-independent function of STR. We have employed two

additional glycine receptor antagonists, Ha966 and L701324. These two chemicals bind to different sites of GlyR, and they exert antagonistic activity via different pharmacological mechanisms [30, 31]. However, these two chemicals did not effect on the MN PCD, even after sub-lethal concentrations of drug treatments (data not shown).

Currently we do not know the target molecules mediating STR action on MN PCD. Considering that the mechanisms how PCD initiates are largely unknown, our current observation may help to identify novel mechanism underlying the control of PCD onset.

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