Distribution of Prospective Glutamatergic, Glycinergic, and GABAergic Neurons in Embryonic and Larval Zebrafish

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ABSTRACT

Zebrafish are an excellent model for studies of the functional organization of neuronal circuits, but little is known regarding the transmitter phenotypes of the neurons in their nervous system. We examined the distribution in spinal cord and hindbrain of neurons expressing markers of transmitter phenotype, including the vesicular glutamate transporter (VGLUT) genes for glutamatergic neurons, the neuronal glycine transporter (GLYT2) for glycinergic neurons, and glutamic acid decarboxylase (GAD65/67) for GABAergic neurons. All three markers were expressed in a large domain in the dorsal two-thirds of spinal cord, with additional, more ventral expression domains for VGLUT2 and GAD/GABA. In the large dorsal domain, dual in situ staining showed that GLYT2-positive cells were intermingled with VGLUT2 cells, with no dual-stained neurons. Many of the neurons in the dorsal expression domain that were positive for GABA markers at embryonic stages were also positive for GLYT2, suggesting that the cells might use both GABA and glycine, at least early in their development. The intermingling of neurons expressing inhibitory and excitatory markers in spinal cord contrasted markedly with the organization in hindbrain, where neurons expressing a particular marker were clustered together to form stripes that were visible running from rostral to caudal in horizontal sections and from dorsomedial to ventrolateral in cross sections. Dual labeling showed that the stripes of neurons labeled with one transmitter marker alternated with stripes of cells labeled for the other transmitter phenotypes. The differences in the distribution of excitatory and inhibitory neurons in spinal cord versus hindbrain may be tied to differences in their patterns of development and functional organization. J. Comp. Neurol. 480:1–18, 2004. © 2004 Wiley-Liss, Inc.

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Zebrafish are an increasingly important model organism for studies of neuronal circuits and behavior. Although much is known about the cell types in their central nervous system (CNS), particularly in spinal cord (Bernhardt et al., 1990; Hale et al., 2001), very little information about the transmitter phenotypes of the cells is available. Such information is critical for understanding normal circuit function as well as for exploring the disruption of function in mutant (Granato et al., 1996) and transgenic lines.

We sought to reveal the distributions of excitatory and inhibitory neurons in zebrafish CNS by examining mRNA distributions of markers of neurotransmitter phenotype including the vesicular glutamate transporters, VGLUT1 and 2, for glutamatergic neurons, the glycine transporter GLYT2 for glycinergic neurons, and glutamic acid decarboxylase (GAD) for GABAergic neurons. VGLUT1 and 2 load glutamate into synaptic vesicles in glutamatergic neurons (Ni et al., 1995; Bellochio et al., 1998, 2000; Lee et al., 1999; Aihara et al., 2000; Takamori et al., 2000, 2001; Bai et al., 2001; Fremeau et al., 2001). GLYT2 is...
expressed in glycinergic boutons in mammals, where it is presumed to function in the reuptake of transmitter at glycinergic synapses, making it an excellent marker for glycinergic neurons (Jursky and Nelson, 1995; Luque et al., 1995; Zafra et al., 1995a, b; Spike et al., 1997; Roux and Supplisson, 2000; Gomeza et al., 2003). Two forms of GAD, GAD65 and GAD67, responsible for synthesis of the inhibitory transmitter GABA, have been identified in vertebrates, and mark GABAergic neurons (Erlander et al., 1991; Kaufman et al., 1991; Martin and Rimvall, 1993). Zebrafish homologs of the GAD genes exist and have been cloned (Martin et al., 1998).

Here, we clone the zebrafish homologs of VGLUT (VGLUT1 and VGLUT2) and GLYT2 by homology-based screening. We use cDNA probes specific for the zebrafish VGLUTs and GLYT2 together with probes for zebrafish GAD to examine their patterns of expression in spinal cord. We focus on spinal cord because it is the place where the neural circuits for rhythmic locomotion are located (Grillner, 1985; Pearson, 1993). Three different time periods (20–24 hours, 30–32 hours, and 4–5 days), which correspond to important time points in the motor development of the fish, were examined. The results document the overall distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in the spinal cord of embryonic and larval zebrafish, providing baseline information for subsequent developmental, functional, and genetic studies. Although our primary focus was spinal cord, we also describe the expression of the genes in hindbrain, where there was a columnar arrangement of stained neurons that was very different from the organization in spinal cord.

MATERIALS AND METHODS

Zebrafish

Embryonic and larval zebrafish (Danio rerio) bred from laboratory stock were maintained at 28.5°C. All experiments were approved by the State University of New York at Stony Brook animal care committee and were in accord with National Institutes of Health guidelines.

Cloning of zebrafish VGLUTs, GLYT, and GADs

Degenerate oligonucleotide primers for polymerase chain reaction (PCR) were designed based on regions of 5 or 6 amino acids in VGLUT that are shared by rat VGLUT1 (Ni et al., 1994) and EAT-4 (Lee et al., 1999), a Caenorhabditis elegans homolog of mammalian VGLUT protein. Complementary DNA from adult brains was used as a PCR template. Different combinations of primers were tested. The corresponding amino acid sequences of successful primers were YAGAV (primer 5′) and GFN-7 (3′) for VGLUT1. The nucleotide sequences of the four primers were: TA(T/C)GC(G/A/T/C)GG(G/A/T/C)GT, TG(G/A)TTG(A/T/C)AC(G/A)TTG(G/A)AAAG(T/C)CC, AT-(A/T/C)GA(G/A)GG(T/C)AT(A/T/C)GT(G/A/T/C)ATG, and GG(G/A/T/C)ATG(G/A/T/C)AC(G/A)AAAG(T/C)CC, respectively. To obtain upstream fragments of VGLUT2.2, PCR was performed with a 3′ primer specific to the VGLUT2.2 gene (AGTACTGACTAATAATCCAGCAGC) and a 5′ degenerate primer corresponding to the amino acid sequence FGIRCN (nucleotide sequence, TT(T/C)GG(G/A/T/C)AT/A/T/C(A/C)(G/A/T/C)TG(T/C)AA). During this PCR, not only VGLUT2.2 but also VGLUT2.1 were obtained due to the high homology between these two. Because the glycine transporters have only been cloned in mammals, to clone GLYT cDNAs from zebrafish we used degenerate oligonucleotide primers designed based on a region of 5 or 6 amino acids that are shared by rat GLYT1 and GLYT2. The amino acid sequences that were used successfully included GLGNVW (5′) and FHNNC (3′) for GLYT2 and CNNPWN (5′) and WGGLT (3′) for GLYT1. The nucleotide sequences of the four primers were: GG(G/A/T/C)AT/CGT(G/A/T/C)GG/AG/AT/C(AAT/T/C)GT(G/A/T/C)TT, TAGA/CAG/AT/TT/GAT(T/C)AT/TC, and GT(G/A/T/C)AT(G/A/T/C)AA (G/A)(G/A/T/C)CC(G/A/T/C)CCC, respectively. The GLYT2 fragment was obtained by using embryonic cDNA as a template while the GLYT1 fragment was obtained from adult brain cDNA. A portion of zebrafish GAD65 and GAD67 (700 bp, each) was amplified with PCR based on the published sequences (Martin et al., 1998). For VGLUT2.1, VGLUT2.2, GLYT2, and GAD67, RACE (rapid amplification of cDNA ends) was performed to obtain the full coding sequence. 5′ RACE was carried out according to Higashijima et al. (1997) by using embryonic cDNA as a template, while 3′ RACE was performed by using adult brain cDNA and a Marathon RACE kit (Clontech, Palo Alto, CA). Both embryonic and adult brain cDNAs were gifts from A. Inoue and H. Okamoto. We did not perform RACE against VGLUT1, GLYT1, and GAD65. Therefore, the present study did not reveal the full-length coding sequences for those genes.

In situ hybridization

For in situ hybridization, animals were fixed overnight in 4% formaldehyde in phosphate-buffered saline. After fixation, in situ hybridization was carried out with a standard protocol (Westefield, 1995). For single-color detection, complementary RNA probes were tagged with digoxigenin (Dig) and Dig was detected by alkaline phosphatase (AP) conjugated anti-Dig antibody (Dig and anti-Dig were from Roche, Penzberg, Germany). For nonfluorescence detection, NBT/BCIP (Roche) was used as the substrate for AP. For fluorescence detection, HNPP/Fast-Red was used as the substrate for AP, generating fluorescent products whose fluorescence spectrum is similar to rhodamine. For dual-color fluorescent in situ hybridization, one probe was Dig-labeled and detected with HNPP/Fast-Red as above. The other probe was tagged with fluorescein (Flu; Roche) and was detected ultimately by a Cy5-conjugated antibody after a series of amplification steps. This was done by using the following antibodies in this order: mouse monoclonal anti-FITC (fluorescein isothiocyanate) (Dak-FITC4 from Dako, Glostrup, Denmark), FITC-conjugated goat antimouse (Cappel, Durham, NC), mouse anti-FITC (the same as above), and Cy5-conjugated Fab fragment of donkey antimouse (Jackson ImmunoResearch, West Grove, PA). For in situ hybridization in older animals (beyond 36 hours postfertilization (hpf)), the ventral region of the body was excised when the samples were in fixative. The sample was first restrained with 1.2% of low-melting-point agarose. After solidification, fixative was added to cover the mounted sample. Then, under a dissecting microscope, a manual cut was made with a #11 scalpel blade at the level of the notochord to remove the ventral portion of the body below spinal cord. In the brain region, probes
and antibodies penetrated without dissection, so these areas were left intact.

Nonfluorescent samples were mounted in 70% glycerol and viewed with standard DIC microscopy. Fluorescent samples were mounted in 1.2% agarose because the fluorescent product from HNPP/Fast-Red is soluble in glycerol. The samples were viewed with a Zeiss 510 confocal laser scanning microscope (Zeiss, Thornwood, NY). The HNPP/Fast-Red signal was detected by 543 nm excitation, while the Cy5 signal was detected by 633 nm excitation. To view the brain, the skin overlying the brain was cut off to remove black pigment in the light path. The dissection was made with a razor blade while holding the fish as described above.

Twenty-micron cryostat sections of in situ samples (NBT/BCIP color development) were obtained by soaking the tissue in 30% sucrose, which was then mounted in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA) and frozen for sectioning. During this procedure the tissue shrank to roughly 70% of its original size. To compare the sectioned images with unsectioned ones, sectioned images were enlarged to match the unsectioned ones. Fixation and in situ hybridization themselves also caused tissue shrinking. The extent of shrinkage was variable from sample to sample, but the size was reduced to 80–95% of the original. We did not compensate for this effect and, thus, the size of the living tissue is slightly bigger than that indicated in the figures.

For each probe at each stage, we examined at least five animals. Based on visual inspection, there was little variation in the expression patterns of each gene at the same developmental time point in different fish.

Probes used for in situ hybridization

The cDNA sequences for VGLUT2.1, VGLUT2.2, GLYT2, and GAD67 genes were obtained as a contig of several subcloned PCR fragments. Dig- or Flu-labeled cRNA probes were generated from each subclone, and then the probes, each representing a different portion of a particular gene, were mixed and used for in situ hybridization. Because 3′ RACE clones for each gene were obtained relatively late in our study, they were not used for the probes. The total length and region used for each gene were as follows. The length used for VGLUT2.1 was 1.4 kb with 0.1 kb of the 5′ untranslated region (UTR) and 1.3 kb of the coding sequence that corresponded to amino acid (aa) residues 1–434 (Fig. 1B). VGLUT2.2 was 1.6 kb with 0.3 kb of the 5′ UTR and 1.3 kb of the coding sequence that corresponded to aa residues 1–435 (Fig. 1B). GLYT2 was 1.7 kb with 0.3 kb of the 5′ UTR and 1.4 kb of the coding sequence that corresponded to aa residues 1–435 (Fig. 1B). GAD67 was 1.5 kb with 0.3 kb of the 5′ UTR and 1.2 kb of the coding sequence that corresponded to aa residues 1–383 (Fig. 3). For VGLUT1, GLYT1, and GAD65, a portion of a cDNA clone obtained from a single PCR was used in each case. The length used for VGLUT1 was 0.5 kb. The corresponding aa sequence is the one shown in Figure 1C with additional 5–6 aa sequences on the N and C terminus sides, derived from PCR primers. The length for GLYT1 was 0.5 kb with the corresponding aa sequence shown in Figure 2C (plus 5–6 aa sequence on both sides). For GAD65 it was 0.7 kb, the same sequence shown in Martin et al. (1998).

The region used for in situ staining for a particular gene had homology with other genes (e.g., VGLUT2.1 with 3EXPRESSION OF VGLUT, GLYT2, AND GAD
VGLUT2.2). Thus, cross-hybridization might occur. However, in our typical experimental conditions, cross-hybridization appeared not to be a problem. For example, as described in Results, only VGLUT2.1 (and not VGLUT2.2) signal was observed in Rohon Beard neurons. This indicates that the VGLUT2.2 probe was not detecting VGLUT2.1 signal in these cells. Furthermore, because the focus of our study was to reveal the distribution of neurons having a particular neurotransmitter phenotype, VGLUT2.1 and VGLUT2.2 probes were mixed together in most of our experiments to reveal the collective pattern of expression of these genes. In these experiments, cross-hybridization between the two corresponding genes would not matter. Similarly, collective expression of GAD65 and GAD67 was examined for GABAergic neurons. The expression patterns of GLYT2 and GLYT1, revealed by in situ hybridization of each gene, were completely different (see Results). Therefore, we are confident that GLYT2 staining described in this study was from GLYT2, with no contamination of GLYT1 signal.

Although there was no clear evidence of cross-hybridization in our typical experimental conditions, when the in situ hybridization signal was substantially increased by prolonged color development and increased sensitivity during image collection on the confocal microscope (higher laser power and increased gain), we observed evidence of likely cross-hybridization. This was found in the case of VGLUT1. VGLUT1 signal was not detected in embryonic and larval spinal cord with our typical staining protocol. We did observe faint signal when...
the sensitivity of the in situ hybridization was substantially enhanced, as above. This staining pattern was similar to VGLUT2.1 or VGLUT2.2. This staining was most likely due to cross-hybridization to VGLUT2.1 and VGLUT2.2 because the staining disappeared with the presence of unlabeled VGLUT2.1 and VGLUT2.2.

Antibody staining

The anti-GABA antibody (rabbit polyclonal) was obtained from Sigma (St. Louis, MO). It was detected by Cy5-conjugated Fab fragment of donkey antirabbit secondary antibody (Jackson Immunoresearch). For dual staining with in situ hybridization, the anti-GABA antibody treatment was carried out after the in situ hybridization.

Figure production

Figures were produced by Adobe Photoshop software (v. 7, San Jose, CA) with adjustments in contrast and brightness.

RESULTS

Zebrafish homologs exist for VGLUT, GLYT, and GAD

Three different VGLUT-like sequences were obtained by PCR (Fig. 1). Comparison of the amino acid sequences of these three (145 amino acids) with the corresponding region of the previously identified rat VGLUT1 and VGLUT2 showed that one is closely related to VGLUT1, while the other two are closely related to VGLUT2 (Fig. 1A). None of the three were as closely related to a recently identified third vesicular glutamate transporter (VGLUT3), whose function in glutamatergic neurotransmission is less clear (Gras et al., 2002). Based on these comparisons, we named the three genes VGLUT1, VGLUT2.1, and VGLUT2.2.

Whole-mount in situ hybridization showed clear expression of VGLUT2.1 and 2.2 in spinal cord and brain of both embryos and larval fish, while VGLUT1 was expressed in a very limited region of the brain and was not expressed in spinal cord in animals up to at least 5 days of age (see below). Given our focus on spinal circuits in embryos and larvae, we further pursued the VGLUT2.1 and 2.2 clones to obtain their entire coding regions. Figure 1B shows the complete deduced amino acid sequence of VGLUT2.1 and 2.2. The deduced amino acid sequence of the identified region of VGLUT1 is shown in Figure 1C. Both VGLUT2.1 and 2.2 are highly homologous to rat VGLUT2 over their entire length (Fig. 1B), with an amino acid identity of 84% (VGLUT2.1 to rat VGLUT2) and 85% (VGLUT2.2 to rat VGLUT2). The amino acid similarities of the VGLUT2.1 and 2.2 to rat VGLUT1 or VGLUT3 are less (72–75%), indicating that both VGLUT2.1 and 2.2 are zebrafish homologs of mammalian VGLUT2. The amino acid similarities of the identified portion of zebrafish VGLUT1 (145 amino acids; Fig. 1C) compared to the corresponding region of the rat VGLUT1, 2, and 3 are 89%, 86%, and 85%, respectively. Thus, the zebrafish VGLUT1 obtained in this study is most likely a homolog of mammalian VGLUT1.

Two different zebrafish GLYT-like sequences were obtained by PCR amplification. One was similar to GLYT2, the neuronal form of the mammalian glycine transporters; the other was similar to GLYT1, the transporter expressed in glial cells in mammals (Fig. 2A) (Adams et al., 1995; Zafra et al., 1995a, b). Whole-mount in situ hybridization against embryonic and larval zebrafish showed that GLYT2 is expressed in a subset of neurons (see below), while GLYT1 is expressed in cells in the ventricular zone in embryonic spinal cord and a subset of cells in larva (data not shown). GLYT1-positive cells in larvae are predominantly located in the medial region of the spinal cord, and we presume them to be glial cells. Thus, the expression pattern of zebrafish GLYT2 and GLYT1 is consistent with that in mammals, where GLYT2 marks neurons and GLYT1 marks glial cells, with expression of GLYT1 in the ventricular zone at early developmental stages (Adams et al., 1995). These similarities suggest that the zebrafish genes are homologs of mammalian GLYT2 and GLYT1.

Given our focus on GLYT2, we further pursued this gene to obtain its entire coding region. Figure 2B shows the deduced full-length amino acid sequence of GLYT2. The amino acid sequence of zebrafish GLYT2 is 69% identical to rat GLYT2, while it is only 40% identical to rat GLYT1. Figure 2C shows a portion of the amino acid sequence of the identified region of GLYT1.

Cloning of portions of zebrafish GAD65 and GAD67 genes (700 bp each) has been reported previously (Martin et al., 1998). In the present study, we used PCR to obtain the same portion of those genes based on the published sequences and used RACE to extend the sequence of GAD67, an abundant form of GAD at embryonic stages. Figure 3 shows the predicted amino acid sequence of GAD67, which is highly homologous to human GAD67 along its entire length.

Expression patterns of the VGLUTs, GLYT2, and GADs in the spinal cord

The expression patterns of the identified genes were examined by whole-mount in situ hybridization at three different time points: 20–24 hours postfertilization (hpf), 30–32 hpf, and 4–5 days postfertilization (dpf). These correspond to times at which the fish show characteristic features of locomotion. The earliest (20–24 hpf) is when the embryos show spontaneous body contractions (Kimmel et al., 1995; Saint-Amant and Drapeau, 1998). At the middle time point (30–32 hpf), the embryos are still in their chorions, but they have acquired the ability to produce alternating bending movements (Saint-Amant and Drapeau, 1998). Thus, the basic circuits for such movements, which are likely to develop into mature swimming, are in place. At the last time point (4–5 dpf), the fish are hatched and freely swimming.

To identify putative glutamatergic neurons, we first performed in situ hybridization using each VGLUT gene as a probe. We found that VGLUT1 was not expressed in the spinal cord at 30–32 hpf or at 4–5 dpf. In contrast, VGLUT2.1 and VGLUT2.2 were both expressed in spinal cord at 30–32 hpf and their expression patterns were very similar to one another except for one region (see below), suggesting that the two genes are coexpressed by the majority of glutamatergic neurons. Thus, we used a mixture of VGLUT2.1 and 2.2 as a probe to investigate the distribution of glutamatergic neurons. This is referred to as VGLUT2 in the following.

For in situ hybridization for glycinergic neurons, we used GLYT2 as a probe. For GABAergic neurons, we used a mixture of GAD65 and 67. GABA can also be used as a
marker for GABAergic neurons. We also stained with antibodies to GABA and found almost complete overlap of GAD and GABA staining (data not shown). We primarily used GAD for describing prospective GABAergic neurons. However, GABA staining was used in a few instances because it was technically easier to apply in double-staining experiments.

**Expression of VGLUT2 and GLYT2 in 20–24 hpf embryos**

There is a rostrocaudal gradient of differentiation in the spinal cord of developing embryos, with rostral regions being more differentiated than caudal ones. This is reflected in a rostrocaudal gradient of expression of VGLUT2 and GLYT2 in 20–24 hpf embryos. We therefore describe the expression patterns of these genes in both rostral and more caudal regions, at about segments 7–9 and segments 15–17, respectively.

In the rostral region of the cord at 20 hpf, VGLUT2 expression is detected in cells scattered from midway along the dorsoventral extent of cord to its dorsalmost region (Fig. 4A). In the caudal portion of the cord, VGLUT2 expression is only detected in cells located in the dorsalmost region (Fig. 4B). These cells (arrows in Fig. 4A or all labeled cells in Fig. 4B) are Rohon Beard (RB) neurons. RB neurons are primary sensory neurons located at the dorsal edge of the spinal cord (Clarke et al., 1984; Bernhardt et al., 1990). They are among the earliest cells to differentiate (Kuwada et al., 1990). Our staining indicates that RB neurons are very likely to be glutamatergic, and that RB neurons already express VGLUT2 at this early stage. The results also indicate that, in addition to RB neurons, several prospective glutamatergic neurons have differentiated in the rostral region of the cord at 20 hpf. These are located in the upper half of spinal cord in a region we call the mediadorsal domain. From 20–24 hpf, the number of labeled cells in the mediadorsal expression domain increases. In the rostral cord, there are more labeled cells in this expression domain at 24 hpf than at 20 hpf (Fig. 4C). In the caudal region of the cord, several labeled cells belonging to the mediadorsal expression domain are recognized at 24 hpf (Fig. 4D), while only RB neurons were labeled at 20 hpf (Fig. 4B). In the spinal cord at these early stages, postmitotic neurons are only present near the pial surface of the cord where the VGLUT2-positive cells were located (see also Fig. 5A).

The distribution pattern of GLYT2-positive cells in 20–24 hpf embryos is very similar to that of the mediadorsal VGLUT2-positive cells. At 20 hpf, a small number of GLYT2-positive cells are recognized in the rostral cord (Fig. 4E). In the caudal cord, no GLYT2-positive cells are detected (Fig. 4F). From 20–24 hpf, GLYT2-positive cells increase in number. In the rostral cord, more positive cells are present (Fig. 4G), while in the caudal cord a small number of positive cells becomes evident (Fig. 4H).

Changes in the number of VGLUT2- and GLYT2-positive cells in the mediadorsal domain (void of RB neurons) were quantified. The number of positive cells on each side of three segments (segments 7–9 for the rostral region and 15–17 for the caudal region) was counted and divided by three. This mean value of the three segments was used to represent the number of positive cells per hemisegment of the corresponding region. Data were collected from three animals for a total of six rostral and six caudal hemisegments. At 20 hpf, the average number of VGLUT2.1-positive cells per segment in the rostral region was 5.5 (standard deviation (SD) = 0.4), while that in the caudal region was 0.1 (SD = 0.2). At 24 hpf, the number increased, with 9.6 (SD = 0.6) in the rostral region and 2.9 (SD = 0.5) in the caudal region. The average number of GLYT2-positive cells per segment at 20 hpf was 2.9 (SD = 1.0) in the rostral region and 0 (SD = 0) in the caudal region. At 24 hpf, the number increased, with 8.4 (SD = 1.2) in the rostral region and 1.7 (SD = 0.3) in the caudal region.

**Expression patterns of VGLUT2 and GLYT2 in 30–32 hpf embryos**

Although a rostrocaudal differentiation gradient is still present in 30–32 hpf embryos, we focused our observations at this stage on a restricted midbody region (segment 6–13) where the gradient was less apparent. The VGLUT2 expression pattern in 30–32 hpf embryos is similar to that seen in 24 hpf embryos. The gene continues to be expressed in RB neurons (arrows in Fig. 5A–C). In the mediadorsal expression domain, more positive cells are recognized (asterisks in Fig. 5A,B), suggesting that more cells have differentiated. Although this dorsal expression domain (RB neurons and the mediadorsal region) is predominant, a second expression domain is present at this stage. This domain was formed by occasional VGLUT2-positive cells located near the ventral edge of the spinal cord (arrowheads in Fig. 5A,C). The number of positive cells in this ventralmost expression domain at this stage is very small compared to the other expression domains, with one cell per hemisegment or even less on average. All of the VGLUT2-positive cells are located near the outer surface of the cord, consistent with the location of postmitotic cells at this stage (Fig. 5A). GLYT2 in 30–32 hpf embryos also shows an expression pattern similar to that in 24 hpf embryos, but with more positive cells. Thus, the gene continues to have a single expression domain in the mediadorsal region of the cord (Fig. 5D,E). The region occupied by cells expressing GLYT2 is very similar to the mediadorsal expression domain of VGLUT2 (compare cells marked with asterisks in Fig. 5A,D). Furthermore, the overall distribution patterns of positive cells within the expression domain are very similar. Both genes show scattered positive cells and the numbers of positive cells appear comparable with the two markers (compare cells in region marked by an asterisk in Fig. 5B,E). It appeared that there were at least 15 positive cells per hemisegment for each gene in the mediadorsal domain of segments 7–9 (six hemisegments were examined). However, positive cells were sometimes stacked, making it difficult to perform accurate counts at this stage (and even more difficult at later stages).

We examined the relationship between VGLUT2-positive cells and GLYT2-positive cells in this expression domain by performing dual-color fluorescent in situ hybridization. We found that VGLUT2 and GLYT2 label different cells, producing mutually exclusive dual labeling patterns (Fig. 5G). No cell was found which was positive for both VGLUT2 and GLYT2. This was expected because VGLUT2 and GLYT2 are markers of glutamatergic neurons and glycinergic neurons, which are generally assumed to be two different populations. Although they are mutually exclusive populations, the VGLUT2-positive and GLYT2-positive cells extensively intermingle with one another, accounting for the scattered expression patterns of...
the individual genes. VGLUT2-positive and GLYT2-positive cells appear to account for a majority of the postmitotic cells in this region. Motoneurons are likely to be located just ventral to this domain. Consistent with this, the vesicular acetylcholine transporter gene, a marker of cholinergic neurons, is expressed in the ventral region, where there is no VGLUT2 and GLYT2 expression (data not shown).

Fig. 4. Expression patterns of VGLUT2 and GLYT2 in the spinal cord at 20–24 hpf. Expression of VGLUT2 (A–D) and GLYT2 (E–H) were detected by whole-mount in situ hybridization. VGLUT2 images were from staining with a mixture of VGLUT2.1 and 2.2 probes. All pictures are lateral views of the spinal cord. In this figure and all other figures, rostral is to the left. Two developmental time points were examined: 20 hpf (A,B,E,F) and 24 hpf (C,D,G,H). At 20 hpf, the rostral images (A,E) are at segments 6–8, while the caudal parts (B,F) are at segments 13–17. At 24 hpf, the rostral images (C,G) are at segments 7–9, while the caudal ones (D,H) are segments 15–17. The lines with double arrows (labeled SC in A) indicate the dorsal and ventral borders of the spinal cord. A: In the rostral spinal cord at 20 hpf, VGLUT2 expression is detected in cells scattered from the middle to the dorsalmost region of the cord. The dorsalmost cells (arrows) are Rohon Beard (RB) neurons (see Fig. 5). B: In the caudal spinal cord, expression was detected in only those cells located most dorsal (RB neurons). C: The rostral region of the cord at 24 hpf shows essentially the same expression pattern as that at 20 hpf. However, more positive cells are evident. Note that many of the dorsalmost RB neurons are out of focus in this picture and, thus, they are not clearly seen. They continue, however, to express VGLUT2 (see Fig. 5). D: In the caudal region at 24 hpf, in addition to RB neurons, a small number of somewhat more ventral cells start to express VGLUT2. E: In the rostral spinal cord at 20 hpf, GLYT2 expression is detected in a small number of cells located in the mediodorsal region of the cord. F: In the caudal region, no GLYT2 expression is detected at 20 hpf. G: In the rostral region at 24 hpf, the number of GLYT2-positive cells increases relative to 20 hpf. H: In the caudal region at 24 hpf, a small number of cells start to express GLYT2. Scale bar = 50 μm.
The previous observations were obtained with a mixture of VGLUT2.1 and VGLUT2.2 as a probe. We also investigated the expression patterns of each of these genes at 30–32 hpf. Only VGLUT2.1 expression was detected in RB neurons (arrows in Fig. 5H), while the VGLUT2.1-positive and VGLUT2.2-positive cells in the mediodorsal expression domain overlapped (dots in Fig. 5H). Low sensitivity of one of the colors in dual in situ hybridization prevented us from concluding that there was 100% overlap in the expression patterns of these two genes in the mediodorsal and ventralmost regions. Nonetheless, the majority, if not all, of the VGLUT2-positive cells in those areas express both VGLUT2.1 and 2.2.

Fig. 5. Expression patterns of VGLUT2 and GLYT2 in the spinal cord at 30–32 hpf. In all figures, rostral to middle body regions (segments 6–13) are illustrated. Except for A, D, and F, pictures are confocal optical sections of the spinal cord in lateral view. The schematic drawing (F) shows regions of the optical sections in each figure. VGLUT2 represents staining with a mixture of VGLUT2.1 and 2.2 probes. A: A cross section showing VGLUT2 expression. VGLUT2 is expressed in cells in the dorsalmost region (RB neurons; arrows), cells in the mediodorsal region (asterisk) and very ventral cells (arrowhead). B: VGLUT2 expression in the lateral region of the cord. At this section, cells belonging to the mediodorsal expression domain are mainly visible (asterisk). The dorsalmost RB neurons (arrows) are visible, although they are more clearly in focus in C. C: VGLUT2 expression in a slightly more medial section. The RB neurons (arrows) and a cell belonging to the very ventral expression domain (arrowhead) are recognized. Small numbers of cells belonging to the mediodorsal expression domain are also visible. The number of the VGLUT2-positive cells belonging to the most ventral expression domain is small. D: A cross section showing GLYT2 expression. GLYT2 is expressed in the mediodorsal region of the cord (asterisk). E: GLYT2 expression in the lateral region of the cord. F: Dual in situ hybridization with GLYT2 and VGLUT2 in the mediodorsal expression domain. Cells expressing GLYT2 and cells expressing VGLUT2 are mutually exclusive. G: Dual in situ hybridization with VGLUT2.1 and VGLUT2.2. The optical section is slightly medial to G (see F). Note that only VGLUT2.1 is expressed in RB neurons (arrows). Other cells belonging to the mediodorsal expression domain (dots) express both VGLUT2.1 and 2.2. I: Dual in situ hybridization with VGLUT2.1 and Islet-1 (Isl1). The dorsalmost cells labeled by VGLUT2.1 (or a mixture of VGLUT2.1 and 2.2) are also positive for Isl1, confirming that these cells are RB neurons. Scale bar = 20 μm.
Expression of VGLUT2, GLYT2, and GAD

The dorsalmost VGLUT2-positive cells are likely to be RB neurons based on morphology. Moreover, dual in situ hybridization of VGLUT2 and Iislet-1 (Isl1), a gene known to be expressed in RB neurons (Korzh et al., 1993; Inoue et al., 1994), indicated that the dorsalmost VGLUT2 (or VGLUT2.1)-expressing cells indeed overlapped with Isl1-positive cells (Fig. 5I).

To summarize, VGLUT2 is expressed in RB neurons, cells in the mediodorsal region, and a small number of cells in ventral spinal cord. Of the two types of VGLUT2, only VGLUT2.1 is expressed in RB neurons, whereas both genes are expressed in the other cells. GLYT2 is expressed in the mediodorsal region of the cord in a pattern similar to the expression of VGLUT2 in the same region. The mediodorsal expression domain of VGLUT2/GLYT2 appears to be located just above the motoneurons. VGLUT2-positive cells and GLYT2-positive cells are mutually exclusive, with extensive intermingling among the two populations.

Expression of GAD/GABA in 30–32 hpf embryos and its relationship to the expression of GLYT2 and VGLUT2

GABA and GAD expression in early stage embryos (up to 27 hpf) has been described (Bernhardt et al., 1992; Martin et al., 1998). We found essentially the same expression pattern at 30–32 hpf. GAD has two expression domains: one is in the mediodorsal region (asterisk in Fig. 6A,B) and the other in ventral cord (ventral cells in Fig. 6A; all labeled cells in Fig. 6C). The cells belonging to the ventral expression domain have been named Kolmer-Agduhr (KA) and are known to contact the central canal (Fig. 6A). The expression level of GAD in the cells in the mediodorsal region is much lower than that in KA neurons (Fig. 6A).

The number of GAD-positive cells in the mediodorsal expression domain (asterisk in Fig. 6B) appeared to be smaller than that of VGLUT2-positive cells (asterisk in Fig. 5B) and GLYT2-positive cells (asterisk in Fig. 5E) in the same region. In addition, the center of the GAD-positive cell population along the dorsoventral axis appeared to be slightly ventrally shifted compared to that of the VGLUT2- or GLYT2-positive cell population. Nonetheless, the general region and pattern of GAD expression was similar to those of VGLUT2 and GLYT2. Therefore, we investigated the relationship between GAD (or GABA)-positive cells and GLYT2- or VGLUT2-positive cells.

Dual-color in situ hybridization with GLYT2 and GAD showed that the majority of GAD-positive cells in this region are also positive for GLYT2 (Fig. 6D). Approximately one-third to one-half of the GLYT2-positive cells were positive for GAD. The same result was obtained when dual staining was performed with GABA immuno-staining and GLYT2 in situ hybridization. The majority of GABA-positive cells are also positive for GLYT2 (Fig. 6E). Dual-positive cells for GABA/GABA and GLYT2 tend to be among the relatively ventrally located GLYT2-positive cells (Fig. 6D,E). However, not all ventrally located GLYT2-positive cells were GABA/GABA-positive, and dually positive cells could sometimes be found among more dorsal GLYT2-positive cells.

The GAD/GABA expression level was highly variable among positive cells. This was especially the case for GABA (Fig. 6E). We often found cells which had very faint GABA staining. We did not consider these cells as GABA-positive, but if they had been included the frequency of dual-positive cells would have increased. Finally, although the majority of GAD/GABA-positive cells in this region are GLYT2-positive, there were GAD/GABA-positive cells which were not accompanied by a detectable level of GLYT2 expression (thin and thick arrows in Fig. 6D,E). Among them, one characteristic cell, which was separable from the others, was consistently negative for GLYT2. These cells were large in size, dorsally located, and heavily stained with GABA (thick arrow in Fig. 6E). A rostrally directed process was often visible from the ventral part of the cell. As shown in the accompanying article, these cells belong to a cell type called the dorsal longitudinal ascending neurons (DoLA).

Dual staining with GABA and VGLUT2 showed that the majority of GABA-positive cells in the mediodorsal region are negative for VGLUT2 (Fig. 6F). This was an expected result because 1) the majority of GABA-positive cells are also positive for GLYT2, and 2) GLYT2 and VGLUT2 expression patterns are mutually exclusive. There were, however, a few cells positive for both GABA and VGLUT2 (dot in Fig. 6F). The ventralmost VGLUT2-positive cells (arrowheads in Fig. 5A,C) are near the location of the GAD/GABA-positive KA neurons (ventral cells in Fig. 6A,C). This led us to investigate the relationship between the two. Dual staining showed that the ventralmost VGLUT2-positive cells (arrowhead in Fig. 6G) are not the KA neurons (cells in red on Fig. 6G).

To summarize, GAD/GABA is expressed in cells in the mediodorsal region and in KA neurons at 30–32 hpf. The majority of GAD/GABA-positive cells in the mediodorsal expression domain are also positive for GLYT2 and negative for VGLUT2.

Expression patterns of VGLUT2, GLYT2, and GAD in 4–5 dpf larvae

For 4–5 dpf fish, the same body region was examined as for 30–32 hpf samples (segments 6–13). In Figure 7, two typical cross sections (Fig. 7A,B,F,G,K,L) and two optical sections in lateral view from lateral and medial locations (Fig. 7D,E,I,J,N,O) are shown for each gene. It is apparent that a greater number of VGLUT2, GLYT2, and GAD-positive cells is present relative to 30–32 hpf (Fig. 7), although the density of cells made it difficult to perform exact counts. An increase in the number of positive cells is especially noticeable for VGLUT2 and GLYT2. Notably, positive cells are now found in the inner region of the spinal cord (Figs. 7A,B,F,G,K,L). It appears that proliferation in the ventricular zone is almost over, and postmitotic neurons are now predominant, even in the inner region of the cord. At this stage, the lateral margin of the spinal cord is largely occupied by axons and dendrites. Consequently, very few labeled cells are found there.

Each gene has overlapping, but different expression domains (Fig. 7C,H,M). VGLUT2 has the most complicated expression pattern (Fig. 7C). The most ventral expression is at the bottom of the cord. The region above this (up to around one-third of the dorsoventral extent of cord) is predominantly occupied by motoneurons and, thus, is devoid of VGLUT2 expression. However, lateral to this motoneuron region, VGLUT2 is expressed by typically only one cell in a cross section and that cell is located near the lateral margin of the spinal cord (see the arrow in Fig. 7A). Among all of the cells labeled by
VGLUT2, GLYT2, and GAD, this unusual VGLUT2 cell type is the only one which was located in the lateral marginal zone.

Dorsal to the motoneurons, many VGLUT2-positive cells are present, and this expression domain continues to near the dorsalmost extent of the cord. Motoneurons and
VGLUT2-positive cells may intermingle in the ventralmost region of this large expression domain. There was an overall tendency for more VGLUT2-positive neurons to be present in the lateral portion of this large expression domain. This tendency is apparent both in cross sections (Fig. 7A,B) and in optical sections from lateral view (number of labeled cells in Fig. 7D is larger than E). In the dorsal region, this tendency goes to the extreme: almost no positive cells were detected in the most medial regions of dorsal cord (Fig. 7C), except at its extreme dorsal edge. These extremely dorsal cells (arrows in Fig. 7E) are likely RB neurons—the characteristic cell shape is evident in the leftmost cell in Figure 7E.

GLYT2 has only one expression domain (Fig. 7F–H), which is located dorsally and encompasses approximately two-thirds of the cross section of the spinal cord. J: GLYT2 expression in a more medial section. At both of the mediolateral levels, GLYT2-positive cells are predominantly located in the dorsal half of the cord. At the medial level, however, GLYT2-positive cells can occasionally be located more ventrally (arrow in J). K,L: Two cross sections showing GAD expression. Arrows in K mark GAD-positive KA neurons which contact the central canal. M: A schematic drawing showing the expression domain of GAD. Arrows labeled N and O show the mediolateral levels of optical sections shown in N and O. N: GAD expression in the lateral region of the cord. At this level, GAD-positive cells are located in the dorsal half of the cord. O: GAD expression in a more medial section. At this level, in addition to cells in the dorsal half, GAD-positive KA neurons are visible in a ventral region of the cord. The expression domain of GAD in the dorsal half in both sections is very similar to that of GLYT2, although the expression domain of GLYT2 is slightly wider. In addition, the density of GLYT2-positive cells is much higher than that of GAD. Scale bar = 20 μm.
positive cells are different from the cells are negative for GLYT2 at 4–5 dpf. Thus, at 4–5 dpf, a large fraction of VGLUT2-positive and GAD-positive were also generally mutually exclusive, although the low sensitivity of this combination of dual in situ prevented a more careful analysis (data not shown).

Dual staining also revealed that cells positive for one gene extensively intermingle with cells positive for the other genes. The expression domains of VGLUT2 and GLYT2 (above the motoneurons) are slightly shifted relative to one another, but largely overlap (Fig. 7C,H). When optical sections were examined along the lateral to medial axis, intermingling was observed at many levels, although the density of positive cells for each gene changed (more VGLUT2-positives laterally and more GLYT2-positive cells medially). The GLYT2 expression domain and the GAD expression domain (excluding the KA domain) mostly overlap (Fig. 7H,M). Like VGLUT2 and GLYT2, GADGLYT2-positive cells and GAD-positive cells intermingled in all of the optical sections.

To summarize, the main expression domain of all three genes at 4–5 dpf is in the dorsal two-thirds of the spinal cord. There, more VGLUT2-positive cells are found in lateral regions, while GLYT2 and GAD-positive cells are more uniformly distributed. Despite this slight differential distribution, the expression domains are largely overlapping. GAD-positive cells are smaller in number. VGLUT2- and GLYT2-positive cells are mutually exclusive, as at earlier stages. Unlike early stages, many of the GAD-positive cells at this stage are not GLYT2-positive. VGLUT2 has two additional expression domains. One is formed by one cell type which lies on the lateral margin at a position of about a quarter of the way up from the bottom of the cord. The other expression domain is at the bottom of the cord. GAD has one additional expression domain formed by the KA neurons, which have contact with the central canal.

Expression patterns of VGLUT1, VGLUT2, GLYT2, and GAD in the midbrain and hindbrain at 4–5 dpf

Although the primary focus of this study is spinal cord, the genes we identified are also expressed in the brain. The expression patterns in the midbrain and hindbrain differed considerably from those in spinal cord. Here, we briefly describe the unique features of these expression patterns.

VGLUT1 expression in the brain is restricted to an area at the rostral edge of the hindbrain near its lateral edge (thick arrows on horizontal optical sections in Fig. 9A). In this expression domain, VGLUT1-positive cells are continuously detected in all of the optical sections along the dorsoventral axis, with gradual expansion of the expression domain ventrally. Outside this domain, a very small number of cells in the medial region of rhombomeres 5 and 6 (r5 and r6) appear to express VGLUT1. Other than these, no clear VGLUT1-positive cells were recognized.

VGLUT2 and GAD expression are found throughout much of the hindbrain and midbrain (Fig. 9B,D). VGLUT2 appears not to be expressed in the area where VGLUT1 is expressed (thick arrows in Fig. 9B). Complementary expression of VGLUT1 and VGLUT2 in prospective glutamatergic neurons is also evident in rodents (Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002). The...
GLYT2 expression pattern differs strikingly from VGLUT2 and GAD. The expression domain has a sharp boundary in the rostral hindbrain (Fig. 9C). The position of the boundary lies near the rhombomere 1 (r1) and r2 border based on staining of preparations with back-filled reticulospinal neurons (data not shown). Rostral to this boundary, almost no GLYT2-positive cells were found except for a very small number of cells in the ventral region of the midbrain (data not shown). The expression of GLYT2 in midbrain and hindbrain is also in accord with the expression pattern of the gene in mammals (Luque et al., 1995; Zafra et al., 1995a). In example, in the middle region of the hindbrain near the border of rhombomeres 1 and 2, GLYT2 expression is absent in those regions, but widespread elsewhere. C: GLYT2 expression. The GLYT2 expression domain has a sharp boundary that appears to lie in the rostral hindbrain near the border of rhombomeres 1 and 2. D: GAD expression. In B, C, and D, small arrows denote stripe-like expression extending along the rostrocaudal axis. Scale bar = 50 μm.

DISCUSSION

VGLUT and GLYT gene families in zebrafish

Our results show that the zebrafish genome contains homologs of both VGLUT1 and VGLUT2. This suggests that VGLUT1 and VGLUT2, whose homology is most likely the result of gene duplication, were already present in the ancestral vertebrate which gave rise to mammals and teleosts. Unlike the case in mammals, two VGLUT2 genes (VGLUT2.1 and 2.2) are present in the zebrafish genome, probably reflecting an additional chromosomal duplication event (Postlethwait et al., 1998). VGLUT2.2 is largely coexpressed, except for the case of RB neurons, where only VGLUT2.1 is evident. The isolated expression of VGLUT2.1 in RB cells may be explained by the loss of regulatory elements of the VGLUT2.2 gene, as suggested for many zebrafish genes (Force et al., 1999).

We found only one VGLUT1 gene and no VGLUT3 gene in zebrafish, but cannot rule out the possibility that there are more VGLUT homologs that were missed.

The majority of prospective glutamatergic neurons in zebrafish spinal cord and hindbrain express VGLUT2 up to 5 days of age, consistent with data from rodents (Aihara et al., 2000; Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002). In rodents, VGLUT1 is eventually upregulated and dominates the expression pattern later in life (Ni et al., 1995). Our studies focused on early stages in zebrafish (up to day 5 in an animal that reaches sexual maturity at 3 months), so we do not know if VGLUT1 is upregulated later, as in rodents.
Fig. 10. Expression patterns of VGLUT2, GLYT2, and GAD in the larval hindbrain. All are cross sections of 4–5 dpf larvae. A–C: VGLUT2 expression. D–F: GLYT2 expression. G–I: GAD expression. A, D, and G are sections of rostral hindbrain (around rhombomere 2). B, E, and H are sections of middle hindbrain (around rhombomere 4–5). C, F, and I are sections of caudal hindbrain. J: Dual staining of GAD and VGLUT2 at the level of caudal hindbrain. K: Dual staining of GLYT2 and VGLUT2 at the level of caudal hindbrain. Note the stripe-like patterns of expression of the various genes and the alternating stripes in the dual stained sections in J and K. Scale bar = 50 μm.
Zebrafish have homologs of mammalian GLYT1 and GLYT2 and their expression patterns match well with those of mammals (Adams et al., 1995; Luque et al., 1995; Zafra et al., 1995a, 1997). GLYT2 is expressed in neurons, while GLYT1 is expressed in cells in the ventricular zone in early stages, and in a subset of cells in larvae, which we presume to be glial cells.

**In situ probes as markers of transmitter phenotype**

The reliability of the genes we studied as markers of transmitter phenotype is important. For glycinergic neurons, we used GLYT2 as a marker. Wherever studied, GLYT2 protein is detected in all glycinergic boutons (Jur-sky and Nelson, 1995; Zafra et al., 1995a; Spike et al., 1997), where it is thought to participate in the reuptake of the neurotransmitter at inhibitory glycinergic synapses (Roux and Supplisson, 2000; Gomez et al., 2003). Thus, the available evidence strongly indicates that GLYT2 is a reliable marker for glycinergic neurons. For glutamatergic neurons, we used VGLUT2 as a marker. In the past several years an array of biochemical, anatomical, electrophysiological, and genetic evidence has unambiguously established that these proteins are the vesicular glutamate transporters that are directly responsible for loading glutamate into synaptic vesicles in glutamatergic neurons (Lee et al., 1999; Bellochio et al., 2000; Takamori et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Takamori et al., 2001). Anatomically, VGLUT1 and VGLUT2 show complementary expression in the mammalian CNS, and their distribution accounts for most of the known glutamatergic neurons of the CNS (Fremeau et al., 2001; Herzog et al., 2001; Hisano and Nogami, 2002; Kaneko and Fujiyama, 2002; Varoqui et al., 2002). Most important, Takamori et al. (2000) have shown that ectopic expression of VGLUT alone can make nonglutamatergic neurons release glutamate. These data strongly indicate that expression of VGLUT serves as a reliable marker for glutamatergic neurons. For GABAergic neurons, we used GABA itself or GAD (a mixture of GAD65 and 67) as markers. The latter encodes a protein that catalyzes the synthesis of GABA (Martin, 1993). The distributions of GABA and GAD are virtually identical in the zebrafish CNS and, thus each serves as a marker for the presence of GABA in the neurons.

Taken together, all of the available evidence strongly supports the conclusion that if a cell is found to be positive for one of the markers we used, it is very likely to have the corresponding neurotransmitter phenotype. Where there was other independent evidence for the transmitter phenotype of neurons (such as the glutamatergic phenotype of Rohon Beard (RB) sensory neurons; Sillar and Roberts, 1988; Li et al., 2003), our data led to a conclusion consistent with the other evidence.

**Expression patterns of VGLUT2 and GLYT2 genes in the spinal cord**

In the following sections, we place the developmental changes in VGLUT2- and GLYT2-positive cells in the context of physiological and behavioral changes in spinal motor systems. We focus on VGLUT2 and GLYT2, as opposed to GAD, because glutamate and glycine are the major neurotransmitters implicated in spinal rhythm generating networks (Roberts et al., 1998; Buss and Drapeau, 2001).

### 20–24 hpf embryos

At 20–24 hpf zebrafish embryos show spontaneous contractions (Kimmel et al., 1995; Saint-Amant and Drapeau, 1998). Electrophysiological recordings from motoneurons and interneurons at this time show periodic bursts of synchronous firing in the cells (Saint-Amant and Drapeau, 2000, 2001). These recordings also demonstrate periodic glycinergic synaptic bursts in motoneurons and prospective premotor interneurons, indicating that there are functional glycinergic synapses at this stage. The observed synaptic bursts grow in their robustness over the time from 20–24 hpf (Saint-Amant and Drapeau, 2000). This increase in inhibition is consistent with our observations that GLYT2-positive cells are present in the rostral region of the spinal cord as early as 20 hpf (Fig. 4E) and increase in number over time (Fig. 4G). The GLYT2-positive cells revealed in this study were very likely the presynaptic source of the glycinergic bursts. The addition of more of these neurons during development may explain the increase in the strength of the synaptic bursts.

In contrast to glycinergic synapses, the presence of glutamatergic synapses onto motoneurons and premotor interneurons at this early stage is less clear. The distribution of VGLUT2-positive cells in the mediodorsal region is similar to that of GLYT2-positive cells. Therefore, glutamatergic synapses might be expected at these stages. Saint-Amant and Drapeau (2001) indicated, however, that electrical coupling among neurons on one side is the primary source of the excitation for the spontaneous contractions. If so, these early glutamatergic neurons may play some other functional role, perhaps in reflex pathways. The RB neurons are positive for VGLUT2, as predicted from studies of other vertebrates (Sillar and Roberts, 1988; Li et al., 2003). Because zebrafish embryos respond to a touch on the body by 24 hpf (unpubl. obs.), glutamatergic synapses from RB neurons are likely to be functional.

### 30–32 hr embryos

By 30–32 hpf, the majority of embryos can produce alternating bending movements, which appear to develop into mature swimming (Saint-Amant and Drapeau, 1998). One of the key neuronal components for rhythmic bending such as swimming is the neurons mediating reciprocal inhibition between the two sides of the body (Grillner, 2003; Roberts et al., 1998). We therefore might expect that the maturation of swimming movements would be associated with a further increase of GLYT2-positive cells by 30–32 hpf. This was indeed the case. Moreover, we examined the morphology of the GLYT2-positive cells at a slightly later stage (32–36 hpf) and found that many of them were indeed commissural interneurons (Higashijima et al., accompanying article). They are likely to be involved in reciprocal inhibition between opposite sides of the body.

Descending excitatory interneurons are important for driving the spinal central pattern generators in swimming vertebrates (Grillner, 2003; Roberts et al., 1998). We observed an increase in VGLUT2-positive spinal neurons in conjunction with the development of swimming movements. Our studies of the identities of VGLUT2-positive cells revealed that they include a population of descending interneurons (accompanying article). Some of these de-
scandial cells may contribute the excitatory drive important for the production of rhythmic swimming movements.

### 4–5 dpf larvae

By 4–5 days, primary neurogenesis in spinal cord appears complete. The larval fish at this stage are free-swimming animals having a complex locomotive repertoire including distinct types of swimming and turning as well as movements associated with prey capture (Budick and O’Malley, 2000). Many of the classes of spinal interneuron at this stage have been previously described. The expression patterns of the genes we studied correspond to the locations of these known classes of spinal neurons (Higashijima et al., accompanying article).

**VGLUT2** has three expression domains. One is near the bottom of the spinal cord. Ventral medial (VeMe) interneurons are included in this expression domain. The second expression domain is located far laterally in ventral spinal cord. Multipolar commissural descending (MCoD) interneurons form this expression domain. The third expression domain is the largest, spanning approximately two-thirds of the spinal cord dorsally. Circumferential descending (CiD) interneurons and unipolar commissural descending (UCoD) interneurons occupy the ventral part of this domain (corresponding to the middle of the spinal cord along its dorsoventral axis). More dorsally, **VGLUT2**-positive cells are restricted to lateral regions. The commissural primary ascending (CoPA) interneurons and commissural secondary ascending glutamatergic (CoSA-glu) interneurons are located in this area. Finally, the glutamatergic RB sensory neurons are located at the top of the spinal cord.

**GLYT2** has a single expression domain, which spans approximately two-thirds of the spinal cord dorsally. The three known classes of glycinergic cells in 4–5 dpf larvae are commissural longitudinal ascending (CoLA) interneurons, commissural bifurcating longitudinal glycinergic (CoBL-gly) interneurons, and circumferential ascending (CiA) interneurons (accompanying article). CoLA interneurons are only found in the relatively lateral and ventral region of the **GLYT2** expression domain. CoBL-gly interneurons appear to be abundant and are distributed throughout the **GLYT2** expression domain. CiA interneurons are also widely distributed, particularly in the dorsal portion of the expression domain (Higashijima et al., 2004).

### Relationship of GAD/GABA and GLYT2 expression

Recent studies have shown that spinal neurons in mammals can coexpress the two main inhibitory neurotransmitters, GABA and glycine (van den Pol and Gorcs, 1988; Todd et al., 1996). In addition, GABA and glycine can be coreleased from the same vesicles (Jonas et al., 1998). We observed cells that were dual positive for **GAD/GABA** and **GLYT2** in zebrafish, consistent with possible cotransmission.

The most striking colocalization was observed in embryonic zebrafish. Except for KA and DoLA interneurons, which were negative for **GLYT2**, the vast majority of **GAD/GABA** cells at 30–32 hpf were positive for **GLYT2**. At larval stages (4–5 dpf), the number of dual-positive cells decreases in the mediodorsal expression domain, suggesting that many of the dual-positive cells become either pure **GAD/GABA**-positives or pure **GLYT2**-positives over time. Which possibility is more likely? Because pure **GAD**-positive cells are often observed in the mediodorsal expression domain at 4–5 dpf (Fig. 8A), the simplest explanation might be that the dual-positive cells turn into pure **GAD/GABA** neurons. Our preferred hypothesis, however, is the opposite, based on the following two observations which suggest that **GAD/GABA** expression in the dual-positive cells in embryos is transient. First, compared to the expression level in KA neurons (pure **GAD/GABA**), the expression level of **GAD/GABA** in the cells in the dual expression domain is always much weaker. In contrast, expression levels of **GLYT2** do not change whether a cell is dual-positive or not. Second, by comparison with previous results examining **GABA**-positive cells at even earlier stages (19–22 hpf; Bernhardt et al., 1992), it appears that the number of **GABA**-positive cells in the dual expression domain does not increase from 19–22 hpf to 30–32 hpf embryos. In contrast, the number of **GLYT2**-positive cells continuously increases during this time period (Figs. 4, 5).

These two pieces of evidence, though indirect, are in accord with the notion that **GAD/GABA** expression in the dual-positive cells is transient, and disappears over time. If this is the case, the pure **GAD**-positive cells observed in 4–5 dpf larvae might be later differentiating neurons, which may be solely **GABA**ergic from the beginning. A transmitter switch from **GABA** to glycine in early-forming **GABA**-immunoreactive interneurons has previously been suggested in studies of the spinal cord of birds (Antal et al., 1994; Berki et al., 1995). A transient **GABA**ergic phenotype in differentiating glycinerigic neurons might therefore be a common phenomenon in the vertebrate spinal cord.

Only a subset of **GLYT2**-positive cells also express **GAD/GABA**. One question is whether or not the dual-positive cells constitute a functional subset among the **GLYT2**-positive cells. A previous immunohistochemical study in early embryos showed that commissural neurons (CoSA), which likely correspond to the glycinerigic CoSA-gly we describe in the accompanying article, were included among **GABA**-positive cells (Bernhardt et al., 1992). Our observations also indicate that some glycinerigic ipsilateral ascending interneurons (CiA) are also **GABA**-positive (Higashijima et al., 2004). Because these two completely different types of neurons (commissural vs. ipsilateral) are included among the dual-positive cells, it appears that **GAD/GABA** expression does not define a functional subclass among **GLYT2**-positive cells. Whether a random subset of **GLYT2**-positive cells express **GAD/GABA** or all of the **GLYT2**-positive cells transiently express **GAD/GABA** during their differentiation remains to be established.
Expression of $\text{VGLUT}, \text{GLYT},$ and $\text{GAD}$

$\text{VGLUT}$ and GABA (Fig. 6P). The glutamatergic VeLD interneurons may temporarily express GABA at very early stages.

Expression patterns of $\text{VGLUT}, \text{GLYT},$ and $\text{GAD}$ in the hindbrain

Striking patterns of expression $\text{VGLUT}, \text{GLYT},$ and $\text{GAD}$ were observed in the hindbrain. Unlike in the spinal cord, where positive cells for each gene intermingled, the positive cells for each gene in the hindbrain formed clusters. Moreover, the clusters or expression domains formed largely nonoverlapping stripes in both horizontal and cross sections. These stripes, which formed columns in three dimensions, were observed for all three genes ($\text{VGLUT}, \text{GLYT},$ and $\text{GAD}$). Although interleaved columns have not been described in the hindbrain of mammals, there is evidence for a stripe-like clustering of neurons expressing GABA, with an appearance similar to the stripes we describe (Katarova et al., 2000). Both a clustering and segregation of neurons expressing cholinergic and GABAergic markers have been described in midbrain (Sanders et al., 2002).

The development and functional consequences of these stripe/columnar patterns are potentially very interesting. It could be that positive cells for each gene are generated sequentially during development, and then migrate laterally in order. Alternatively, positive cells for each gene might be generated randomly, but subsequent complex migration could form the segregated distributions. Unfortunately, little is known about development of interneurons in the hindbrain, in contrast to the extensive studies of spinal cord (Jessell, 2000). Nonetheless, the alternating distribution of neurons with different neurotransmitter phenotypes could have important implications for how the neuronal circuits are wired during development. Understanding the implications for circuit organization will depend on establishing the relationships between neurons in the stripes and known functional classes of hindbrain neurons (Liu and Fetcho, 1999; Gahtan et al., 2002). Studies exploiting transgenic lines expressing GFP under control of the $\text{VGLUT}, \text{GLYT},$ and $\text{GAD}$ genes should prove very useful for addressing the developmental and functional implications of the transmitter columns (e.g., Higashijima et al., 2000).


