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Brief Embryonic Strychnine Exposure in Zebrafish Causes Long-Term Adult Behavioral Impairment with Indications of Embryonic Synaptic Changes

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Abstract

Zebrafish provide a powerful model of the impacts of embryonic toxicant exposure on neural development that may result in long-term behavioral dysfunction. In this study, zebrafish embryos were treated with 1.5 mM strychnine for short embryonic time windows to induce transient changes in inhibitory neural signaling, and were subsequently raised in untreated water until adulthood. PCR analysis showed indications that strychnine exposure altered expression of some genes related to glycinergic, GABAergic and glutamatergic neuronal synapses during embryonic development. In adulthood, treated fish showed significant changes in swimming speed and tank diving behavior compared to controls. Taken together, these data show that a short embryonic exposure to a neurotoxicant can alter development of neural synapses and lead to changes in adult behavior.

Keywords

Zebrafish; Development; Strychnine; Synaptic; Behavior; Novel Tank Diving Task

1. Introduction

Many environmental chemicals have been linked to childhood and adolescent behavioral deficits and studies have demonstrated the negative effects of environmental toxicants on developmental disabilities (Bellinger, Stiles et al. 1992; Lanphear, Dietrich et al. 2000; Mendola, Selevan et al. 2002; Stein, Schettler et al. 2002; Needleman 2004; Slotkin 2004; Koger, Schettler et al. 2005; Slotkin, Levin et al. 2006). One of the difficulties when investigating environmental toxicants is that many cause no overt morphological deformities, but demonstrate behavioral abnormalities. A study on the organophosphate pesticide chlorpyrifos (Dursban®) in zebrafish embryos demonstrated that adults exposed to

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Note: Sadly, Dr. Cerutti recently passed away.

low doses at embryonic stages where morphologically normal, but demonstrated learning deficiencies (Levin, Swain et al. 2004; Roy and Linney 2007). While this study showed that toxicant exposure during early development can lead to adult behavioral deficits, it did not relate those deficits to specific perturbation of neuronal signaling in the developing embryonic nervous system. Furthermore, the behavioral tests were conducted infancy, and so do not address the issue of the persistence of the effect of treatment. Studies with ethanol and lead have also demonstrated early embryonic exposure affects neurobehavioral function in zebrafish, but again, no early neural molecular changes are demonstrated (Carvan, Loucks et al. 2004) and the behavioral testing was rudimentary. These studies suggested the hypothesis that chemical perturbation of the developing nervous system alters the delicate balance of neurotransmitters in the developing fetal brain, which can be observed as long-lasting effects on behavior (Roy and Linney, 2007).

A neurotransmitter important to neural development is glycine, which inhibits firing of brainstem and spinal cord motor neuron (Legendre 2001). This is a critical process to counterbalance the firing of glutamatergic excitatory neurons and is essential for regulating motor rhythm which underlies locomotive behavior (Legendre 2001). The inhibitory actions of glycine on the brain and spinal cord result from an increase in chloride conductance. Additional glycine left in the cleft is reabsorbed into the presynaptic neuron by glycine transporters embedded in the membrane surface of the presynaptic neuron (glycine transporter 2) or in glial processes in close association with these neurons (glycine transporter 1). Studies in knockout mice, which lack the glycine transporter 1 and cannot reabsorb glycine suffer severe sensorimotor deficits. GLYT1$^{-/-}$ mice demonstrate hyporesponsiveness to tactile stimuli due to hyperactive glycine-mediated signaling (Gomeza, Hulsmann et al. 2003; Aragon and Lopez-Corcuera 2005). Additionally, knockout mutations of glycine transporter 2 lead to high levels of glycine in the synaptic cleft resulting in muscular spasticity, impaired motor coordination and tremor (Eulenburg, Armsen et al. 2005). In other vertebrate species, like zebrafish, mutations in glycine transporter 1 lead to the *shocked* phenotype which results in motor deficits, spontaneous coiling of the trunk, an absence of the fish escape response and a lack of swimming behavior (Cui, Low et al. 2005). Furthermore, mutations in the postsynaptic glycine receptor subunits have also demonstrated defects. Mutations in the glycine receptor beta-subunit results in a loss of glycinergic inhibitory neural signaling resulting in the *bandoneon* phenotype which causes trunk muscles to fire excitatory neurotransmitters concurrently (Hirata, Saint-Amant et al. 2005).

Strychnine antagonizes the inhibitory actions of glycine (Legendre 2001; Hirata, Saint-Amant et al. 2005) by blocking the binding of glycine to its postsynaptic chloride channel receptor (Hirata, Saint-Amant et al. 2004; Hirata, Saint-Amant et al. 2005). Thus the channel does not open, preventing propagation of the inhibitory signal. In zebrafish, strychnine exposure demonstrates an “accordion” phenotype (Hirata, Saint-Amant et al. 2004), characterized by simultaneous contraction of the bilateral trunk muscles along the length of the body. Studies of behavior in 30 day old Japanese medaka (*Oryzias latipes*) exposed to strychnine demonstrate changes in equilibrium, general activity, and startle response (Rice, Drewes et al. 1997).

The zebrafish model system provides a powerful tool to study early neurotoxic challenge and the impact on adult behavior. Zebrafish develop *ex utero*, allowing toxicant treatment at time windows beginning immediately post-fertilization. Once treated, embryos can be removed from the toxicant and raised in non-treated water to adulthood (3 months). Behavioral testing can be performed at any time during development. Most importantly, however, zebrafish are vertebrate organisms that develop in accordance with the vertebrate plan like humans. Thus, research investigating how environmental toxicants affect zebrafish neural development and influence juvenile and adult behavior is applicable to human development.

Here, we treated zebrafish embryos for short embryonic time windows with strychnine. We examined changes in neurons by analyzing the expression of synaptic transporter, receptor and processing enzymes for various neurotransmitters at embryonic stages via PCR analysis and demonstrate changes in expression of glycinergic, GABAergic and glutamatergic related genes between 24 and 96 hours after treatment. Treated siblings were raised in normal fish water until adult stages where they were tested for behavioral deficits using test methods developed in our labs. The adult fish showed significant changes in swimming speed and tank diving behavior in a novel tank diving test. Taken together, our data suggest that a short embryonic exposure to a toxic chemical is significant enough to cause changes in embryonic neural synapses and lead to deficits in adult behavior.

2. Methods

2.1 Embryo handling

Embryos were generated by natural pair-wise mating in zebrafish mating boxes (Westerfield 1994). Embryos were placed in Petri dishes in 30% Danieau Buffer (50X Danieau's Solution [169.475g NaCl, 2.61g KCl, 4.93g $MgSO_4$ 7H20, 7.085g $Ca(NO_3)2$ $4H_20$, 0.5M Hepes at a pH of 7.6, autoclaved]. 30% Danieau's solution was prepared by mixing 6ml of the 50X concentrated solution into 1L of dH_2O) at 28° C for 5hrs before moving into drug treatment. Zebrafish were staged in accordance with standard staging series (Kimmel, Ballard et al. 1995).

2.2 Drug administration

Strychnine (Sigma, St. Louis, MO, USA) was dissolved in distilled water to a stock concentration of 10mM. A 1.5mM working stock was made by dilution in 30% Danieau Buffer. Strychnine dose was chosen from previous studies (Granato, van Eeden et al. 1996; Hirata, Saint-Amant et al. 2005). Although our dose was higher, in verbal communications with Granato, we used a different form of strychnine salt. However, we increased the dose until our embryos mimicked their published "accordion" phenotype without any morphological abnormalities. At 5 hpf (hours post fertilization) embryos were moved from 30% Danieau Buffer to working concentration of 1.5mM strychnine. At 24 hpf, embryos were manually dechorionated with forceps. To verify strychnine was effective, the "accordion" phenotype was checked by manually touching embryos delicately with forceps (Hirata, Saint-Amant et al. 2004). Embryos were left in strychnine solution for 24, 48, 72 and 96 hour time periods until they were subjected to RNA extraction. Increased time

exposures were used to determine if longer treatments resulted in stronger phenotypes. Treatment in strychnine for longer time periods resulted in death. At the 96hr time point, the longest treatment window, the embryos appeared healthy with no morphological defects, yet displayed the “accordion” phenotype. Embryos to be raised to adulthood for behavioral testing were treated for 18 and 29hrs, removed from strychnine treatment, washed with 30% Danieau Buffer and placed in Danieau Buffer until they were 5 days old after which they were raised in standard fish water (Westerfield 1994). Control embryos were collected and raised in 30% Danieau Buffer only. At 24, 48, 72 and 96 hours, they were subjected to RNA extraction. Siblings raised for behavioral testing were kept in 30% Danieau buffer until they were 5 days old after which they were raised in standard fish water as described above for the strychnine treatments.

2.3 Molecular Biology Methods

RNA Extraction was performed with a Qiagen RNEasy RNA Extraction kit, protocol for purification of total RNA from animal cells using spin technology was used (catalogue #: 74104) with the following modifications. Control and drug treated embryos were placed in a 15ml snap-cap tube and residual water was removed. 700μl RTL + BME was added to each tube and homogenized for 30 seconds. After standard processing the RNA was eluted off the column by adding 20μl of RNAse free water directly to the membrane. After sitting for 2 minutes, the samples were spun for 1 minute at 10,000rpm to elute the RNA which was quantified and stored at −80°. For embryos at 24hpf, 75 embryos were homogenized, at 48hpf, 60 embryos and at 72 and 96hpf, 40 embryos were homogenized for RNA extraction.

cDNA Preparation was conducted with a Bio-Rad iScript cDNA synthesis kit (catalogue #: 170-8890) according to the manufacturers specifications. 1μg/μl of RNA sample was used for each reaction.

PCR and Gel Electrophoresis was conducted with a Platinum Blue PCR Supermix from Invitrogen (catalogue #: 12580-015) and used according to the manufacturers specifications. Primers were all ordered from IDT Technologies (www.idtdna.com) and designed by IDT SciTools PrimerQuest from sequences imported from PubMed. Primer sequences and Tm’s are shown in Table 1. A standard 1% agarose gel was performed.

2.4 Novel Tank Dive Test

Adult zebrafish, 3 months of age, were placed individually in either of two unfamiliar (“novel”) 1.5-liter plastic tanks filled with 1350 ml of tank water. The tanks were trapezoidal in shape and extended 22.9 cm along the bottom and 27.9 cm in length cross the top. The diagonal side of the tank was approximately 15.9 cm in length and the opposing side was 15.2 cm long (Figure 1). The novel tank dive test has been described previously (Levin, Bencan et al. 2007). The two tanks had the short side next to each other with a barrier between them and a solid white 60 cm x 122 cm plastic board that was positioned behind the tanks. The two tanks were separated from each other in terms of water contact and visual access as shown in Figure 1. Thus, each fish was tested independently. The video image was divided into lower, middle, and top swimming areas using the EthoVision™ program (Noldus Information and Technology, Wageningen, Netherlands). The trial series

was started with a five-minute trial duration for testing. The video signal was transmitted through an 8 mm Samsung Camcorder. The video was then transmitted to the computer for analysis. There was one 5-min. trial per fish.

2.6 Data Analysis

The behavioral data from the novel tank diving task (seconds/min. in the bottom of the tank and cm/min traveled were evaluated with the analysis of variance. Strychnine exposure was the between subjects factor and minute of the 5-min test was a repeated measure. The group sizes were as follows: Control N=19, Strychnine 18 N=17 and Strychnine 29 N=21. A p-value of 0.05 was used as the threshold for a significant effect.

3. Results

3.1. Strychnine effects glycinergic synapses

To explore possible changes in the glycinergic neural synapses in response to strychnine toxicant exposure, we performed PCR analysis using primers for genes that mark the glycinergic synapse. Figure 2.A. shows a time course of gene expression changes in control and strychnine treated embryos at 24, 48, 72 and 96-hrs time periods. *Beta-actin* served as a loading control with an equivalent amount of DNA for each reaction. Genes analyzed for the glycinergic synapse included glycine transporters 1 and 2 (*glyT1, glyT2*), glycine receptors (*glyRα1, glyRα3, glyRα4* and *glyβ2*) and serine methyltransferase (*smet*), an enzyme that plays an important role metabolizing glycine. This exploratory investigation suggested that embryos exposed to strychnine had a slight downregulation of *glyRα4* at 24 and 48 hrs, but not at the later time points. Treated embryos appeared to show a stronger downregulation of *glyβ2* at 24 and 48 hrs, but again a recovery was observed at the later time points.

3.2 Effects of strychnine on glutamatergic and GABAergic synapses

To explore possible changes in the glutamatergic and GABAergic synapses in response to strychnine exposure during development, we performed PCR analysis using primers for genes that mark the glutamatergic and GABAergic synapses. Figure 2.B. shows a time course of gene expression changes in control and strychnine-treated embryos at 24, 48, 72 and 96 hr time periods. Again, *Beta-actin* served as a loading control with an equivalent amount of DNA for each reaction. There was an indication of a downregulation of *gad67 (glutamate decarboxylase)* at each time point. Indications of a downregulation of *vglut2.1* and *vglut2.2* (vesicular glutamate transporters) were also seen at 48 hrs with a recovery at later time points.

3.3 Effects of strychnine on the novel tank diving task

Developmental strychnine exposure affected behavior in the novel tank diving task. There was a significant ($F(2,54)=3.42$, $p<0.05$) main effect of strychnine on the diving response (Figure 3). Dunnett's post hoc tests showed that the strychnine 29 group (N=21) spent significantly ($p<0.05$) less time in the bottom third of the tank compared with controls (N=19), while the lower dose strychnine 18 group (N=17) did not differ from controls (Control = 57.0±1.3 seconds/minute, Strychnine 18 = 54.6±2.5 seconds/minute, Strychnine 29 = 49.5±2.4 seconds/minute). There was a significant main effect of minute

(F(4,216)=8.76, p<0.0005) with a consistent decrease in bottom dwelling over the five minutes of the test, which is typical for this test (Levin, Bencan et al. 2007). The strychnine x minute interaction was not significant.

With speed of swimming in the novel tank diving test, there was a significant (F(2,54)=6.48, p<0.005) main effect of strychnine (Figure 4). Dunnett's post hoc comparisons of the treated groups to controls showed that both the strychnine 18 and strychnine 29 groups had significant (p<0.01) increases in swimming speed (Control = 157±24 cm/min, Strychnine 18 = 256±28 cm/min, Strychnine 29 = 262±73 cm/min). There was a significant main effect of minute (F(4,216)=12.25, p<0.0005) with a consistent increase in swimming speed over the five minutes of the test, which is typical for this test (Levin, Bencan et al. 2007). The strychnine x minute interaction was not significant.

4. Discussion

In this study, we demonstrated that brief strychnine exposure during early neural development causes long-term behavioral dysfunction detected by the novel tank diving task both in terms of attenuated normal diving response and hyperactive swimming months after developmental exposure. There were also suggestions of short-term alterations in glycine, GABA and glutamate receptors just after strychnine exposure. Short-term strychnine-induced synaptic changes may be important for setting up the abnormal brain development underlying the strychnine-induced behavioral alterations which are clearly seen in adulthood.

The novel tank diving task assesses the species-specific predatory avoidance response (diving) as opposed to foraging strategies (completely exploring the tank). The thigmotaxis of the fish diving to the floor of the novel tank is quite similar to the thigmotaxis of rodents for the wall of an open field or closed arms of an elevated plus maze. Attenuation of this normal diving response would put a fish at predatory risk in a novel environment. We have previously seen developmental neurotoxicity cause long-term attenuation of the novel tank diving response with low-level exposure of zebrafish to the organophosphate pesticide chlorpyrifos (Sledge et al., 2011). Like with the current study, the chlorpyrifos-treated fish also showed a significant degree of hyperactivity in this task. We also found that treatment of zebrafish with methylphenidate, medication used to treat attention deficit hyperactivity, also significantly attenuated the novel tank diving response (Levin et al, 2011). However, with methylphenidate a long-term increase in swimming activity was not seen. It is apparent that diverse developmental toxicants can impair the novel tank diving response. This task is a sensitive measure of long-term neurobehavioral toxicity but it is not selective to a particular mechanism of toxic effect.

In the current study with strychnine the long-term impairment in the diving response and hyperactivity may be related to effects of strychnine on glycinergic, GABA and/or glutamate receptors. The PCR data are preliminary in nature but do suggest developmental effects on these three receptor systems. Interestingly, most of the synaptic effects seem to be transient whereas the behavioral effects are seen well into adulthood. It appears that the disrupted neurodevelopment mediated through glycinergic, GABA and/or glutamate receptor systems

caused a cascade of abnormal development which persisted after these synaptic systems returned to normal. We previously found that methylphenidate exposure during development in zebrafish caused only a transient elevation of dopamine, norepinephrine and serotonin concentrations but, like strychnine, caused a long-term impairment in the novel tank diving task in adulthood. Work from the Spitzer laboratory has demonstrated that perturbation of developing Xenopus neurons caused a neurotransmitter switching event, demonstrating how perturbation could alter the plasticity of the early nervous system (Borodinsky, Root et al. 2004; Roy and Linney 2007). However, these studies were limited to early embryonic neurons and did not asses adult behavioral consequences of these events. Early neural development is a very delicate time period where neurons are being born, dividing, migrating, differentiating and establishing synaptic connections in a highly organized fashion. These delicate events are controlled by neurotransmitters and neurotrophic factors (Schettler 2001) and wire the nervous system for later stage behavior.

Future investigation can also take advantage of pharmacological probes to determine the specificity of strychnine's effects. Specific receptor agonist and antagonist treatments could be used to potentiate or block the effects of strychnine on locomotor behavior. In prior research embryonic exposure to strychnine in the chick has been found to also increase locomotor activity (Oppenheim and Reitzel 1975; Reitzel and Oppenheim 1980; Sedlacek 1992). This suggests a more general action of glycinergic receptors in the development of locomotor function across vertebrate species.

Here we demonstrate a novel study that shows early, embryonic molecular changes in neural synapses in response to strychnine. We further demonstrate adult stage behavioral deficits in fish that were treated for short embryonic exposures. This study demonstrates novel behavioral testing methods that may be useful for future work. With industrialization, the increase in pharmaceutical effluent and pesticide contamination, we are exposing developing organisms to compounds that can perturb early neural development and impact adolescent and adult behavior and thus, this is an important area of vertebrate study.

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Figure 1.

A. The novel tank diving task apparatus with digital video camera.

B. Image of the swimming track taken for a fish over the course of a 5-min. session.

Figure 2 A.

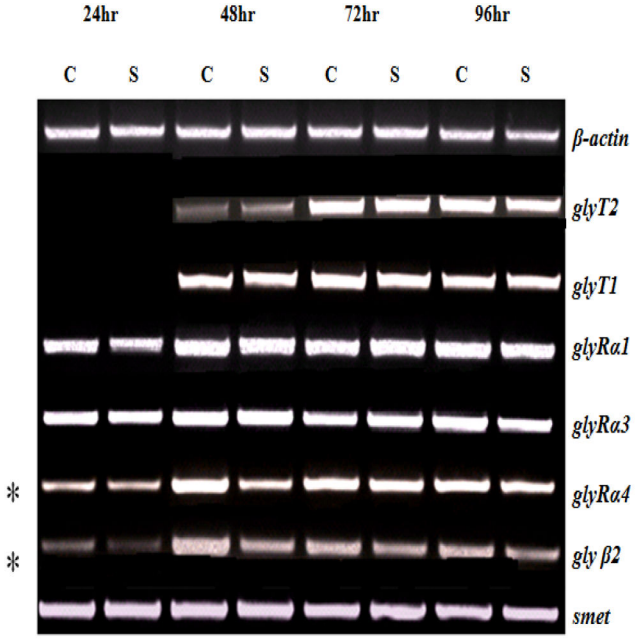

Figure 2 B.

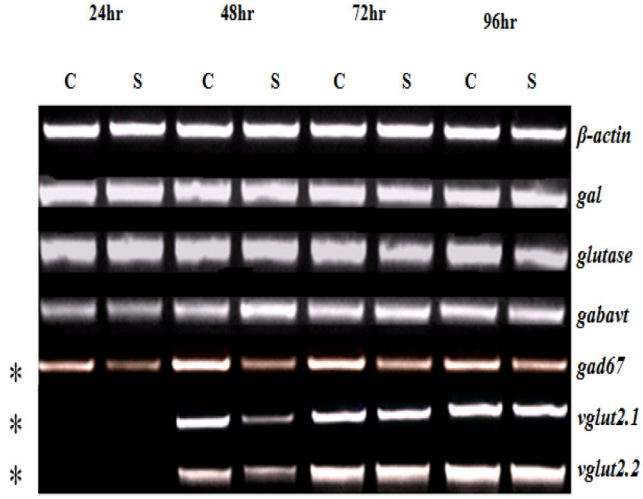

Figure 2.

(A). PCR results analyzed on 1% agarose gel of glycinergic genes demonstrating downregulation in *glyRa4* and *glyβ2* at 24 and 48hrs. Both genes demonstrate a recovery at later time points. *glyT2* and *glyT1* gene expression is too weak for detection at 24hpf **(B).** PCR results analyzed on 1% agarose gel of glutamatergic and GABAergic genes demonstrating downregulation in *gad67* at 24 and 48hrs with a recovery at later time points. *vglut2.1* and *vglut2.2* gene expression is too weak for detection at 24hpf, but demonstrates downregulation at 48hrs with recovery at later time points. *β-actin* is the loading control. C: control, S: strychnine. Time points tested at top.

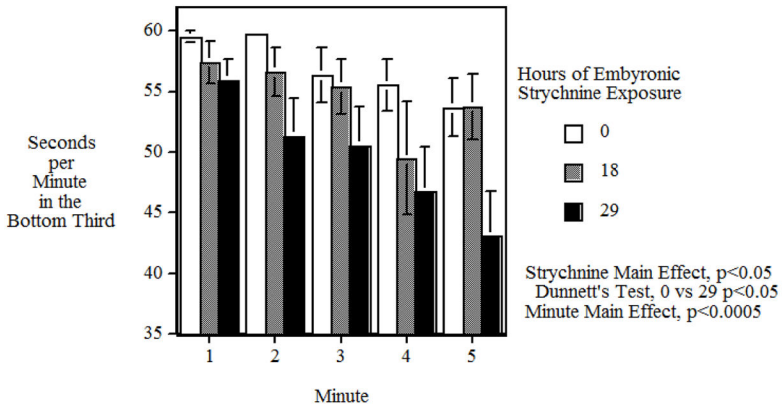

Figure 3.

Effect of embryonic strychnine exposure on novel tank diving behavior, seconds per minute in the bottom third of the tank for each of the five minutes of the test (mean±sem).

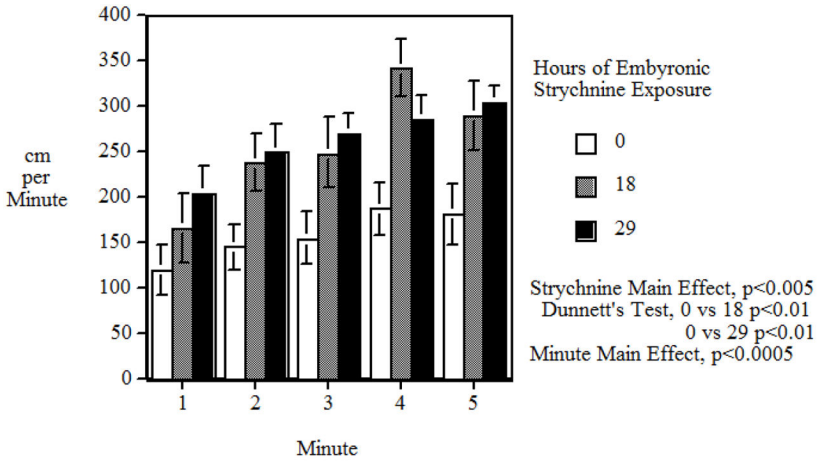

Figure 4.

Effect of embryonic strychnine exposure on swimming speed in the novel tank diving for each of the five minutes of the test (mean±sem).

Table 1

Primer sequences used for PCR

gene	gene	forward sequence 5′-3′	reverse sequence 5′-3′
β-actin	*beta-actin*	TTG GCA TGG GAC AGA AAG ACT CCT	ACC GCA AGA TTC CAT ACC CAG GAA
glyT2	*glycine transporter 2*	GGT TTT GTG AGG ACA TTG AG	GAA TGT GAG AAT GGT TGG AG
glyT1	*glycine transporter 1*	AGG TGT GGG CTA TGG AAT GAT GGT	ACT GAG TGC CCA AAC CCA ATA GGA
glyRα1	*glycine receptor alpha 1*	GCT GAC CAT GAC CAC CCA GA	GAA GGC ACA CTG CCA TCC AA
glyRα3	*glycine receptor alpha 3*	AGG TCA CCA CTG ACA ACA AGC TCT	AAC AGC AAA CAC ACA GCC ATC CAG
glyRα4	*glycine receptor alpha 4*	ACT TTC CGG GCT TTG GGA TAG ACA	AAG GTC AGG AGT GAA TCA GCC GTT
glyβ2	*glycine receptor beta 2*	CAC GTG CGT GGA GGT GAT TT	GCC AGG AAA GGA CAA CGA TGA G
smet	*serine hydroxymethyltransferase*	AGT GTT GAT GCA AAG ACC GGC AAG	CCA AAG TCA ACA CAA TGC CCT GGT
gal	*glutamate ammonia ligase*	ACC GGA GAG GGA TTG AGA TGC AAA	TGT GCA GGC ATG ACT TCA GCA TTG
glutase	*glutaminase*	TGC ACC GTT TCA TTG GGA AAG AGC	AGC ACA TGA TGC CCA TCA CAT TGG
gabavt	*gaba vesicular transporter*	TCT CCA TCG GCA TCA TCG TGT TCA	ATA TGG CGA CGT CGA AGA ACA CCT
gad67	*glutamate decarboxylase*	ATT GCG CCT GTG TTT GTC CTG ATG	TGT TTG TCC GGC TGG AAG AGG TAA
vglut2.1	*vesicular glutamate transporter 2.1*	AGT ACA CGG GCT GGT CTT CTG TTT	TAC TGG CAT AGC GAG GAG CAA TGT
vglut2.2	*vesicular glutamate transporter 2.2*	ACT ACG GCT GTG TGA TGT TCG TGA	TGA CGG TGG TGG TGG TTA AGA TGT