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RESEARCH ARTICLE

The HMG box transcription factors Sox1a and Sox1b specify a new class of glycinergic interneuron in the spinal cord of zebrafish embryos

Vanessa Gerber[1,*], Lixin Yang[1,2,*], Masanari Takamiya[1], Vanessa Ribes[3], Victor Gourain[1], Ravindra Peravali[1], Johannes Stegmaier[4,5], Ralf Mikut[4], Markus Reischl[4], Marco Ferg[1], Sepand Rastegar[1,‡] and Uwe Strähle[1,‡]

ABSTRACT

Specification of neurons in the spinal cord relies on extrinsic and intrinsic signals, which in turn are interpreted by expression of transcription factors. V2 interneurons develop from the ventral aspects of the spinal cord. We report here a novel neuronal V2 subtype, named V2s, in zebrafish embryos. Formation of these neurons depends on the transcription factors *sox1a* and *sox1b*. They develop from common *gata2a*- and *gata3*-dependent precursors co-expressing markers of V2b and V2s interneurons. Chemical blockage of Notch signalling causes a decrease in V2s and an increase in V2b cells. Our results are consistent with the existence of at least two types of precursor arranged in a hierarchical manner in the V2 domain. V2s neurons grow long ipsilateral descending axonal projections with a short branch at the ventral midline. They acquire a glycinergic neurotransmitter type during the second day of development. Unilateral ablation of V2s interneurons causes a delay in touch-provoked escape behaviour, suggesting that V2s interneurons are involved in fast motor responses.

KEY WORDS: *sox1a*, *sox1b*, Spinal cord, Mouse V2c interneuron, Transcriptional network, Development, Zebrafish, Notch, *gata2a*, *gata3*, Glycinergic neurotransmission, Axonal projections

INTRODUCTION

The spinal cord of vertebrates consists of multiple classes of neurons and glial cells, which differentiate from distinct progenitor domains along the dorsoventral (DV) axis of the embryonic spinal cord (Dessaud et al., 2008). Initial differences along the DV axis are triggered by signals like *sonic hedgehog* from the notochord and floor plate forming a gradient in the ventral neural tube. Dorsally, other signals, like BMPs and Wnts, act to instruct neurogenesis. These external signals trigger the expression of combinations of transcription factors in the progenitor domains at distinct DV levels along the spinal cord. As a consequence, these levels produce different types of postmitotic neurons at different DV positions of the spinal cord (Goulding, 2009).

Further subdivisions into neuronal subtypes occur within the initially specified domains. V2 interneurons located in the ventral half of the spinal cord are derived from the p2 progenitor domain. In mouse, three different subtypes of V2 interneurons (termed V2a, V2b and V2c) are generated in this domain (Karunaratne et al., 2002; Li et al., 2005; Panayi et al., 2010; Smith et al., 2002; Zhou et al., 2000). Postmitotic V2a interneurons are characterized by expression of the homeobox transcription factor Vsx2 (Chx10) (Ericson et al., 1997; Kimura et al., 2006). V2b interneurons express the zinc-finger transcription factors Gata2 and Gata3, as well as the bHLH transcription factor Tal1 (Scl) (Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002), and V2c interneurons express the HMG group transcription factor Sox1 (Panayi et al., 2010). V2a neurons are glutamatergic excitatory neurons, whereas V2b cells are GABAergic inhibitory neurons. No neurotransmitter type has been reported for V2c interneurons. Studies in mouse support a signalling cascade in which *Foxn4* acts upstream of *Gata2*. Gata2 in turn activates *Tal1* (also known as *Scl*), which finally induces *Gata3*, specifying V2b interneurons (Del Barrio et al., 2007; Muroyama et al., 2005; Nardelli et al., 1999). Sox1 is required for V2c development (Panayi et al., 2010).

In the spinal cord of zebrafish, the glutamatergic V2a and the GABAergic V2b interneurons are also present, suggesting a conserved structure of the vertebrate spinal cord with respect to these V2 interneurons (Batista et al., 2008). Because of their distinct axonal projections, V2a and V2b interneuron homologues were previously referred to as CiD and VeLD, respectively (Batista et al., 2008; Bernhardt et al., 1992). They express markers related to those of the mouse V2a and V2b cells (Batista et al., 2008; Kimura et al., 2008), and they differentiate at the p2 level by asymmetric divisions of a basally located V2a/b progenitor. The orientation of the division planes is stochastic with respect to the axes of the spinal cord (Kimura et al., 2008). The expression of *vsx1* and *vsx2* is detected in mature V2a interneurons, whereas mature V2b interneurons express *gata2a* (previously *gata2*), *gata3*, *tal1* and *tal2* (Batista et al., 2008; Kimura et al., 2006). Immediately after birth, however, the precursors can share gene expression patterns, e.g. *vsx1* and *gata2a* are co-expressed in these precursors. Moreover, the persistence of GFP expressed from a *vsx1:GFP* transgene is an ideal tracer of cell lineage, as V2b cells also continue to harbour fluorescence, albeit at lower levels, for a significant time after birth (Kimura et al., 2008). The decision to develop into a V2a or V2b interneuron is affected by Notch signalling (Batista et al., 2008; Del Barrio et al., 2007; Kimura et al., 2008; Peng et al., 2007). Genetic inhibition of Notch signalling in homozygous *mindbomb* zebrafish mutants causes the V2a/b precursor cells to develop into

[1]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), 76021 Karlsruhe, Germany. [2]State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, 100012 Beijing, China. [3]Institute Jacques Monod, CNRS UMR7592, Université Paris Diderot, Sorbonne Paris Cité, 75205 Paris Cedex, France. [4]Institute for Automation and Applied Informatics, Karlsruhe Institute of Technology (KIT), 76021 Karlsruhe, Germany. [5]Institute of Imaging and Computer Vision, RWTH Aachen University, 52074 Aachen, Germany.
*These authors contributed equally to this work

‡Authors for correspondence (sepand.rastegar@kit.edu; uwe.straehle@kit.edu)

V.R., 0000-0001-7016-9192; S.R., 0000-0003-4411-5646; U.S., 0000-0002-4062-9431

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V2a at the expense of V2b cells (Batista et al., 2008). By contrast, forced activation of Notch signalling shifts cell fate towards the V2b fate (Kimura et al., 2006). In the mouse, V2c neurons are derived from Gata3^{+} cells in the V2 domain (Panayi et al., 2010). It is not clear whether V2c interneurons exist in the zebrafish spinal cord, even though expression of *sox1a* and *sox1b* has been detected (Andrzejczuk et al., 2018; Armant et al., 2013; https://itgmv3.itg.kit.edu/ffdb/index.html). Interestingly, V2b regulatory genes are also expressed in more ventrally located, cerebrospinal fluid-contacting Kolmer-Agduhr interneurons in the zebrafish (Andrzejczuk et al., 2018; Yang et al., 2010). However, the jointly expressed transcription regulators appear to be connected into distinct regulatory networks (Yang et al., 2010).

Studies in mice and zebrafish indicate that V2 interneurons fine-tune the output of motor neurons (Ampatzis et al., 2014; Zhong et al., 2011). V2a neurons organized in different units activate distinct motor neuron pools during fast locomotion, such as gallop in the mouse or escape behaviour in zebrafish (El Manira, 2014; Song et al., 2018). V2b neurons in mouse are involved in limb-flexion movements (Britz et al., 2015). So far, nothing is known about the function of V2c interneurons.

Here, we have investigated the role of *sox1a* and *sox1b* in the specification of V2 interneurons in the zebrafish spinal cord. We provide evidence for the existence of a third V2 interneuron type in the zebrafish spinal cord, which is distinct from V2a and V2b. We refer to this neuronal cell type as V2s. This neuronal subtype initially and transiently shares marker expression with V2b cells. During the second day of development, however, V2s cells develop into glycinergic cells and present axonal projections distinct from V2a and V2b cells. Both *sox1a* and *sox1b* genes are required for specifying V2s interneurons. This dependence on *sox1* gene activity is reminiscent of mouse V2c interneurons. Our data are consistent with a hierarchical model of two precursor types producing first a pair of cells giving rise to V2a cells and V2b,s precursors (*gata2a*$^{+}$*; gata3*$^{+}$*; tal1*$^{+}$*; tal2*$^{+}$*; gad1b*$^{+}$*; sox1a/b*$^{+}$) followed by a second fate decision giving rise to V2b and V2s cells. V2s cells require Notch signalling to differentiate. Unilateral ablation of V2s cells in zebrafish embryos causes a delay in startle movements upon touch, suggesting that V2s cells are required for fast escape responses.

RESULTS

sox1a and *sox1b* expression delineates a V2 interneuron

In an expression screen of transcription regulators in the 24 h post fertilization (hpf) zebrafish embryo (Armant et al., 2013), we detected expression of two closely related HMG box transcription factor genes, *sox1a* and *sox1b*, in the brain and in neurons of the spinal cord (https://itgmv3.itg.kit.edu/ffdb/index.html). *sox1a* and *sox1b* mRNAs are expressed in the ventral telencephalon, ventral diencephalon, the lens, the spinal cord and the lateral line (Fig. 1A,B, Fig. S1A-C′, data not shown). In transverse sections through the spinal cord of 24 hpf embryos, the mRNA of *sox1a* and *sox1b* was detectable in single cells in and next to the lateral floor plate, and in slightly more dorsal cells (Fig. S1C,C′). The ventral cells are in contact with the ventricle and therefore represent Kolmer-Agduhr KA′ (Fig. S1C,C′) and KA″ (Fig. S1C,C″) interneurons. A third type of *sox1a*- and *sox1b*-expressing cell was located more dorsally, close to the pial surface (Fig. S1C,C′). We mapped the dorsal *sox1a*- and *sox1b*-expressing cells relative to the V1/V0 and the motor neuron domain by staining *Tg(dbx1b:eGFP)* and *Tg(olig2:eGFP)* transgenic animals using *sox1a* and *sox1b* antisense mRNA (Fig. S1D-E″). The location of *sox1a*- and *sox1b*-expressing cells relative to *olig2* and *dbx1b* markers suggests that the dorsal *sox1a*$^{+}$ and *sox1b*$^{+}$ cells belong to the V2 class of interneuron. We next assessed whether *sox1a* and *sox1b* are co-expressed in KA′, KA″ and V2 domain cells by staining embryos with *sox1a* and *sox1b* antisense mRNA. Although differences in

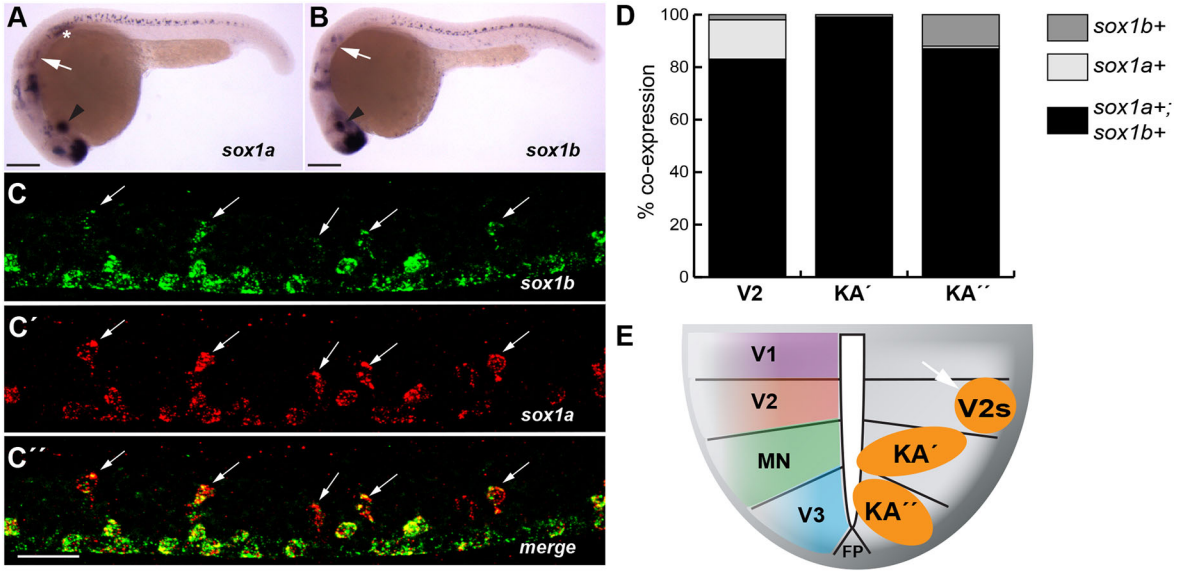

Fig. 1. *sox1a* and *sox1b* are co-expressed in the zebrafish spinal cord. (A,B) Embryos at 24 hpf hybridized to *sox1a* (A) and *sox1b* (B) probes. Both genes are expressed in the forebrain, hindbrain and lens (arrowheads), in the otic vesicle (arrows), and in cells along the spinal cord. In addition, *sox1a* is strongly expressed in the lateral line primordium (asterisk). (C-C″) Fluorescence *in situ* hybridization (FISH) for *sox1a* and *sox1b* mRNA at 24 hpf shows co-expression in V2 (white arrows) and KA neurons. (D) Cells were counted over the yolk extension in a five-somite-long segment. Data are mean±s.d. In the V2 domain, 83±2% of cells (*n*=103) co-express *sox1a* and *sox1b* mRNA, 15±3% of cells express only *sox1a*, and 2±1% of cells express only *sox1b*. Similar counts were obtained for KA′ (*sox1a/b*, 99±4%; *sox1a*, 0%; *sox1b*, 1±0%, *n*=86) or KA″(*sox1a/b*, 87±3%; *sox1a*, 1±0%; *sox1b*, 12±3%, *n*=115). Six embryos from two independent experiments were counted. (E) Ventral spinal cord domains V1 to V3 with the locations of *sox1a*$^{+}$ and *sox1b*$^{+}$ KA′ and KA″ neurons (orange circles), and neurons in the V2 domain (V2s, arrow) indicated. Embryos are at 24 hpf. Dorsal is upwards; anterior is leftwards. Scale bars: 200 µm in A,B; 25 µm in C-C″.

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onset of expression in the spinal cord were noted during earlier development (Fig. S1B,B′), *sox1a* and *sox1b* have similar patterns of expression in the spinal cords of 24 hpf embryos (Fig. 1C-E). Among all *sox1a*- and *sox1b*-expressing cells in the V2 domain, around 80% co-express *sox1a* and *sox1b* mRNA. Similar percentages of co-expression were noted for KA′ and KA″ cells (Fig. 1D).

We next investigated whether *sox1a*$^+$ and *sox1b*$^+$ cells express markers of the known V2 neuronal subtypes, V2a and V2b, in the zebrafish spinal cord (Table S1). The *sox1a*$^+$ and *sox1b*$^+$ cells only rarely express the mRNA of the V2a interneuron marker *vsx2* (Fig. S2A-A″,F-F″) (Batista et al., 2008; Ericson et al., 1997). At 24 hpf, *sox1a*$^+$ and *sox1b*$^+$ cells partially co-express the transcription factors *gata2a*, *gata3* and *tal2*, which are known to be expressed in V2b cells (Batista et al., 2008; Yang et al., 2010) (Table S1; Fig. S2B-D″,G-I″). Glutamic acid decarboxylase (*gad1b*), a marker for GABAergic neurons such as V2b interneurons, was expressed in 90% of *sox1a*$^+$ and 80% of *sox1b*$^+$ cells in the V2 domain (Fig. S2E-E″,J-J″). Thus, *sox1a*$^+$ and *sox1b*$^+$ cells do not express markers of V2a cells but share the expression of markers characteristic for V2b cells at 24 hpf (Table S1).

Reporter labelling identifies new neuronal subtype in the V2 domain

In a functional screen of conserved regulatory sequences in genes expressed in the central nervous system (Naville et al., 2015; data not shown), we noted that an integrated *Tg(dmrt3a-gata2a:eGFP)* chimeric reporter expressed eGFP in a pattern very similar (Fig. S3A) to that seen for the *sox1a* and *sox1b* genes (Fig. 1A,B). We verified this notion by staining transgenic embryos with *sox1a* and *sox1b* mRNAs. The overall pattern of expression matches that of *sox1a* and *sox1b* (Fig. S3C-F). This pattern, however, does not match that of the *dmrt3a* gene (https://itgmv3.itg.kit.edu/ffdb/index.html) (Satou et al., 2013). Sequencing of the genome of the transgenic embryos revealed an insertion of the transgene into the exon of the *sox1a* gene (Fig. S3B,B′). The reporter therefore appears to be expressed under the control of the endogenous *sox1a* gene.

In time-lapse studies of *sox1a:eGFP* embryos, eGFP expression is initiated in single cells in the V2 domain that did not divide anymore prior to extension of axons (Movie 1). We next assessed the morphology of transgene expressing cells in the V2 domain at 60 hpf *sox1a:eGFP* embryos. The eGFP-expressing cells in the ventral spinal cord are too densely packed to assess the projections of individual neurons (Fig. 2A). We thus reduced the number of eGFP-expressing cells by knocking out *eGFP* with a guide RNA/Cas9 approach. The *eGFP*$^+$ cells in the V2 domain have an oval cell nucleus located at the pial surface and extend an axon ventrally (Fig. 2B,D, 100%, *n*=50 cells). This axon bifurcates in all examined cases with a short branch ending at the ventral region of the spinal cord (Fig. 2B′-D′, yellow arrows). The other branch descends ipsilaterally for five or six somites rising to an intermediate dorsoventral level (Fig. 2D, arrowheads). This pattern of axon branching and extension is different from the patterns of the V2a (CiD) and V2b (VeLD) interneurons (Batista et al., 2008; Kuwada et al., 1990).

We verified these data by a different way of cell labelling to exclude adverse effects of the transgene integration or the CRISPR/Cas9 approach to reduce the number of eGFP-expressing cells. We labelled a *sox1a*-encoding BAC clone by recombineering with a GFP reporter cassette [*TgBAC(sox1a:eGFP)*]. After microinjection of *TgBAC(sox1a:eGFP)* into fertilized eggs, transgene expression was obtained in single V2 domain cells in the spinal cord of the G0

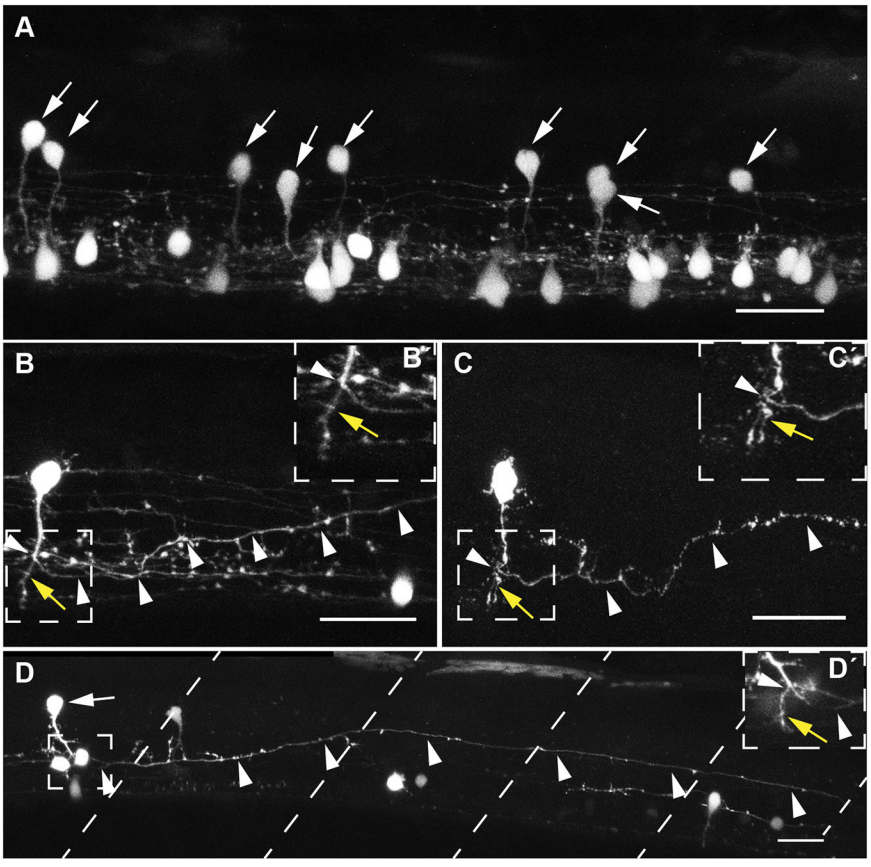

Fig. 2. Morphology of V2s neurons. (A) Spinal cord of a *sox1a:eGFP* embryo with eGFP$^+$ neurons in the V2 domain (arrows) at 60 hpf over a section of three somites above the yolk extension. Ventrally located *sox1a:eGFP*$^+$ cells without arrows are KA′ and KA″ neurons. *sox1a:eGFP*$^+$ V2 neurons show an oval-shaped soma at an intermediate spinal cord position and an axon extending ventrally towards the floor plate. (B,B′) *sox1a:eGFP*$^+$ V2 neuron in a mosaic eGFP knockout embryo at 60 hpf. The ventrally extending axon branches over the floorplate into a long axon descending ipsilaterally (arrowheads) and a short axon branch ending ventrally (B′, yellow arrow). (C,C′) A *sox1a*$^+$ neuron labelled transiently by *TgBAC(sox1a:eGFP)* at 48 hpf with an oval-shaped soma and ventrally extending axon that branches into a short axon ending ventrally (C′, yellow arrow) and a long axon descending and rising to an intermediate DV level of the spinal cord (arrowheads). (D,D′) The *sox1a:eGFP*$^+$ V2 neuron (arrow) extends a long axon ipsilaterally over five somites (somite boundaries are indicated by dashed lines). (D′) Different focal plane showing the short axon branch (yellow arrow) ending ventrally and the main axon branching and descending (arrowheads). Dorsal is upwards; anterior is leftwards. Data are derived from at least two independent experiments. Scale bars: 25 µm in A-C; 100 µm in D.

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generation at 48 hpf (Fig. 2C). The eGFP-expressing V2 cell showed an oval-shaped soma and a ventrally extending axon with a short branch stretched towards the ventral midline of the spinal cord and a long ipsilaterally descending axon branch (Fig. 2C,C′, *n*=20 cells). These data fully support the observations obtained with the stably integrated reporter in the *sox1a:eGFP* transgenic line. The distinct patterns of axonal projections suggest that the *sox1a*- and *sox1b*-expressing neurons in the V2 domain are different from V2a and V2b interneurons described previously (Bernhardt et al., 1992; Kimura et al., 2006) at this location of the zebrafish spinal cord. We will refer to these cells as V2s cells for their distinguishing expression of *sox1a* and *sox1b*.

Mature V2s interneurons are glycinergic

We next assessed whether the V2s cells can be distinguished from V2a and V2b cells by their neurotransmitter production. V2a and V2b interneurons are glutamatergic and GABAergic, respectively (Batista et al., 2008; Kimura et al., 2008). *sox1a*- and *sox1b*-expressing cells do not significantly co-express markers of glutamatergic V2a cells (Fig. S2A-A″,F-F″). About 78% of *sox1a:eGFP*$^+$ cells co-expressed *gad1b*, a marker for GABAergic cells, at 24 hpf (Fig. S4A-A″). Comparable results (92%) were scored in double-labelling experiments using *sox1a* antisense RNA instead of the eGFP fluorescence as a readout of *sox1a*$^+$ cells in the V2 domain (Fig. S2E-E″). We also noted, however, that about 64% of *sox1a*$^+$ cells co-expressed solute carrier family 6 (neurotransmitter transporter), member 5 (*slc6a5*, previously *glyt2*), a marker for glycinergic inhibitory neurons (Fig. 3A-A‴, Fig. S4B-B″). Thus, at 24 hpf a large number of *sox1a*-expressing cells are both GABAergic and glycinergic. This ratio changed over the next few hours of development (Fig. 3B, Fig. S4). At 30 hpf, about 45% of the *sox1a*$^+$ cells expressed *gad1b* and about 70% expressed *slc6a5* (Fig. S4C-D″). However, by 48 hpf, the majority of *sox1a*$^+$ cells have stopped to express the GABAergic marker *gad1b* (Fig. S4E-E″) and have turned on expression of the glycinergic marker *slc6a5* (Fig. 3B, Fig. S4F-F″). This suggests that mature V2s cells are inhibitory neurons employing the neurotransmitter glycine. Altogether, these results bring further support for the notion that V2s cells are different from the previously described V2a and V2b interneurons (Bernhardt et al., 1992; Kimura et al., 2006).

We next wondered whether such glycinergic V2 interneurons could be found in the spinal cord of mouse embryos. We performed immunodetection of Sox1 and *in situ* hybridization with *Gad1* and *Slc6a5* probes on adjacent transverse sections of embryonic day E11.5 and E12.5 mouse embryos. As shown previously (Adams et al., 1995; Katarova et al., 2000), *Slc6a5* and *Gad1* mRNAs were not detected in the ventral part of the spinal cord at E11.5, and hence in Sox1$^+$ cells (data not shown). Conversely, at E12.5, *Gad1* was detected in clusters of ventral cells that match those where Sox1$^+$ interneurons (INs) were located (Fig. S4i-ii′,iv-v′). *Slc6a5* mRNA was also detected in these clusters but only at more developed brachial levels (Fig. S4iii,iii′,vi,vi′). Taken together, these data support the idea that the mouse spinal cord contains glycinergic Sox1$^+$ V2 cells.

Notch signalling is required to specify V2s cell fate from a common V2 precursor pool

Precursor cells at 24 hpf appear to co-express to a large degree markers of V2b cell fates (Andrzejczuk et al., 2018; this study). Our data suggest, however, that mature V2s cells differentiate from these co-expressing progenitors. To address this issue further, we double stained 24 and 30 hpf embryos with *gata2a* and *sox1a* probes, and counted *sox1a*$^+$*;gata2a*$^+$ double-positive cells in a five-somite-long segment of the V2 domain of the spinal cord over the yolk extension. At 24 hpf, 65% of *sox1a*$^+$ cells (*n*=71) co-express *gata2a* (Fig. 4A, Fig. S5A-A″). In contrast, only 32% cells (*n*=34) are still positive for both markers at 30 hpf (Fig. 4A, Fig. S5B-B″). Over the same time course, the total number of cells expressing *sox1a* or *gata2a* are increased by 92% (*n*=111) and 62% (*n*=244), respectively. Thus, between 24 and 30 hpf, *sox1a*$^+$ cells stop co-expressing the V2b marker *gata2a*, consistent with the appearance

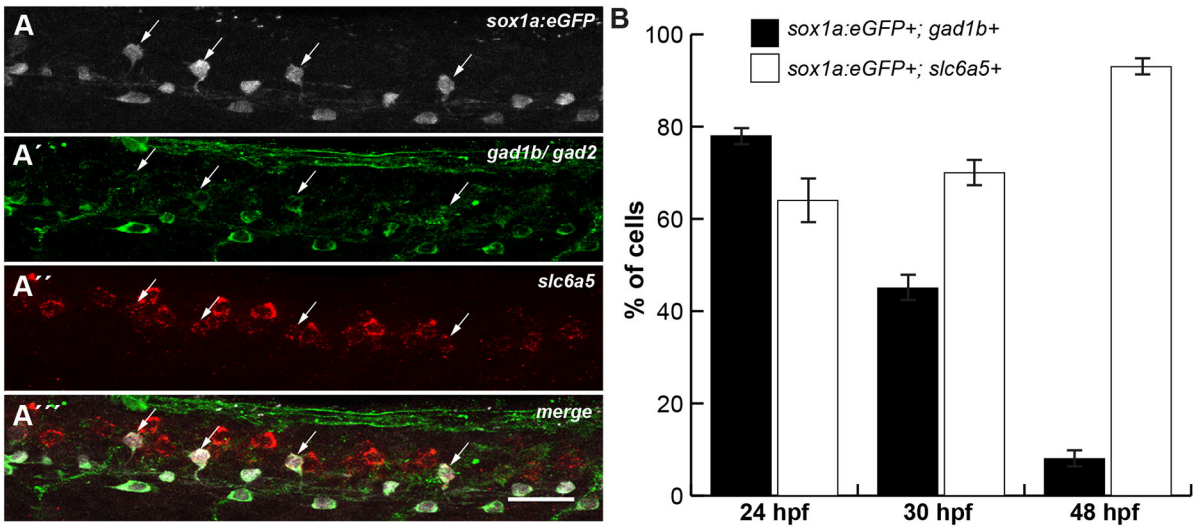

Fig. 3. V2s cells develop into glycinergic interneurons. (A-A‴) Multicolour-labelling of a 24 hpf *sox1a:eGFP* transgenic embryo using FISH with a *slc6a5* probe (red), and immunohistochemistry (IHC) with anti-eGFP (white) and anti-Gad1b/Gad2 (green) antibodies. Many *sox1a:eGFP*$^+$ cells in the V2 domain are both GABA- and glycinergic at 24 hpf (arrows). (B) Percentage of cells expressing *sox1a:eGFP* and *gad1b* (black) or *sox1a:eGFP* and *slc6a5* (white) calculated from FISH with a *gad1b* or *slc6a5* probe, and IHC with anti-eGFP antibody at 24, 30 and 48 hpf (Fig. S4). At 24 hpf, 78% of *sox1a:eGFP*$^+$ cells are GABAergic (*n*=91 of 117) and 64% are glycinergic (*n*=34 of 53). At 30 hpf, 45% of *sox1a:eGFP*$^+$ cells (*n*=51 of 114) are GABAergic and 70% are glycinergic (*n*=56 of 80). At 48 hpf, only a minor fraction (8%) still expresses *gad1b* (*n*=8 of 98). The majority of *sox1a:eGFP* cells (93%) have turned on the glycinergic marker by 48 hpf (*n*=84 of 90). Counts of five to eight embryos from two independent experiments. Data are mean±s.d. Views of spinal cord over yolk extension: dorsal is upwards; anterior is leftwards. Scale bar: 25 µm.

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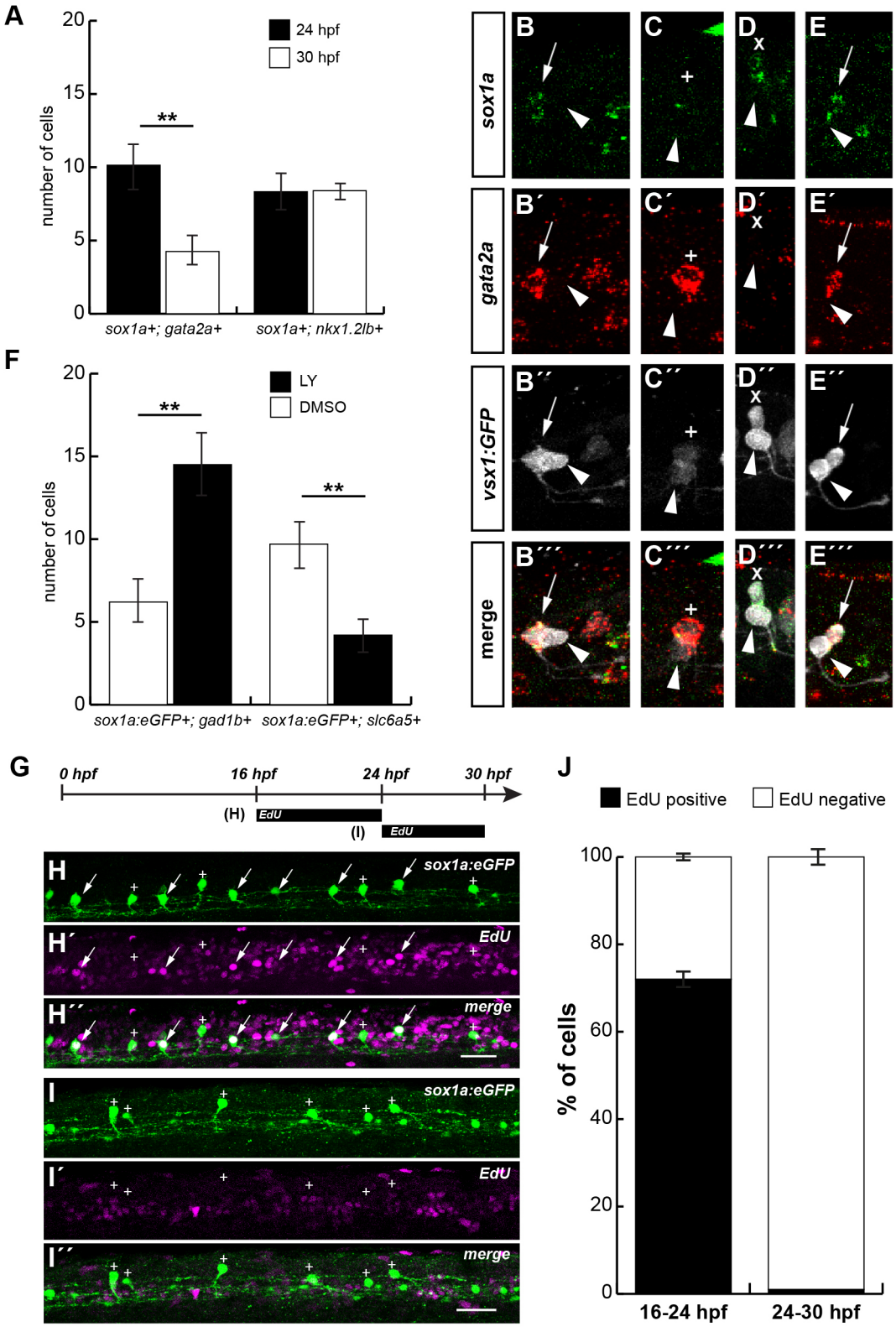

Fig. 4. See next page for legend.

of glycinergic *sox1a*$^{+}$ cells that no longer express the neurotransmitter marker *gad1b* (Fig. 3B). In contrast to these V2b markers, the gene NK1 transcription factor related 2-like,b (*nkx1.2lb*, also known as *sax2*; Bae et al., 2004) tightly follows *sox1a* expression. In 80% of cases, *sox1a*-expressing cells are positive for *nkx1.2lb* mRNA at both 24 hpf and 30 hpf (Fig. 4A, Fig. S5C-D″). Thus, *nkx1.2lb* is tightly linked to *sox1a* expression and V2s differentiation.

As V2s cells express initially V2b markers, V2s cells appear to derive from a common V2b,s precursor pool. We took advantage of

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Fig. 4. V2s neurons depend on Notch signalling. (A) Mean number of cells expressing *sox1a* and *gata2a* mRNA at 24 hpf (black) and 30 hpf (white) determined by FISH (Fig. S5A-B″). Cells expressing *sox1a* and *gata2a* mRNA decreased by about 48% from 24 (black, *n*=71) to 30 hpf (white, *n*=34), whereas *sox1a:eGFP*$^+$ V2 cells co-expressed *nkx1.2lb* mRNA (Fig. S5C-D″) at similar levels at both stages. (B-E‴) Examples of co-expression of *vsx1:GFP*, *gata2a* and *sox1a* mRNA. (B-B‴) A pair of *vsx1:GFP*$^+$ cells with one cell being *vsx1:GFP*$^+$;*gata2a*$^+$;*sox1a*$^+$ (arrows) extending axons at 24 hpf. Committed V2a cells express only *vsx1:GFP* (arrowheads). (C-C‴) Example of a *vsx1:GFP*$^+$ cell co-expressing *gata2a* (+) at 24 hpf. (D-D‴) A V2s cell that is still *vsx1:GFP*$^+$ and expresses *sox1a* mRNA (x) at 26 hpf. (E-E‴) *sox1a* is co-expressed with *gata2a* in one cell of a V2a/V2b,s pair (arrows) at 22 hpf. Arrowheads indicate *vsx1:GFP*$^+$ V2a neurons. (F) Disruption of Notch signalling in *sox1a:eGFP* embryos from 16 to 24 hpf and assessment of neurotransmitter type at 30 hpf shows a 2.3-fold increase in *sox1a:eGFP*$^+$ neurons expressing the GABAergic marker *gad1b*. In contrast, blocking of Notch signalling leads to a 2.3-fold decrease in *sox1a:eGFP*$^+$;*slc6a5*$^+$ neurons (DMSO-treated control, white; LY treated, black; for original data, see Fig. S5E-H″). (G-J) *sox1a* precursor cells divide largely before 24 hpf. *sox1a:eGFP*$^+$ (green) embryos were treated with EdU (magenta) during two different time windows before processing with EdU click-chemistry at 30 hpf (G). *sox1a:eGFP* embryos treated from 16 to 24 hpf (H-H″) or from 24 to 30 hpf (I-I″) showing a five-somite spinal cord segment at 30 hpf (*sox1a:eGFP*$^+$; EdU$^+$ cells, arrows; *sox1a:eGFP*$^+$; EdU$^-$ cells, +). (J) Percentages of EdU$^+$ and EdU$^-$; *sox1a:eGFP*$^+$ cells. Most cells divided before 24 hpf. (A,F) Counts of six to nine embryos from two independent experiments in the V2 domain of the spinal cord above the yolk extension over a five-somite distance. Dorsal is upwards; anterior is leftwards. Data are mean±s.e.m. (A,F) or mean±s.d. (J). Statistical significance was assessed using the unpaired two-tailed Student's *t*-test. $^{**}P \leq 0.01$. Scale bars: 25 µm.

the persistence of *vsx1:GFP* expression, which marks the V2a/V2b progenitors (Kimura et al., 2008) and their derivatives for some time, to assess the expression of *sox1a* and *gata2a* at 22, 24 and 26 hpf (Fig. 4B-E‴, Fig. S6). We detected four different combinations of marker gene expression (Fig. 4B-E‴, Fig. S6). All four classes of cells expressed *vsx1:GFP*, although at varying levels (Fig. 4B-E‴, Fig. S6), which presumably reflects the common origin but also the difference between cells actively expressing the transgene at this stage and cells in which transcription of the transgene has been stopped (Fig. 4B-E‴). Cells expressing *vsx1:GFP* only represent most likely terminally committed V2a interneurons (Fig. 4B-E‴). Except for a slight increase in *vsx1:GFP*$^+$; *sox1a*$^+$ cells (Fig. 4D-D‴, Fig. S6) the overall proportion of the four types of cells does not differ strongly across the short period of observation. The group of increasing *vsx1:GFP*$^+$; *sox1a*$^+$ cells are most likely committed V2s cells (Fig. 4D-D‴). In addition, we detected *vsx1:GFP*$^+$; *gata2a*$^+$ cells (Fig. 4C-C″) and cells that co-expressed *vsx1:GFP*, *gata2a* and *sox1a* (Fig. 4E-E‴). These patterns represent committed V2b and uncommitted V2b,s precursors, respectively. Thus, V2s cells derive from the V2 domain. These data are consistent with the hypothesis that the V2 domain harbours two precursor types: a V2a/V2b and a V2b,s precursor.

Forced activation of Notch signalling abolished the V2a fate (Kimura et al., 2008), whereas loss of Notch signalling in the *mindbomb* mutant shifted cell fate towards V2a cells at the expense of V2b cells (Batista et al., 2008). Thus, we asked whether Notch signalling is also involved in specifying V2s cells. We reasoned that this second window of Notch requirement is delayed with respect to the first one. We therefore chose a conditional approach by exposing *sox1a:eGFP* transgenic embryos to the γ-secretase inhibitor LY411575, which blocks Notch processing after ligand binding (Fauq et al., 2007). We determined the number of *sox1a:eGFP/gad1b* or *sox1a:eGFP/slc6a5* co-expressing cells at 30 hpf in embryos exposed to LY411575 from 16 to 24 hpf (Fig. 4F). Blocking Notch signalling in this time window increased the number of GABAergic *sox1a:eGFP*$^+$;*gad1b*$^+$ cells by 2.3-fold (Fig. 4F, Fig. S5E-F″). At the same time, it reduced the number of glycinergic *sox1a:eGFP*$^+$;*slc6a5*$^+$ cells by 43% (Fig. 4F, Fig. S5G-H″). Thus, specification of V2s cells depends on Notch signalling.

We next tested when precursors of V2s cells become post-mitotic by monitoring incorporation of the nucleotide analogue EdU. We exposed embryos to EdU either between 16 and 24 hpf or between 24 and 30 hpf (Fig. 4G). *Sox1a:eGFP* transgenic embryos were labelled for incorporated EdU and cells were counted in a five-somite-spanning segment of the V2 domain in the spinal cord over the yolk extension (Fig. 4H-I″,J). More than 70% of *sox1a:eGFP*$^+$ cells were positive for EdU incorporation in embryos exposed to the nucleotide analogue from 16 to 24 hpf (Fig. 4H-H″,J). In contrast, when embryos were exposed from 24 to 30 hpf, only 1% of the *sox1a:eGFP*$^+$ cells had incorporated EdU (Fig. 4I-I″,J). Thus, the *sox1a:eGFP*$^+$ cells that became gradually negative for V2b markers between 24 and 30 hpf were born before 24 hpf.

V2b,s precursors require Gata2a/3 activity

Disrupting either *gata2a* or *gata3* translation alone produced at best a marginal effect on V2b marker expression (Yang et al., 2010). However, double knockdown of *gata2a* and *gata3* reduced *tal2*-expressing V2b interneurons (Fig. 5A-A″) and expression of *gad1b* (Fig. 5B-B″) was also decreased in the V2 domain. Thus, *gata2a* and *gata3* are required for differentiation of V2b cells. Reduced *gad1b* expression was recently also reported in *gata2a;gata3* double mutants (Andrzejczuk et al., 2018). These genetic data underscore the specificity of our *gata2a* and *gata3* morpholino knockdown approaches employed in this and a previous report (Yang et al., 2010). The expression of *sox1a* and *sox1b* in the V2 region was significantly reduced in the absence of Gata2a and Gata3 (Fig. 5C-D″). Thus, *gata2a* and *gata3* are also required for *sox1a* and *sox1b* expression.

As V2b and V2s cells are derived from preceding fate decisions of common V2a/b,s progenitors that also transiently express *gata2a* (Batista et al., 2008; Kimura et al., 2008), we asked whether the V2a cells were affected by loss of Gata factors. Knockdown of *gata2a* and *gata3* did not affect expression of *vsx2*, a marker of V2a cells (Fig. 5E-E″). Thus, Gata2a and Gata3 appear to be essential for controlling the V2b and V2s fates without influencing V2a interneuron differentiation. The distinct and specific effects on different neurons (Fig. 5A″-E″) underscore the specificity of the *gata2a* and *gata3* morpholinos. This is further supported by the analysis of *gata2a* and *gata3* mutants (Andrzejczuk et al., 2018).

Mutation of *sox1a* and *sox1b* reduces V2s and increases V2b cells

We next assessed the function of *sox1a* and *sox1b* by CRISPR/Cas9-directed gene knockout (Fig. S7A,B). Homozygous single mutants of both Sox1 genes were viable and produced offspring with apparent normal spinal cord patterning. We therefore bred double-homozygous mutant embryos. Double mutants failed to inflate their swim bladders (Fig. S7C,D) and died during early larval stages. *sox1a*$^{-/-}$;*sox1b*$^{-/-}$ mutant embryos showed differences in expression patterns of V2b-specific genes (Fig. 6A-D″). Counts of *gata2a*$^+$ cells in whole spinal cords showed an increase of 22% in *sox1a*$^{-/-}$; *sox1b*$^{-/-}$ mutants (Fig. 6A-A″). The numbers of cells expressing *tal1*, *tal2* and *gata3* in the V2 domain at 24 hpf increased by 83%, 75% and 35%, respectively (Fig. 6B-D″). One interpretation of this increase in V2b cells is that V2s cells fail to form, thereby increasing the number of V2b,s precursors differentiating into *tal1*$^+$, *tal2*$^+$ and *gata3*$^+$ V2b cells. In agreement, the *sox1a*$^{-/-}$;*sox1b*$^{-/-}$ mutants show a 2.3-fold increase in the number of *gata3*-expressing cells

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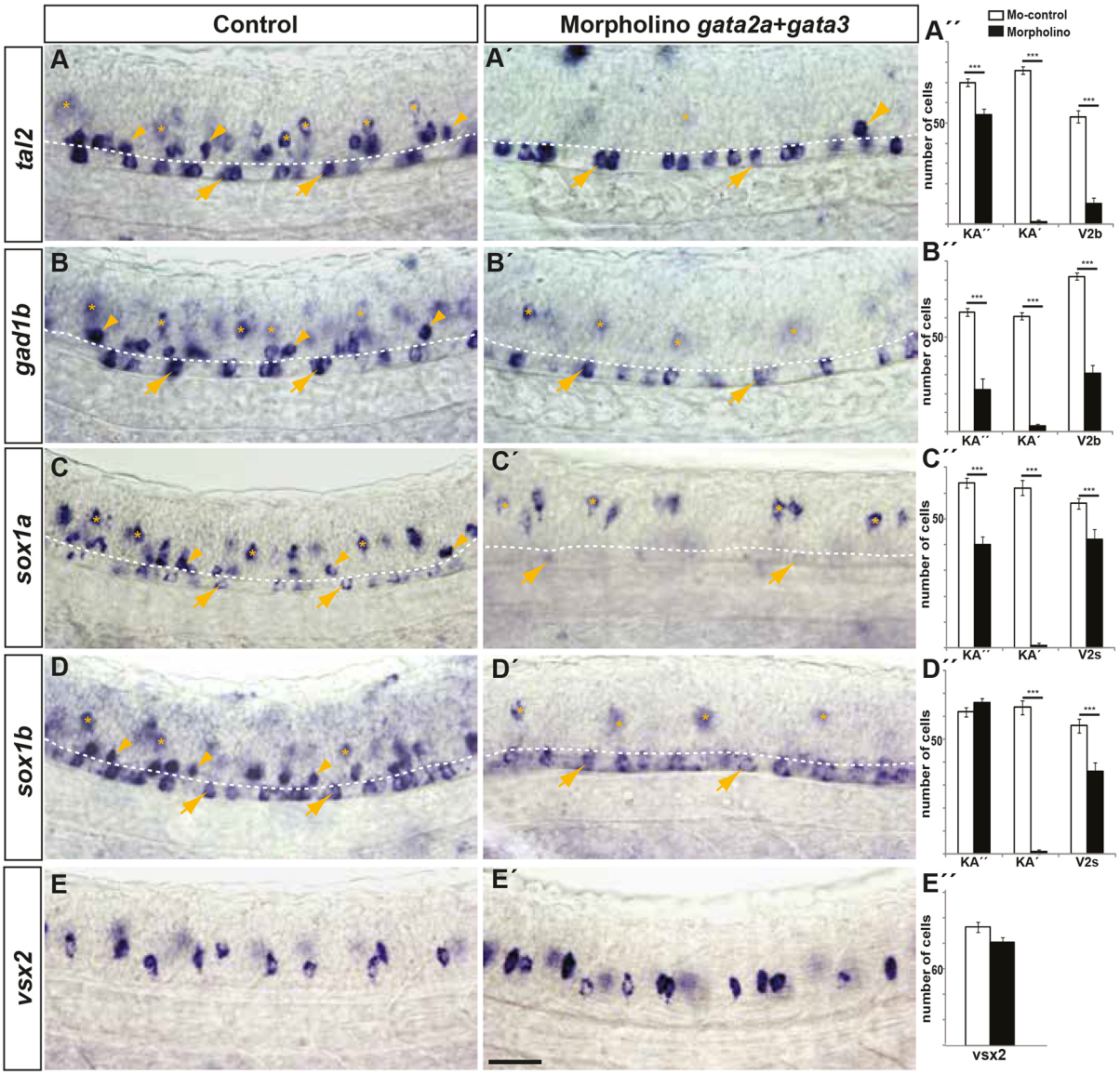

Fig. 5. Gata2a and Gata3 are required for expression of *sox1a* and *sox1b* in V2 interneurons. (A-E′) Embryos injected with mismatch morpholinos (control, A-E) or with a mixture of morpholinos directed against *gata2a* and *gata3* mRNA (A′-E′). Cells in the V2 domain are indicated by asterisks, KA′ cells by arrowheads and KA″ cells by arrows. (A-A″) Loss of function of Gata2a and Gata3 resulted in a reduction of *tal2*-expressing KA″ cells, an elimination of KA′ and decrease of *tal2*$^{+}$ V2b cells. (B-B″) Reduction of *gad67*-expressing V2b and KA″ cells was noted in morpholino-injected embryos, whereas loss of function of Gata2a and Gata3 almost abolished *gad67*$^{+}$ KA′ cells. (C-C″) Knockdown of Gata2a and Gata3 decreased *sox1a*$^{+}$ V2 cells by 25% (*n*=650 cells) and almost abolished *sox1a*-expressing KA′. (D-D″) Loss of function of Gata2a and Gata3 decreased *sox1b*$^{+}$ V2 cells by 35% (*n*=2016 cells) and almost eliminated *sox1b*-expressing KA′. (E-E″) V2a cells (*vsx2*$^{+}$) were not affected in knockdown embryos. Data are mean±s.e.m. from 22 to 42 embryos from at least two independent experiments. Cells were counted from the yolk extension to the tail on both sides of the spinal cord. Statistical significance was assessed using the unpaired two-tailed Student's *t*-test. ***$P \leq 0.001$. Scale bar: 25 µm.

(Fig. 6F-F″) and a decrease of *nkx1.2lb*$^{+}$, *slc6a5*$^{+}$ co-expressing cells by 22% (Fig. 6G-I) in the V2 domain at 30 hpf. The expression of the V2a marker *vsx2* was unchanged in the mutants (Fig. 6E-E‴), suggesting that *sox1a* and *sox1b* only affect the fate decision between V2b and V2s.

We next tested whether injection of morpholinos directed against *sox1a* and *sox1b* would result in the same effects. The two single exon genes are so similar that it is impossible to achieve gene-specific effects. Knockdown of *sox1a/b* translation resulted in a significant increase of *tal2*-, *gad1b*-, *gata2a*- and *gata3*-expressing cells in the V2 domain at 24 hpf (Fig. S8A-D″). Thus, overall the morpholinos reproduced the effect seen in the double mutants. The only exception was a significant increase of *gad1b* expression in the morphants at 24 hpf (Fig. S8B-B″), which we could not measure in the double mutants at the similar stage (data not shown). In addition, the number of *vsx2*-expressing V2a cells was unaffected (Fig. S8E-E″). This agrees with the conclusion that *sox1a/b* is involved in the V2b/V2s fate decision. This dependence on *sox1* activity is reminiscent of V2c cells of the mouse spinal cord.

To verify that lack of *sox1a* and *sox1b* leads to loss of V2s cells, we knocked down Sox1a and Sox1b protein translation by injecting the morpholinos, and asked whether the numbers of *sox1a* or *sox1b* mRNA-expressing cells were reduced. A sixfold reduction in the number of *sox1a*$^{+}$;*gad1b*$^{-}$ cells was noted (P<0.006; n>8 animals for control and knockdown). At the same time, a twofold increase in the number of *sox1a*$^{+}$;*gad1b*$^{+}$ cells was counted (P<0.005, n=9

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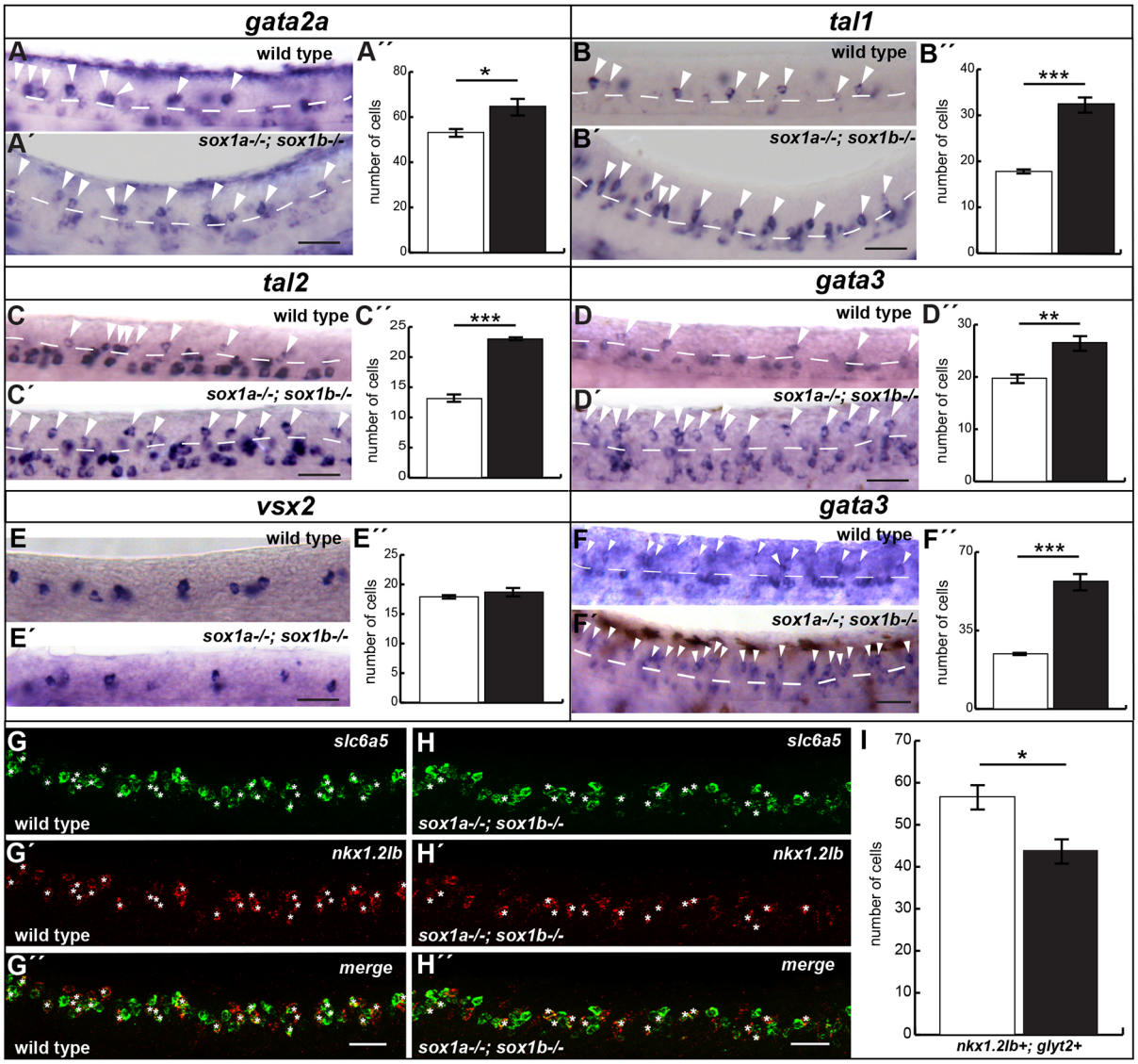

Fig. 6. Knockout of *sox1a* and *sox1b* causes increase of V2b cells. (A-A″) Knockout of *sox1a* and *sox1b* increases *gata2a*$^{+}$ cells in the V2 domain by 22%. (B-B″) *tal1*$^{+}$ cells are increased by 83% in *sox1a*$^{-/-}$*; sox1b*$^{-/-}$ embryos. (C-C″) Lack of *sox1a/b* results in an increase of *tal2*$^{+}$ cells by 75%. (D-D″) The *sox1a/b* mutant has 35% more *gata3*$^{+}$ cells. (E-E″) *vsx2*$^{+}$ cells are not affected by the mutations. (F-F″) At 30 hpf, the *gata3*$^{+}$ cells in the V2 domain are increased by 2.3-fold. (G-I) Double FISH of *nkx1.2lb* (red) and *slc6a5* (green) at 30 hpf and quantification of *nkx1.2lb*$^{+}$; *slc6a5*$^{+}$ cells in I. Glycinergic *nkx1.2lb*$^{+}$ cells were decreased by 22% in the mutant. Asterisks indicate double-labelled cells. Counts of *gata2a*$^{+}$ cells (A-A″) were derived from the fourth somite to the tail on both spinal cord sides from five embryos. Counts of *gata3*-, *tal2*-, *tal1*-, *vsx2*- and *nkx1.2lb; slc6a5*-expressing cells were derived from the spinal cord above the yolk extension over five somites from three to eight embryos (B-F″). V2 cells, white arrowheads (A-F′). Wild-type (white) and mutant (black) values are presented as mean±s.e.m. in A″-F″,I from at least two independent experiments. Statistical significance was assessed using the unpaired two-tailed Student's *t*-test. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$. Lateral views at 24 hpf (A-E′) and 30 hpf (F-H″). Dorsal is upwards; anterior is leftwards. Scale bars: 25 µm.

animals for control and knockdown). A 3.2-fold increase was noted for *sox1a*$^{+}$*;gata3*$^{+}$ cells (P=0.001, n>8 animals for control and knockdown) and a 2.4-fold increase in the number of *sox1b*$^{+}$*; gad1b*$^{+}$ double-labelled cells (P=0.004, n>10 animals for control and knockdown) in the *sox1a* and *sox1b* morphants. Thus, precursors develop into V2b cells (*gata3*$^{+}$, *gad1b*$^{+}$) in the absence of *sox1a* and *sox1b* function. This is consistent with the notion that *sox1a* and *sox1b* specifies V2s cell fate from a common V2b,s precursor that follows the V2b fate as default in the absence of *sox1a* and *sox1b* activity.

Sox1-mutant and V2s-ablated embryos show motility defects

Given the predominant expression of *sox1a* and *sox1b* in the central nervous system, we assessed whether *sox1a* and *sox1b* double mutants show aberrant movement behaviour. We recorded spontaneous movement every 2 h between 96 and 108 hpf. By using an automated imaging system, minimum disturbance of embryos during the period of recording was assured. Double mutants moved less frequently than wild-type or heterozygous siblings (Fig. 7A). They tend to lie at the bottom of the Petri dish for longer periods than their wild-type or heterozygous siblings.

sox1a and *sox1b* genes are not only expressed in multiple neuronal subtypes in the spinal cord but also in the brain (Fig. 1, Fig. S1). The functional relevance of V2s neurons may therefore not be detectable in a phenotypic analysis of double mutants. To address more directly the role of V2s neurons, we ablated the V2s neurons on one side of the spinal cord of wild-type embryos. We used the *sox1a:eGFP* line to specifically identify the V2s neurons at 2 days

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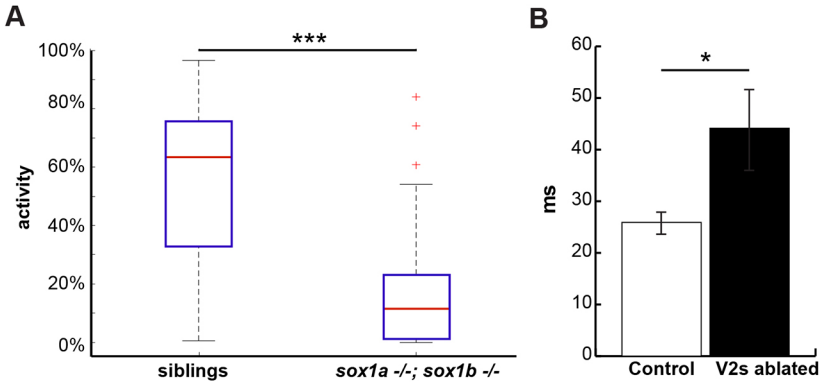

Fig. 7. Behavioural assessment of *sox1a*$^{-/-}$*; sox1b*$^{-/-}$ mutants and V2s-ablated larvae. (A) Comparison of the percentage activity between heterozygous siblings and *sox1a*$^{-/-}$;*sox1b*$^{-/-}$ mutant larvae. Spontaneous locomotion was recorded over 1.5 h starting at three different time points during the daytime between 96 and 108 hpf. A custom algorithm was used to extract the kinematic properties of the locomotion activity. The activity percentage indicates the percentage of time the larvae were moving. There is a significant difference in the activity of the larvae, with the siblings being more active than the mutant larvae (siblings, *n*=10; mutants, *n*=6). Red crosses indicate outliers. The lowest and highest points of the whisker indicate the extreme values in the data not considered as outliers. The box indicates the 25th-75th percentile of the data. The red line in the box is the median of the data. (B) Ablation of V2s neurons. *sox1a:eGFP* embryos were embedded in agarose at 2 dpf and V2s neurons were laser-ablated on one side of the spinal cord. Controls were mock ablated. At 4 dpf touch-evoked escape response of individual embryos was measured. Latency was increased (control, *n*=11; V2s ablated, *n*=7). Data are mean±s.e.m. from three experiments. Statistical significance was assessed using the unpaired two-tailed Student's *t*-test. *$P \leq 0.05$, ***$P \leq 0.001$.

of development. All *sox1a:eGFP*-expressing V2s neurons on one side of the spinal cord were ablated using a two-photon laser. These treated embryos, as well as mock-treated embryos, were subjected to analysis of swimming movement upon touch stimulation using a high-speed camera. The overall swimming movements were indistinguishable between ablated, mock-ablated and untreated embryos (data not shown). However, ablated embryos showed a significant delay in response to touch relative to mock-treated embryos (Fig. 7B). This suggests that V2s neurons are required for a fast escape reaction to touch-evoked stimuli.

DISCUSSION

We report here the identification of a novel type of V2 interneuron in the spinal cord of the zebrafish embryo. This V2s interneuron depends on *sox1a* and *sox1b*, has a unique pattern of axonal projections and is glycinergic. It differentiates from a common pool of V2 progenitors during the second day of development. Moreover, ablation of V2s interneurons delays the touch-provoked escape reaction, suggesting that the neurons are required for a fast motor response.

V2a, V2b and V2s interneurons develop from common precursors

The two Sox1 genes are expressed in overlapping patterns in the spinal cord, including neurons in the V2 domain. At around 24 hpf, precursors share expression of *sox1a*, *sox1b* and V2b-specific markers. During the second day of development, however, cells acquire the glycinergic V2s fate characterized by expression of *slc6a5* and loss of the GABAergic neuron marker *gad1b*. At the same time, the number of V2b neurons that express only *gad1b* and other V2b markers, such as *tal1* and *gata3*, but not *sox1a/b* increases. Hence, the V2s and the V2b neurons start to differentiate into their terminal neuronal subtypes during the second day. Clearly at 24 hpf, a large proportion of *gata2a*$^{+}$ and *gad1b*$^{+}$ cells express *sox1a* and *slc6a5*, the markers of V2s cells. Knockdown of *gata2a* and *gata3* abolished differentiation of both V2b and V2s interneuronal types. These observations are in agreement with the recent analysis of *gata2a/gata3* double mutants (Andrzejczuk et al., 2018). Thus, a common *gata2a/gata3*-dependent precursor referred to as V2b,s expresses a chimeric gene programme that is representative of both V2 subtypes. Lack of *sox1a* and *sox1b* function leads to an increase in the number of V2b cells and a loss of V2s cells. This suggests that *sox1a* and *sox1b* are required to shift a proportion of cells from the initial chimeric precursor V2b,s state (*gad67*$^{+}$*; gata3*$^{+}$*; gata2a*$^{+}$*; sox1a/b*$^{+}$*; slc6a5*$^{+}$) to a V2s cell state (*gad67*$^{-}$*; gata3*$^{-}$*; gata2a*$^{-}$*; sox1a/b*$^{+}$*; slc6a5*$^{+}$). In the absence of *sox1a* and *sox1b*, more precursors develop into V2b cells (*gad67*$^{+}$*; gata3*$^{+}$*; gata2a*$^{+}$*; sox1a/b*$^{-}$*; slc6a5*$^{-}$). Markers for V2a neurons (*vsx1*$^{+}$ and *vsx2*$^{+}$) were unaffected in *sox1* mutants/morphants, showing that *sox1a* and *sox1b* are not required for differentiation of V2a interneurons.

Differentiation of V2s from the precursor pools requires Notch signalling. Our inhibition experiments suggest a differential temporal requirement of Notch signalling for specification of the different V2 domain interneurons. Conditional inhibition of Notch signalling between 16 and 24 hpf reduced the number of V2s cells, while V2b-expressing cells were increased. Inhibition of Notch signalling before 16 hpf resulted in a shift towards V2a differentiation with loss of V2b cell fate (Batista et al., 2008), whereas forced activation of Notch signalling shifted cells towards a V2b fate (Kimura et al., 2008). V2s cells are predominantly born between 16 and 24 hpf. However, the differentiation from the chimeric V2b,s precursor state into the distinct V2b and V2s subtypes appears to require most of the second day of development. Our data are consistent with the notion that Notch-dependent cell fate specifications are required at least twice consecutively in the V2 domain: first, in the decision of the V2a/V2b precursor cell (Batista et al., 2008; Kimura et al., 2008) and then in that of the V2b,s precursor (Fig. 8). At 22 to 26 hpf, many V2b,s precursors express traces of *vsx1:GFP*, suggesting that these precursors are derived from the *vsx1:GFP*-expressing V2a/b progenitors (Kimura et al., 2008). At least some of the primary progenitors may therefore be referred to as V2a/b,s progenitors. Symmetric cell fate decisions were also noted for some of the primary V2a progenitors (Kimura et al., 2008). A key question is: are V2b and V2s cells generated by division of the V2b,s cell? Our time lapse analysis of *sox1a:eGFP*-expressing V2 cells suggests that cells marked by the transgene did not divide. We therefore favour a model where V2s and V2b cells arise post-mitotically from the V2b,s precursor without cell division

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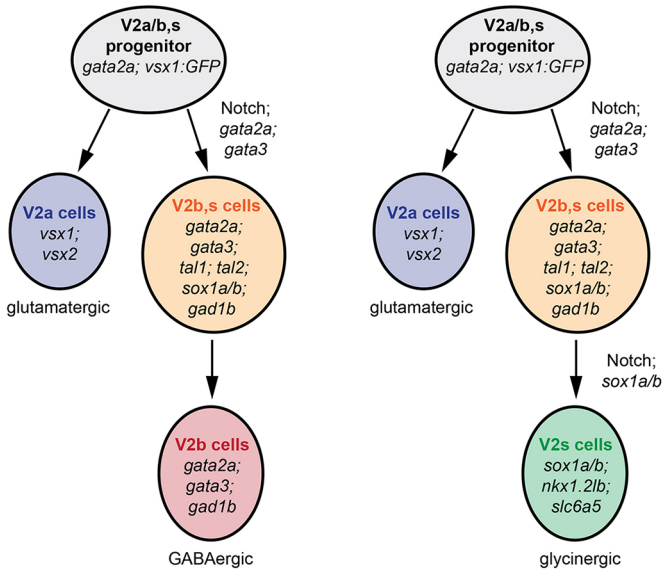

Fig. 8. Model of V2s cell development. A V2a/b,s progenitor (*gata2a*$^{+}$*; vsx1:GFP*$^{+}$) gives rise to a *vsx1*$^{+}$*; vsx2*$^{+}$ V2a cell and to a V2b,s precursor (*gata2a*$^{+}$*; gata3*$^{+}$*; tal1*$^{+}$*; tal2*$^{+}$*; gad1b*$^{+}$*; sox1a/b*$^{+}$). V2b,s precursors start to differentiate either to a GABAergic *gata2a*$^{+}$*; sox1a*$^{-}$ V2b cell or to a glycinergic *gata2a*$^{-}$*; sox1a/b*$^{+}$ V2s cell. Notch signalling is required for the V2b,s precursor and for V2s differentiation. Gata2a/Gata3 are required for V2b and V2s differentiation. Sox1a and Sox1b are needed for V2s differentiation. In *sox1a/b* mutants, V2b cells increase in number.

(Fig. 8). However, eGFP expression slightly lags behind endogenous *sox1a* mRNA expression. Thus, we cannot totally exclude the possibility that V2b/V2s differentiation is initiated by division of the progenitor. To address these issues, sophisticated cell-tracing experiments will be required.

Are zebrafish V2s interneurons homologues of V2c interneurons in the mouse spinal cord?

The zebrafish spinal cord shares many similarities with the organization of the mouse spinal cord, including aspects of the underlying mechanisms of cell specification (Jessell, 2000; Lewis, 2006). The developmental path leading to differentiation of V2s interneurons has remarkable resemblance to the pathway outlined for the development of V2c interneurons in the mouse (Panayi et al., 2010). Like murine V2c neurons (Panayi et al., 2010), zebrafish V2s interneurons require *sox1* activity for differentiation. V2b and V2c interneurons are derived from common precursors in the mouse. Moreover, the V2c progenitors express transiently *gata3* in both organisms. In contrast to mouse (Panayi et al., 2010), however, both *gata3* and *gata2a* are required for expression of the V2s marker *sox1a* in the zebrafish spinal cord. V2s cells are glycinergic. This trait is likely to be also conserved as some Sox1^{+} cells expressed glycinergic markers at brachial levels in the E12.5 mouse spinal cord. V2s cells could thus be homologues of V2c cells of the murine spinal cord. However, functions and axonal projections of murine V2c cells have not been characterized. Although the expression domains of *sox1a* and *sox1b* largely overlap, there is a proportion of cells in the V2 domain that expresses only *sox1a* or *sox1b*. In addition, in the mouse spinal cord not all Sox1^{+} cells are glycinergic (V.R., unpublished). The possibility cannot be excluded that more V2 interneuron subtypes exist in the spinal cord of fish and mice (e.g. murine V2d; Francius et al., 2016). It is currently also not possible to unequivocally answer the question of whether V2s cells are the zebrafish homologues of mouse V2c interneurons, even though some similarities of the developmental path leading to differentiation of V2c and V2s cells in the two organisms may suggest so. Clearly, *sox1* is co-expressed with *gad1* and *slc6a5* in a large proportion immediately after birth of *sox1*$^{+}$ cells in the zebrafish, whereas post-mitotic Sox1^{+} cells in the murine E11.5 spinal cord do not seem to express glycinergic markers until E12.5 in anterior brachial levels. Thus, the mechanism of differentiation is either different between zebrafish and mice or we are looking at non-homologous V2 IN cell types.

The function of V2s interneurons

Concomitant with the development of a V2s-specific gene expression programme by loss of V2b-specific gene expressions, axons grow out from V2s interneurons during the second day of development, forming stereotypic patterns of projections. A ventrally growing axon splits into a short side branch before growing ipsilaterally and caudally over the next five to six somites. This long descending axon gradually rises from a ventral to a mid-spinal cord level. This pattern of axonal projection resembles that of V2a (CiD) interneurons, which sometimes also develop a short ventral branch (Kimura et al., 2006). However, in contrast to V2a cells, which develop an ascending axon branch (Kimura et al., 2006), we never observed ascending branches in V2s cells. Clearly, the V2s axonal projections are also different from those of V2b (VeLD) neurons (Batista et al., 2008; Bernhardt et al., 1992). V2s neurons are glycinergic and therefore inhibitory, like the GABAergic V2b neuron, unlike the glutamatergic V2a interneurons. The axonal connections of V2s cells remain to be determined in detail.

sox1a and *sox1b* double mutants are significantly less active in unprovoked swimming tests than wild-type embryos. Double mutants fail to inflate the swim bladder, which may be an indirect consequence of the reduced motility, as immotile mutants fail to gulp air from the surface to fill their swim bladder (Nicolson et al., 1998; Schoppik et al., 2017). *sox1a* and *sox1b* genes are expressed in several different structures (telencephalon, cerebellum, spinal cord, lateral line, etc.) in the nervous system. It is therefore likely that the reduced motility is not only due to impaired activity of the spinal cord but also to defects in higher order brain structures. This notion is supported by our ablation experiments, where we ablated the V2s neurons unilaterally in the spinal cord. These embryos show normal unprovoked swimming behaviour. However, upon touch, the V2s ablated larvae show significantly longer delays of escape movements in comparison with mock-ablated controls. Thus, V2s neurons are required in a circuit for efficient touch-evoked escape.

MATERIALS AND METHODS

Fish stocks

Wild-type zebrafish were obtained from the ABO line, an inbred line initially derived from an intercross between the AB and OX lines (European Zebrafish Resource Centre). Wild-type and transgenic zebrafish [*Tg(olig2:eGFP)*^{VU12} (Shin et al., 2003), *Tg(dbx1b:eGFP)* and *sox1a*$^{Tg(dmrt3a-gata2a:eGFP)ka705}$ (this study)] were maintained on a 14 h/10 h light-dark cycle at 28.5°C in a recirculation system (Schwarz) and fed commercial food and in-house hatched brine shrimp as described previously (Westerfield, 2000). Embryos were cultured in embryo medium and staged according to Kimmel et al. (1995).

Knockout of *sox1a* and *sox1b*

sox1a^{ka701} and *sox1b*^{ka702} mutant alleles were generated using CRISPR/Cas9 genome editing (Jao et al., 2013) by co-injection of *sox1a* guide RNA (target sequence GGGGCAAACGGGTCCAAATT) and *sox1b* guide RNA (target sequence GGAGTGGAAACTCATGTCCG) with Cas9 mRNA into one-cell stage embryos. A G1 founder with a 7 bp deletion in the *sox1a* gene 72 bp downstream of the ATG and a 2 bp indel in the *sox1b* gene 233 bp downstream of the ATG was identified by fin clipping (Fig. S7A,B) and

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sequencing of the subcloned PCR product amplified from the target region of the guide RNA. This results in frameshifts upstream the HMG and the SOX domain (interaction site for partner proteins), suggesting that these alleles are likely to cause a complete loss of function (Fig. S7A,B). The G1 founder with both mutations in *sox1a* and *sox1b* was outcrossed with wild-type fish and genotyped (Etard et al., 2017). G2 fish carrying $sox1a^{ka701}$ and $sox1b^{ka702}$ alleles were incrossed and $sox1a^{ka701/ka701};sox1b^{ka702/ka702}$ double mutants were identified by genotyping.

Genotyping

DNA was isolated from anesthetized adults via fin biopsy or from fixed embryos via dissection of the head. Fin biopsy of adults was performed as previously described (Etard et al., 2017) and genomic DNA was extracted using the HotSHOT method using 100 µl of 50 mM NaOH and 10 µl Tris-HCl (pH 7.5). Heads of fixed embryos were dissected in PTW (PBS, 0.1% Tween 20) with pins, and embryo trunks were stored in PTW at 4°C for later analysis. The $sox1a^{ka701}$ and $sox1b^{ka702}$ alleles were identified by restriction enzyme digestion, as both mutations disrupt endogenous restriction enzyme sites. Genomic DNA extraction from dissected heads was performed as described previously (Juárez-Morales et al., 2016) or by directly inserting parts of dissected heads into the PCR reaction mix. The genomic region encompassing the mutation site was PCR amplified using the following conditions: initial denaturation step at 94°C for 7 min; 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s; and a final elongation step at 72°C for 2 min. For *sox1a*, a 349 bp amplicon encompassing the mutation site was generated using the following primers: forward, TTCCACACTTCATCGGAGCT; reverse, TGCTGAGTGGAAGGTGATGT. This amplicon was digested with *Apo*I-HF to yield fragments of 191 bp and 158 bp (wild-type allele) or 342 bp (mutant allele). For *sox1b*, a 346 bp amplicon encompassing the mutation site was generated using the following primers: forward, AGAGACCCATGAACGCCTTT; reverse, GGCCAGAGGTTAGAGAGTCC. This amplicon was digested with *Nla*III to yield 168 bp, 111 bp and 54 bp fragments (wild-type allele) or 168 bp and 166 bp fragments (mutant allele).

In situ hybridization, immunohistochemistry, sectioning and cell counting

Whole-mount *in situ* hybridization was performed as described previously (Yang et al., 2010). Antisense RNA probes for *tal2* (Pinheiro et al., 2004), *gata2a* (Detrich et al., 1995), *gad1b* (previously known as *gad67*, a mix of *GAD67a* and *GAD67b* probes), *slc6a5* (previously known as *glyt2*, a mix of *glyt2a* and *glyt2b* probes) (Higashijima et al., 2004), *gata3* (Yang et al., 2010), *vsx2* (Passini et al., 1997), and *sox1a* and *sox1b* (Armant et al., 2013) (Table S2) were labelled with digoxigenin (Roche) or DNP-11-UTP (PerkinElmer).

In double fluorescent *in situ* hybridizations, the RNA probes were detected using anti-digoxigenin-POD (Roche, 1:250) and anti-DNP-HRP (PerkinElmer, 1:200). The DNP-probe was stained first with either TSA Plus Cyanine 5 Tyramide Reagent (PerkinElmer) or Alexa Fluor 488 Tyramide Reagent (Invitrogen) as a substrate following the manufacturers' instructions. For the second staining, the embryos were incubated in 1% hydrogen peroxide for 1 h to quench horseradish peroxidase followed by washes in 1× PBS, 1% Tween-20. The second staining (Cyanine 3) was developed following the manufacturer's instructions for the TSA Plus Cyanine 3 (Cy3) kit (PerkinElmer).

Chicken anti-GFP (1:1000, Aves Labs, GFP-1020) or rabbit anti-GAD65+GAD67 (anti-Gad1b/Gad2, 1:500, Abcam, ab11070) antibodies were added to embryos with blocking buffer (PerkinElmer blocking buffer of the TSA Plus Cy3 kit) overnight at 4°C followed by incubation with goat anti-chicken or anti-rabbit antibody conjugated with Alexa Fluor 488 or Alexa Fluor 647 (1:1000, Invitrogen). The yolk sac of stained embryos was removed manually, and the embryos were embedded laterally in Aqua-Poly/Mount (Polysciences) followed by analysis with a TCS SP5 confocal microscope (Leica).

For sectioning, stained embryos were embedded in 3% agarose and 25 µm sections were cut using a vibratome (Leica). Sections and whole embryos (without yolk sac) were mounted in 100% glycerol and imaged under a compound microscope (DM5000B, Leica) with DIC optics. The intensity, contrast and exposure time of the pictures were adjusted using Photoshop and ImageJ software.

Counts of *gad1b*-, *gata2a*-, *gata3*-, *sox1a*-, *sox1b*-, *tal2*- and *vsx2*-positive cells in whole-mount embryos were derived from the entire trunk or from the first somite (V2 interneuron domain) to the tail on both sides of the spinal cord (Fig. 5, Fig. S8). Counts of *gata2a*-positive cells in the $sox1a^{-/-};sox1b^{-/-}$ mutant and wild type were derived from the fourth somite to the tail on both sides of the spinal cord. Counts of *tal1*-, *tal2*-, *gata3*- and *vsx2*-positive cells in the *nkx1.2lb; scl6a5* double FISH experiment analysing the $sox1a^{-/-};sox1b^{-/-}$ mutant and wild type were derived from both sides of the spinal cord above the yolk extension over a five-somite distance (Fig. 6).

Mouse spinal cord immunofluorescence

Mouse embryo fixation, embedding, cryo-sectioning and immunolabelling protocols have been previously described (Briscoe et al., 2000). For further details, see the supplementary Materials and Methods

Morpholino knockdown

Injections were performed using a gas-driven microinjector (Tritech Research) as described previously (Muller et al., 1999). The antisense morpholino directed against *sox1a* and *sox1b* (5′-CCGTTTCCATCATCATGCTATACAT-3′) was designed by GeneTools. Morpholinos containing five mismatches (mismatches indicated by small letters) were used as controls: 5′-CCcTTTgCATgATgATGgTATACAT-3′. The efficiency of morpholinos was examined by knockdown of GFP expressed from a reporter construct (data not shown). Other morpholinos used in this study have been described previously (Yang et al., 2010). The morpholinos were resuspended in water with 0.1% Phenol Red to 0.25 or 0.5 mM and were injected into the yolk of one- to two-cell stage embryos. For single gene knockdown experiments, morpholinos were injected at a concentration of 0.5 mM. In double knockdown experiments, individual morpholinos were used at a concentration of 0.5 mM and 0.25 mM, respectively. For controls, the same concentrations of the respective five mismatch control morpholinos were injected.

Generation of *Tg(dbx1b:eGFP)* and *TgBAC(sox1a:eGFP)* transgenes

The zebrafish bacterial artificial chromosome (BAC) DNA for *dbx1b* (CH211-7E10) and for *sox1a* (CH211-207-L5) was purchased from Children's Hospital Oakland Research Institute. The BAC modification strategy for transgenesis was adapted from Shin et al. (2003) and Lam et al. (2009). In the first homologous recombination, the first exon of *dbx1b* and *sox1a* was replaced with *eGFP*. In the second homologous recombination, an ampicillin resistance gene flanked by *Tol2* sites was introduced into the modified BAC, as previously described (Suster et al., 2011). The modification of BAC DNA was confirmed by PCR, and bacteria colonies positive for eGFP and *Tol2* arms were selected for culture and further BAC DNA extraction using a Nucleobond AX-100 maxi-prep kit (Macherey-Nagel). The modified BACs were injected into one-cell stage embryos at 10 ng/µl. Embryos expressing GFP at 24 h post fertilization (hpf) were raised to maturity. The stable transgenic lines (F0) were identified by out-crossing injected fish with wild-type fish. Identified F0 were out-crossed with wild-type fish to obtain stable progenies that express the transgene in the F1 generation. We obtained a stable line for *Tg(dbx1b:eGFP)*. *TgBAC(sox1a:eGFP)* was used only in transient expression experiments.

Creation of the gene trap line $sox1a^{Tg(dmrt3a\text{-}gata2a:eGFP)ka705}$

$sox1a^{Tg(dmrt3a\text{-}gata2a:EGFP)ka705}$ (*sox1a:eGFP* for short) is a gene trap allele in which a *Tol2*-based Gateway destination enhancer test vector (Navratilova et al., 2009) was inserted fortuitously into the 5′ untranslated region of the *sox1a* gene (Fig. S3B). The integration was verified by whole-genome sequencing with an Illumina Hiseq1500 at a coverage of 7.1× (see supplementary Materials and Methods).

Knockout of eGFP in *sox1a:eGFP* embryos

sox1a:eGFP transgenic embryos were injected at the one-cell stage with a guide RNA (final concentration: ∼100 ng/µl) against eGFP (Auer et al.,

2014) and Cas9 protein (Invitrogen, final concentration: ∼300 ng/µl), resulting in embryos with mosaic eGFP expression that were raised under normal conditions until imaging.

LY411575 treatment

LY411575 (Fauq et al., 2007) was reconstituted with dimethyl sulfoxide (DMSO) to a stock concentration of 25 mM. Embryos raised in embryo medium were dechorionated manually and placed in agarose-coated Petri dishes. The embryos were incubated in embryo medium containing 10 µM LY411575, 0.04% DMSO or 0.04% DMSO alone from 16 to 24 hpf, then washed with embryo medium containing 0.04% DMSO and raised until 30 hpf.

Time-lapse imaging

For long-term *in vivo* time lapse imaging (more than 16 h), *sox1a:eGFP* embryos were embedded as previously described (Middel et al., 2016) in LMP agarose (0.5%) with agarose removed from the tail (for elongation) in a 6 cm Petri dish and covered with 10 ml 1× E3 medium (60× stock: 5 mM NaCl, 0.17 mM KCl, 10 mM HEPES, 0.33 mM $MgSO_4 7H_2O$ and 0.33 mM $CaCl_2 6H_2O$) containing 0.02% MESAB. Multiple positions of the lateral spinal cords were imaged under a Leica TCS SP5 upright microscope (HCX APO L 63×/1.2 W U-V-I objectives) using the bright field and 488 nm channel. *Z*-stacks covering 40-90 µm were acquired every 0.8 µm at each position (two positions per spinal cord). Each position was imaged every 10 min for 16 h in 512×512 pixel format with a depth of 8-bit by bidirectional resonant scanning at 8 kHz. Images with an extended depth of field were generated in Fiji (Schindelin et al., 2012) through maximum projections for the temporal analysis of the fluorescence.

Ablation of V2s neurons

For ablation of V2s neurons, *sox1a:eGFP* embryos were incubated in 0.003% phenylthiourea (PTU) containing E3 medium from 24 hpf onwards and were laterally embedded at 2 dpf in LMP agarose (0.5%) as previously described (Middel et al., 2016). Using a confocal microscope (TCS SP2, Leica), single V2s neurons on one side of the spinal cord were damaged at 32× zoom using a Ti:Sa laser (MaiTai, Spectra Physics) set to 864 nm (gain 65%, offset 26%) using the region-of-interest (ROI) set to 0.25-2 µm. Control and V2s-ablated embryos were removed from the agarose and incubated in E3 medium for recovery. At 4 dpf, single embryos were transferred to 6 cm Petri dishes and a touch evoked escape response was stimulated with a pin and recorded with a high-speed camera.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V. Gerber, L.Y., S.R., U.S.; Methodology: V. Gerber, L.Y., M.T., V. Gourain, R.P., J.S., R.M.; Software: V. Gourain, R.P., J.S., R.M.; Validation: V. Gerber, L.Y., V.R., M.R.; Formal analysis: V. Gourain, R.P., J.S., R.M., M.R.; Investigation: V. Gerber, L.Y., M.T., V.R., V. Gourain, M.F.; Resources: V.R., R.P., S.R., U.S.; Data curation: V. Gourain, R.P., J.S., R.M.; Writing - original draft: L.Y., S.R., U.S.; Writing - review & editing: V. Gerber, L.Y., V.R., S.R., U.S.; Visualization: V. Gerber, L.Y., V.R.; Supervision: S.R., U.S.; Project administration: S.R., U.S.; Funding acquisition: U.S.

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Data availability

Whole-genome sequencing data of the *sox1a:eGFP* line have been deposited in NCBI's Sequence Read Archive (SRA) under accession number SRR8237123.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.172510.supplemental

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