Regulation of Spinal Interneuron Differentiation by the Paracrine Action of Glycine

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Abstract

Glycine and γ -aminobutyric acid (GABA) are depolarizing during early development but the purpose is unclear. We tested the effect of altering glycine signaling in zebrafish embryos by overexpressing the potassium-chloride co-transporter type 2 (KCC2) to reverse the chloride gradient or by blocking glycine receptors with strychnine or by selectively knocking down the embryonic glycine receptor (GlyR KD). Using a variety of markers we observed in all three cases a reduction of all types of spinal interneuron populations examined, indicating that glycine modulates their overall differentiation rather than choice of cell fate. Other cell populations (motor, sensory, and glial cells) were unaffected. As glycine appeared to act preceding neural and synaptic development, we examined the *bandoneon* (*beo*) mutant in which glycine receptors are functional but not clustered at synapses. Neural populations in *beo* embryos appeared normal, suggesting a paracrine action of circulating glycine in promoting interneuron differentiation.

Keywords

glycine receptors; KCC2; interneurons; motor neurons; sensory neurons

INTRODUCTION

In contrast to the well-known roles of γ -aminobutyric acid (GABA) and glycine in the adult nervous system as hyperpolarizing inhibitory neurotransmitters, at an early developmental stage neurotransmission in the embryo largely relies on their depolarizing excitatory neurotransmission (Ben-Ari, 2002). The role of this paradoxical chloride-mediated depolarization is unclear and has been attributed a variety of trophic functions (Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005; Ben-Ari and Spitzer, 2010). The switch from excitation to inhibition (Blaesse et al., 2009) comes about just prior to birth in mammals when the chloride gradient is altered upon delayed expression of the neural-specific KCl co-transporter type 2 (KCC2; Ben-Ari, 2002; Blaesse et al., 2009). We reported previously that blockage of glycinergic transmission from the beginning of zebrafish development by embryonic glycine receptor knockdown (*GlyR* KD) (McDeamid et al., 2006) or by reversing of the depolarizing chloride gradient by overexpression of human *KCC2*

(Reynolds et al., 2008) resulted in a selective loss of interneurons with minimal changes in motoneuron and spinal sensory neuron populations.

The embryonic zebrafish spinal cord shares a common vertebrate developmental plan (Lewis, 2006) including genetic specification of neuronal subtypes by a combinatorial code of transcription factors (Jessell, 2000). Here, we examined the glycinergic phenotype using a variety of selective markers available in zebrafish in order to determine whether all types of interneurons or just a subset was affected as this could indicate whether early differentiation or later cell fate choices were dependent on glycine signaling. We also examined mutant *bandoneon* (*beo*) embryos in which glycine receptors are functional but not clustered at synapses, thus allowing for paracrine (rather than synaptic) glycinergic activity. As neurons in *beo* embryos seemed unaffected, our results indicate that paracrine release of glycine selectively mediates differentiation of all types of interneurons. Interestingly, neural progenitors express receptors for GABA and glycine (Belachew and Gallo, 2004), indicating a propensity for neurotransmitter signaling early during the period of neurogenesis (Cicirata et al., 1991; Miranda-Contreras et al., 1998, 1999).

METHODS

Animals

Zebrafish (*Danio rerio*) were bred and maintained according to standard procedures (Westerfield, 1995). All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and the University of Montreal. To study the importance of depolarizing chloride homeostasis in the development of a particular population of spinal neurons, we used embryos from transgenic lines in which enhanced green fluorescent protein (EGFP) expression is driven under the control of a cell type specific promoter. The *Alx* transgenic line was used to identify glutamatergic ipsilateral descending interneurons (Kimura et al., 2006) and the *Evx* line was used to visualize commissural interneurons (Suster et al., 2009). In addition, some of the experiments were repeated in the bandoneon [*beo*^{p221}] mutant line (Haffter et al., 1996; Hirata et al., 2005). The *Neurogenin1* (*Ngn1*) transgenic line (Blader et al., 2003) was used to study differentiated neurons in general and sensory neurons in particular; motoneurons were visualized using the *Isl1* line (Uemura et al., 2005) and the *PLP* line (Yoshida and Macklin, 2005) was used to assess oligodendrocyte progenitors. We thank Drs. Higashijima, Kuwada, Blader, Okamoto, and Yoshida for their lines.

Manipulations of Glycine Signaling

To perturb glycinergic signaling from the beginning of development, three types of experiments were carried out. Embryos with severe developmental defects under any experimental condition were excluded from the analysis (<10%).

1. *Glycine receptor knockdown*: Morpholino (Gene Tools) against the embryonic $\alpha 2$ and $\alpha 4$ subunits of the zebrafish glycine receptor was injected (0.25 mM) into 1–2 cell stage blastocysts, as previously described (McDermid et al., 2006).

2. *KCC2 overexpression*: To reverse the chloride gradient from the beginning of development, mRNA of human KCC2 (100–150 ng) was injected into 1 cell stage blastocytes as previously described (Reynolds et al., 2008).
3. *Chronic drug application*: Wild-type or transgenic embryos were placed in Evans solution (for details see calcium imaging section) containing 100 μ M strychnine (Gibco BRL) for 20–40 h, starting 8-h post-fertilization.

Whole-Mount Immunohistochemistry

Standard fixation and immunolabeling protocols were used as previously described (Reynolds et al., 2008). Briefly, 48 hpf embryos were anesthetized (0.02% tricaine, Sigma), dechlorinated and subsequently fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature or overnight at 4 °C. Embryos were then washed in phosphate buffer, permeabilized in distilled water for 30 min and incubated for an hour in blocking solution (2% goat serum, 1% BSA, 1% DMSO, 0.1% triton X-100 in PBS). Then, embryos were soaked overnight at 4 °C in primary antibody and blocking solution. The following primary antibodies were used: anti-HuC (1:50; Molecular Probes), anti-Pax2 (1:100; Covance), anti-GFP (1:100; Molecular Probes), anti-GAD65/67 (Biomol; 1:100), anti-PH3 (Upstate; 1:100), and anti En1 (Sigma; 1:50). The next day embryos were washed repeatedly and incubated overnight in secondary antibody anti-mouse or anti rabbit conjugated with Alexa488 or Alexa568 (1:1000; Molecular probes).

Confocal Microscopy and Data Analysis

Fluorescent images of fixed embryos were taken using a Quorum Technologies spinning-disk confocal microscope mounted on an upright Olympus BX61W1 fluorescence microscope equipped with an Hamamatsu ORCA-ER camera. Image acquisition was performed with Volocity software (Perkin–Elmer) and the number of neurons stained at over twice the background level in 100 μ m sections at the 14–18 somites region were counted from 1 μ m stacked images using imageJ software (NIH). Changes in the number of neurons were compared to the control, non-treated group, using Student's *t*-test.

RESULTS

Blocking Glycinergic Signaling Selectively Reduces All Types of Interneuron Populations

Previous reports demonstrated that blocking depolarizing glycinergic transmission in zebrafish embryos from the beginning of development by *GlyR* KD (with an antisense morpholino oligonucleotide targeting the embryonic alpha subunit; McDearmid et al., 2006) or by overexpression of human *KCC2* mRNA (Reynolds et al., 2008) resulted in the loss of half of the interneuron population with minimal changes in motoneuron and spinal sensory neuron populations. We examined this phenotype in greater detail in order to determine whether all types of interneurons or selective subsets were affected as this could indicate whether early progenitor differentiation or later cell fate choice, respectively, was dependent on glycine signaling. We repeated these manipulations (*GlyR* KD or *KCC2* overexpression) and as a third manipulation we chronically treated embryos with the GlyR antagonist strychnine. To evaluate the interneuron populations, we used a variety of markers for

different subtypes of spinal interneurons (see “Control” column in Fig. 1). The Pax2 antibody was used to label mostly glycinergic and many GABAergic neurons (Batista and Lewis, 2008) [Fig. 1(A)]. We also stained GABAergic interneurons with an antibody to GAD 65/67 (Batista and Lewis, 2008) [Fig. 1(F)]. We then examined a transgenic *Evx1:GFP* line expressing GFP from the *Evx* promoter in mostly commissural interneurons (Suster et al., 2009) [Fig. 1(K)] and the *Alx* (also called *Vsx2*, a homolog of *CHX10*) transgenic *Alx:GFP* line expressing GFP in mostly glutamatergic neurons (Kimura et al., 2006) [Fig. 1(P)]. Finally, the En antibody was used to mark the glutamatergic circumferential ascending interneurons (Fjose et al., 1988; Ekker et al., 1992) [Fig. 1(U)]. For all three treatments (strychnine, *GlyR* KD, or *KCC2* overexpression), we observed a major and statistically significant reduction (30–50%) in the number of cells labeled (at developmental day 2, around hatching) with each marker [Pax2—Fig. 1(B–D), GAD—Fig. 1(G–I), *Evx*—Fig. 1(L–N), *Alx*—Fig. 1(Q–S), En—Fig. 1(V–X)], as summarized in the bar graphs of Figure 1 [Pax2—Fig. 1(E), GAD—Fig. 1(J), *Evx*—Fig. 1(O), *Alx*—Fig. 1(T), and En—Fig. 1(Y)]. We conclude that all types of interneurons examined, representing the majority of interneuron subtypes, were significantly and equally reduced in number following disruption of normal glycinergic activity.

We also confirmed (Fig. 2) that the populations of motoneurons [*Isl1* transgenic line expressing *GFP* from the *Isl1* promoter mostly in motoneurons; Uemura et al., 2005; Fig. 2(A–E)], sensory Rohon-Beard neurons [*Ngn1* transgenic line, which expresses at later stages selectively in Rohon-Beard sensory neurons; Blader et al., 2003; Fig. 2(F–J)], and glial cells [*PLP* transgenic line which expresses GFP from the *PLP* promoter; Yoshida and Macklin, 2005; Fig. 2(K–O)] were not significantly affected by all three treatments, except for a decrease in sensory neuron numbers in the presence of strychnine but not upon the other treatments. Thus not only was the interneuron population reduced by all treatments without apparently being translocated to other common spinal cord cell types but every (and likely all) types of interneurons examined were reduced in number (as opposed to a generalized developmental delay).

Glycine May Act in a Paracrine Manner

We next examined overall spinal neuron populations in the *beo* mutant in which the GlyR β subunit involved in synaptic clustering is lost and thus lacks synaptic but preserves extrasynaptic or paracrine glycinergic signaling (Haffter et al., 1996; Hirata et al., 2005). Previous studies of *beo* did not report any obvious anomalies in the gross morphology of the embryos though they showed dramatic synaptic and behavioral deficits. We examined the levels of HU (McDermid et al., 2006; Reynolds et al. 2008) and Pax2 (Batista and Lewis, 2008) expression in broad populations of newborn and mostly glycinergic interneurons, respectively. As shown in Figure 3, neither population was significantly affected in *beo* $-/-$ embryos [Fig. 3(C,G)] compared to heterozygous siblings [$+/?$, consisting of mixed $+/+$ and $+/-$, Fig. 3(F)] and wild type embryos at developmental day 2 [$+/+$, Fig. 3(A,E); statistics provided in figure legends], indicating that a paracrine action of glycine may be sufficient to promote normal interneuron differentiation.

DISCUSSION

We blocked depolarizing glycinergic signaling from the onset of embryonic development in zebrafish by three independent means: pharmacologically with strychnine or genetically by *GlyR* KD (McDermid et al., 2006) or by overexpressing *KCC2* in order to prematurely reverse the chloride gradient (Reynolds et al., 2008). We then examined various cellular populations of the embryonic spinal cord using selective markers for different populations (Pax2, GAD, Evx, Alx, En, Isl1, Ngn1, Plp, and HU). For all three manipulations of glycine signaling, the interneuron populations were equally and selectively reduced as motor, sensory and glial cells were unaffected. In contrast, the *beo* mutant lacking synaptic but not extra-synaptic, paracrine glycinergic signaling (Hirata et al., 2005) showed no obvious differences in neural populations (stained for Pax2 and HU), indicating that a paracrine effect of glycine was sufficient to maintain appropriate interneuron generation.

The absence of significant cell death reported previously following alteration of glycinergic signaling (McDermid et al., 2006; Reynolds et al., 2008) and the lack of transfecting to other cell types observed in this study indicate that the core interneuron neurogenic program (Jessell, 2000) is normally enhanced (doubled) by depolarizing (epigenetic) glycinergic signaling in interneuron progenitors. As *beo* mutants showed no obvious differences in neuronal populations, this indicates a paracrine effect of glycine at a stage preceding neurogenesis and confirms previous studies in other preparations (LoTurco et al., 1995; Owens et al., 1999; Scaini et al. 2010). Glycine and GABA are present early in the embryo, long before synaptic transmission is established (Cicirata et al., 1991; Miranda-Contreras et al., 1998, 1999; Owens et al., 1999), and their receptors are also present before synapse formation (Belachew and Gallo, 2004) and numerous studies have highlighted their trophic roles (Represa and Ben-Ari, 2005). Thus, the presence of circulating glycine and GABA at an early stage allows them to act as neurogenic trophic substances if progenitors in zebrafish respond to glycine as they do in mammals.

Interestingly, previous work in *Xenopus* has indicated that altering the frequency of spontaneous calcium transients can change the fate of newly born neurons (Ben-Ari and Spitzer, 2010). Our results attribute to glycine a new and fundamental role at the earlier stage of progenitor differentiation. Spontaneous (Ashworth and Bolsover, 2002) and glycine-dependent (Brustein et al., 2003) Ca^{2+} transients were detected in the zebrafish embryonic spinal cord previously and cell differentiation is known to require Ca^{2+} influx from both extracellular and intracellular Ca^{2+} sources (Resende et al., 2010). Thus, paracrine glycinergic depolarization may regulate the core neurogenic program for interneuron differentiation. Presumably the pattern of calcium transients, such as their strength, duration, and frequency, determines the extent (rate) of early interneuron progenitor differentiation and the later fate of terminally differentiated neurons. Thus glycinergic and GABAergic activity is not only a consequence but also a cause of neural differentiation.

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Figure 1.

All spinal interneuron populations are reduced by blocking embryonic glycinergic signaling. The effect of manipulating glycinergic signaling from the beginning of development on the number of spinal interneurons population was evaluated in 48-h old embryos raised in strychnine (Strych) to block glycinergic activity, after glycine embryonic receptor knockdown (*GlyR* KD) or subsequent to over-expression of the extruding chloride-cotransporter *KCC2* to reverse the chloride gradient. The treatment group results were compared to the control and are summarized in bar graphs (averages $\pm$ SD, *t*-test, ** $p < 0.0001$, * $p < 0.05$). The various interneurons were distinguished using the following markers: (A–E) Pax2 antibody ($n = 6–10$), (F–J) GAD 65/67 antibody ($n = 6–13$), (K–O) *Evx1* transgenic line expressing GFP from the *Evx1* promoter ($n = 4–22$), (P–T) *Alx2* transgenic line expressing GFP from the *Alx* promoter ($n = 6–20$), (U–Y) En antibody (7–15). Scale bar: 20 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

Motor neurons, spinal sensory Rohon-Beard neurons, and glial cells were not affected by blocking glycinergic signaling. The effect of manipulating glycinergic signaling from the beginning of development on the number of motor neurons, central sensory Rohon-Beard neurons, and glial cells was evaluated in 48-h old embryos raised in strychnine (Strych), after *GlyR* KD or subsequent to overexpression of *KCC2*. The treatment group results were compared to the control and are summarized in bar graphs (Averages $\pm$ Std, *t*-test, ** $p < 0.0001$, * $p < 0.05$). The various cell types were distinguished using the following markers: (A–E) *Isl* transgenic line expressing *GFP* from the *Isl* promotor mostly in motoneurons, which are the ventral population (below the dashed line; $n = 6–24$). F–J: *Ngn* transgenic line, which expresses at later stages selectively in Rohon-Beard sensory neurons (above the dashed line $n = 5–10$). K–O: *PLP* transgenic line which express *GFP* from the *PLP* promoter specifically in glia ($n = 4–8$). Scale bar: 20 μm . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

Glycine does not affect neural differentiation in the *beo* mutant. We examined the number of newborn and mostly glycinergic neurons using HU (A–D) and Pax2 (E–H), respectively, in the 48-h old *beo* mutant which lacks synaptically clustered glycine receptors. No statistical difference (Average $\pm$ SD, *t*-test) was found between these neural populations in wild type embryos (+/+, A, E, D, H, total *n* = 24), heterozygous siblings (+/?, B, F, D, H, total *n* = 29), and mutant embryos (*beo*^{-/-}, C, G, D, H, total *n* = 28). Scale bar: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]