Proper migration and axon outgrowth of zebrafish cranial motoneuron subpopulations require the cell adhesion molecule MDGA2A

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ABSTRACT

The formation of functional neuronal circuits relies on accurate migration and proper axonal outgrowth of neuronal precursors. On the route to their targets migrating cells and growing axons depend on both, directional information from neurotropic cues and adhesive interactions mediated via extracellular matrix molecules or neighbouring cells. The inactivation of guidance cues or the interference with cell adhesion can cause severe defects in neuronal migration and axon guidance. In this study we have analyzed the function of the MAM domain containing glycosylphosphatidylinositol anchor 2A (MDGA2A) protein in zebrafish cranial motoneuron development. MDGA2A is prominently expressed in distinct clusters of cranial motoneurons, especially in the ones of the trigeminal and facial nerves. Analyses of MDGA2A knockdown embryos by light sheet and confocal microscopy revealed impaired migration and aberrant axonal outgrowth of these neurons; suggesting that adhesive interactions mediated by MDGA2A are required for the proper arrangement and outgrowth of cranial motoneuron subtypes.

KEY WORDS: Zebrafish, MDGA, Cranial motoneurons, Digital light sheet microscopy, Cell migration, Cell adhesion, Axon guidance

INTRODUCTION

During the formation of the nervous system subsets of postmitotic neuroblasts delaminate from primary neuroepithelia and migrate over considerable distances to settle in different regions of the growing organism. These migratory cells follow stereotypic pathways detecting even minute concentration differences of attractive and repulsive neurotropic cues or changes in the adhesive properties of the surrounding tissue. Therefore miss-regulation of either neurotropic or adhesive molecules often leads to aberrant migration and ectopic clustering of neuronal populations.

Following neuronal migration and terminal differentiation, neurons have to properly connect with corresponding targets in order to integrate the information flow throughout the nervous system. In analogy to neuronal migration, elongating neurites also grow along predetermined pathways relying on instructional information from attractive and repulsive cues and growth promoting adhesive environments. Cell-cell and cell-substrate interactions via adhesion molecules, especially by molecules belonging to the immunoglobulin superfamily have been shown to be crucial for nervous system development (Maness and Schachner, 2007). This has been demonstrated by countless in vitro assays, antibody perturbation assays as well as loss and gain of function experiments (Bingham et al., 2002).

Due to its relatively simple segmental organization, the developing hindbrain has been the focus of many studies (Bingham et al., 2010). As hindbrain development is essentially conserved among vertebrates, knowledge derived from one species can potentially give insights into hindbrain development in other species (Gilland and Baker, 1993; Moens et al., 1998; Moens and Prince, 2002; Gilland and Baker, 2005). In our study we have focused on the development of the zebrafish hindbrain, especially studying migration and axonal outgrowth of branchiomotoneurons (Drapeau et al., 2002). The concise and segmental organization as well as the stereotype migration and axonal outgrowth pattern have made branchiomotoneurons an attractive model system. Branchio-as well as somato- and viscera-motoneurons represent subgroups of cranial motoneurons whose axons exit the CNS at predetermined exit points (for reviews, see Chandrasekhar, 2004; Song, 2007).

Neurons from specific nuclei form different cranial nerve bundles innervating the muscle masses of the branchial (pharyngeal) arches. While somatomotoneurons, innervating extraocular muscles, cluster in the oculomotor (cranial nerve III), the trochlear (IV) and the abducens (VI) motor nuclei; branchiomotoneurons (BMN) build up the trigeminal (V), facial (VII) glossopharyngeal (IX) and vagal (X) nuclei. In zebrafish BMN migration and axon outgrowth is initiated within the first 24 h of development. BMN precursors are generated in specific rhombomeres, which subsequently migrate towards their final destination at characteristic dorsolateral and rostrocaudal positions within the developing hindbrain. For example motoneuron precursors of the facial nerve originating in rhombomere 4 migrate as far as rhombomeres 6 and 7 (Chandrasekhar, 2004; Song, 2007). These cells project axons via specific motor nerves into the periphery. The generation of transgenic zebrafish where GFP expression is driven by the islet1 promoter has proven valuable to study the generation, positioning and axon outgrowth of branchiomotoneurons (Higashijima et al., 2000).
Using this Isl1-GFP transgenic line the involvement of planar cell polarity (PCP) pathway genes such as Sbnm/Vangl2/tri (Jessen et al., 2002; Sittaramane et al., 2009), prickle1a (Carreira-Barbosa et al., 2003), prickle1b (Rohrschneider et al., 2007), scribble1 (Wada et al., 2005), Celsr2 and Frizzled3a (Wada et al., 2006), col/hdac1 (Nambiar et al., 2007), as well as the PCP effector gene Nhs1b (Walsh et al., 2011) in the migration of branchiomotoneurons has already been demonstrated. However, besides the genes from the planar cell polarity pathway, other factors must be involved in motoneuron migration in the hindbrain, as several aspects of the migration appear normal in vangl2 mutants (Bingham et al., 2010). Furthermore, a “collective mode” of migration that requires the interaction between migrating facial BMNs themselves and is independent of PCP proteins has been suggested to work together with PCP-dependent mechanisms to drive directed migration of facial BMNs in vivo (Walsh et al., 2011). Recent studies also suggest that fucosylated glycans, such as gmds/twd expressed by neuroepithelial cells (Ohata et al., 2009), may repulse migrating vagal motoneurons preventing radial/apical migration (Ohata et al., 2011). Moreover, TAG1, laminin and cadherin mediated signals have been shown to be involved in guiding branchiomotoneurons (Sittaramane et al., 2009; Grant and Moens, 2010; Stockinger et al., 2011). In addition, interaction between motor nerves and sensory nerves are required for the proper axonal growth of trigeminal but not facial nerves (Cox et al., 2011), but the molecules mediating this interaction remain unknown. Interestingly, recently it has been shown that facial branchiomotoneuron migration also depends on the interaction of migrating neurons with axons of the medial longitudinal fascicle (MLF), as preventing MLF axons from entering the hindbrain results in staling of FBMN migration (Wanner and Prince 2013).

We have recently identified a novel group of cell adhesion molecules, called MDGAs (for MAM domain containing glycosylphosphatidylinositol anchor proteins) (Gesemann et al., 2001; Litwack et al., 2004), MDGAs, which belong to the immunoglobulin superfamily of cell adhesion molecules (for review, see Maness and Schachner, 2007), have been shown to be expressed in the spinal cord of different species including rat (Litwack et al., 2004), chicken (Joset et al., 2011) and medaka (Sano et al., 2009). For chicken it has been demonstrated that inactivation of MDGA2 by RNA interference or function blocking antibodies leads to outgrowth defects of commissural interneurons (Joset et al., 2011). Subsequent experiments have demonstrated that MDGA2 interacts homophilically and that interactions between commissural interneurons and MDGA2 positive ipsilateral projecting neurons are important for proper rostral growth of commissural interneurons (Joset et al., 2011).

In order to explore additional roles of MDGA in nervous system development, we have now analyzed MDGAs in the zebrafish Danio rerio. In zebrafish three different MDGAs are present; MDGA1, MDGA2A and MDGA2B. Of these, MDGA2A is highly expressed in subsets of migrating cranial motoneurons. Using morpholino mediated knockdown experiments, we could demonstrate that the absence of MDGA2A leads to migration defects of trigeminal neurons as well as aberrant axonal growth and defasciculation of facial branchiomotor axons.

RESULTS

Through comparative database searches with rat, human and chicken MDGA sequences, we found and subsequently cloned three orthologous zebrafish MDGA genes, namely MDGA1, MDGA2A and MDGA2B (for a phylogenetic comparison, see supplementary material Fig. S1). The amino acid sequence of zebrafish MDGA1 shows 59% and the one of zebrafish MDGA2A and -2B 76% and 74% identity with the corresponding rat orthologs. As the homology between individual domains of MDGAs varies significantly, a detailed homology analysis is given in supplementary material Fig. S1. While some data about the RNA distribution and functional properties of rat (Litwack et al., 2004; Takeuchi and O’Leary, 2006; Takeuchi et al., 2007), mice (Ishikawa et al., 2011), chicken (Fujimura et al., 2006; Joset et al., 2011) and medaka (Sano et al., 2009) MDGAs is available, no such information exists for the zebrafish embryo. We therefore performed in situ hybridization assays in zebrafish embryos at different stages of development, to test whether MDGA gene expression correlates with specific aspects of nervous system development.

Zebrafish MDGAs are expressed in the spinal cord and in defined brain areas

Since rat and chicken MDGAs are highly expressed in the developing spinal cord (Litwack et al., 2004; Joset et al., 2011), we started our analysis in the corresponding region of the zebrafish embryo. As expected from rat and chicken studies, zebrafish MDGA transcripts are expressed in distinct interneuron subpopulations within the dorsal and mediolateral part of the embryonic spinal cord (Fig. 1A–C). MDGA1 and MDGA2B transcripts can be observed at regions where dorsal commissural interneurons are located. In addition, MDGA2A and MDGA2B messages are expressed in cell pools that coincide with the location of intermediate and ventral interneuron subpopulations. In the spinal cord MDGA1 positive cells can further be found in a narrow band of mediolateral located interneurons as well as within the dorsal ventricular zone (Fig. 1A), from where newborn cells spread laterally.

Interestingly, MDGA transcripts are also abundantly expressed in the zebrafish brain. At two days post fertilization, MDGA1 riboprobes label distinct clusters of neurons that stretch bilaterally alongside the medial border of the eyes (Fig. 1D,G). The anterior region encompasses the ventral thalamus (asterisks in Fig. 1D,G) and the hypothalamus (arrow in Fig. 1D,G), whereas the posterior cell clusters represent low level staining of branchiomotoneurons. MDGA1 transcripts are also highly expressed in cells of the peripheral nervous system. Three well discernible cell clusters, the anterior and posterior lateral line ganglia as well as cells associated with the otic placode such as cells of the statoacoustic ganglion (gVIII) prominently express MDGA1 (arrowheads in Fig. 1D,G).

MDGA2A transcripts are localized in distinct neuronal clusters corresponding to motoneurons of several cranial nerves (Fig. 1E,H). Among them are motoneurons of the oculomotor (nIII) and trochlear (nIV) nerve in the midbrain (asterisks in Fig. 1E,H), as well as BMNs of the trigeminal, facial and vagal nerve in the hindbrain (Fig. 1E,H, nV/nVII/nX). In addition, MDGA2A transcripts are also weakly expressed in muscle tissue, such as the sternohyoideus (sh) and in mandibular muscles (data not shown). Expression in head muscles can also be seen for MDGA2B transcript. In addition, MDGA2B is expressed in the telencephalon, the ventral thalamus, the tegmentum, the hypothalamus and at low levels also in subpopulation of cranial motoneurons (Fig. 1F,J).
MDGA2A is expressed in axonal tracts of cranial motoneurons during development

The concise and segmentally arranged cell bodies and their stereotypical axonal course have made BMNs an easily accessible system for studying different aspects of neuronal development, such as tangential migration and axon pathfinding (Bingham et al., 2002). Moreover, transgenic zebrafish lines with BMNs expressing GFP under the control of the Islet 1 promoter have substantially contributed to deciphering the molecular aspects of their development. In this transgenic zebrafish line, the Islet1 promoter/enhancer sequence drives GFP expression in cranial motoneurons, some of the cranial sensory neurons, and several other groups of cells (Fig. 2E,G; Higashijima et al., 2000). As MDGA2A is highly expressed in cranial motoneurons, we generated peptide antibodies against MDGA2A to analyze the distribution and potential role of MDGA2A in developing cranial neurons. In agreement with the RNA distribution pattern, MDGA2A antibodies stained neuronal cell bodies and axonal tracts and to a lesser extend head muscles. In the hindbrain, branchiomotoneurons are stained by MDGA2A antibodies (Fig. 2A,B). Among the MDGA2A positive cranial nerves are tracts of the oculomotor (nIII) and trochlear (nIV) nerves. The ciliary nerve (cn, part of nIII) innervating the lens muscle as well as a branch of nIV that innervates the superior oblique muscle are clearly MDGA2A positive (Fig. 2C). Additional axons expressing MDGA2A are the trigeminal (nV) and facial (nVII) motor nerves, innervating muscles in the jaw and jaw-support structures (Schilling and Kimmel, 1997; Chandrasekhar, 2004), as well as axons within the vagal motor nerve (nX) (Fig. 2D,H).
RoL1 neurons as well as the large Mauthner neurons. Moreover, the lateral line system and components of the statoacoustic ganglion, both of which innervate hair cells of the head, trunk, tail and the inner ear are MDGA2A positive (for more information, see supplementary material Fig. S2).

In summary, MDGA2A protein is prominently expressed in several neuronal subpopulations and their axons during the period of neuronal migration and axon outgrowth, suggesting a potential role for this molecule in mediating adhesive interactions during these processes. To test such a hypothesis, we selectively downregulated MDGA2A protein synthesis using morpholino antisense oligonucleotides and analyzed occurring phenotypes.

MDGA2A deprivation leads to impaired migration and axonal growth of branchiomotoneurons

Since MDGA2A message is present in subgroups of branchiomotoneurons, namely the trigeminal, facial and vagal motoneurons, we further analyzed the effect of MDGA2A protein knockdown in Islet1-GFP transgenic zebrafish embryos. To analyze the efficiency of protein knockdown in MDGA2A morpholino treated zebrafish we compared antibody stainings in wildtype (wt) and MDGA2A knockdown animals. Neurons and axons that are clearly stained in wt fish, lack corresponding immunoreactivity in MDGA2A knockdown zebrafish (Fig. 3A–D; supplementary material Fig. S4C). Interestingly, several cranial motoneuron migration and axon outgrowth defects could be observed in Islet1-GFP MDGA2A knockdown animals. While in wt zebrafish migration of trigeminal BMNs occurs within the limits of their rhombomere (r) of origin resulting in the formation of an anterior and posterior trigeminal motor nucleus in r2 and r3, respectively; in MDGA2A morphants, trigeminal motoneurons (V) fail to undergo their proper migration pattern and settle at various ectopic positions (arrows in Fig. 3F). Also, neurons within clusters appear disorganized, accumulating in a pile, instead of settling in the characteristic lateral trigeminal clusters. Interestingly, motoneurons of the facial nerve (VII) have left their place of origin in r4 and undergone appropriate caudal migration. However, their number in the forming clusters seems to be reduced. Moreover, the bilateral branches of the vagal motor nucleus appear more prominent, as if containing more cells than normal.

Examination of the corresponding axon tracts in uninjected and morphant embryos suggest that fewer axons run in axonal tracts of MDGA2A deprived embryos (Fig. 3G,H). Interestingly, their course and innervation pattern is still maintained in MDGA2A knockdown animals, indicating that proper pathfinding of growing trigeminal and facial motoneurons can still occur. Nevertheless, many axons seem to stall along the axonal path, leaving a reduced number to reach their muscle targets. While the reduced number of axons running in these tracts may be caused by errors originating from the absence of MDGA2A, this phenotype may also be caused by migration defects of the mentioned branchiomotoneurons. In order to study these phenotypes in more detail in vivo, we performed time-lapse light sheet microscopy.

Live light sheet microscopy of zebrafish branchiomotoneuron development confirms MDGA2A knockdown phenotypes

To observe branchiomotoneuron migration and axon outgrowth in real time, Isl1-GFP fish were imaged by digital scanned light sheet microscopy (DSLM) between 24–36 hpf. Image stacks that covered the region of the nV, nVI and nVII axons were recorded every 16 min (supplementary material Movie 1). In wt control embryos trigeminal neurons formed compact clusters and stayed together throughout the period of observation (24–36 hpf; Fig. 4A, w1–w5; supplementary material Movie 2). Interestingly, in MDGA2A morpholino treated embryos, single trigeminal neurons started to move along the axon bundle instead of remaining in a stable cell cluster (Fig. 4A, m1–m5; supplementary material Movie 2). To quantify this phenotype we measured the fluorescence intensity along the trigeminal nerve. At 28 hpf the fluorescence intensity along the axons started to increase in MDGA2A knockdown animals compared to control embryos and remained higher than control values during the rest of the observation period. During this period continuous ectopic migration takes place in morpholino injected animals.
Regular confocal pictures taken from 34 hpf zebrafish embryos treated with 3 different MDGA2A morpholino oligonucleotides, confirmed that cohesion between trigeminal neurons is weakened in MDGA2A knockdown fish and that some cells undergo ectopic migration (supplementary material Fig. S4A,D). While in confocal stacks of wt and control morpholino injected embryos an average of 12.35 ± 0.21 neurons can be seen in the trigeminal nerve cluster, MDGA2A knockdown fish have a significantly reduced number (9.66 ± 0.27; p < 0.005). These missing cells can be found at ectopic positions often along the trigeminal nerve (arrows in supplementary material Fig. S4A), a situation never observed in wt embryos.

Axonal outgrowth of nVII axons started around 28 hpf and 3 h later a characteristic 60° turn could be observed, as previously reported by Higashijima et al. (Higashijima et al., 2000) (Fig. 5, w2–w4, asterisks; supplementary material Movie 1). At the turning point continuous outgrowth of short protrusions were observed, but they readily retracted and the main axon bundle stayed fasciculated (Fig. 5, w2–w4; supplementary material Movie 1). By 34 hpf, a stable bundle of axons all showing the 60° turn was established. However, upon morpholino mediated knockdown of MDGA2A, the turn of the axon was less pronounced and the turning angle was decreased (Fig. 5, mA2–4, mB2–4; supplementary material Movie 3). As for control embryos, axons in the region of the turning point started to protrude in random directions, but in MDGA2A knockdown

(supplementary material Figs S4B, S3; Movie 2). Regular confocal
animals these axons did less frequently retract, and some even continued growing. In addition, the axon bundles in MDGA2A morpholino treated embryos were less organized and often highly defasciculated compared to control embryos (supplementary material Movie 3). To quantify this axon outgrowth phenotype, we measured the fluorescence intensity in an area below the axon in the DSLM images (supplementary material Fig. S3). In animals older than 30 hpf the total fluorescence intensity in this area was slightly higher for MDGA2A morpholino treated embryos compared to control. Confocal pictures of embryos treated with 3 different MDGA2A morpholinos showed extensive protrusions at the choice point and a significantly reduced turning angle (32.75±1.53°) compared to control fish (58.15±1.11, p<0.005; supplementary material Fig. S4B,D).

In summary, in MDGA2A morpholino treated embryos several subpopulations of branchiomotoneurons display errors in the predetermined migration pattern and/or their axons show increased collateral branch formation and less intense bundling.

**DISCUSSION**

MDGA proteins have been studied in humans (De Juan et al., 2002; Díaz-López et al., 2005), rats (Litwack et al., 2004), mice (Takeuchi et al., 2007), chickens (Fujimura et al., 2006) and medaka (Sano et al., 2009). Here we identified and cloned three MDGA orthologs in zebrafish, MDGA1, MDGA2A and MDGA2B. We found MDGA2A to be expressed in a subset of motoneurons, especially in the ones of the cranial, trigeminal and facial nerves. Morpholino mediated knockdown of MDGA2A led to aberrant cell migration of trigeminal neurons and to defasciculation and increased branch formation of the trigeminal as well as facial nerve. These results demonstrate that MDGA2A interactions are necessary for proper migration, axon outgrowth and bundling in cranial motoneurons.

In agreement with our current findings, MDGAs in other species have already been implicated in neuronal migration and axon guidance. In rats, MDGA positive cells were found in the pontine migratory stream, suggesting that these circumferentially migrating neurons may rely on this cell adhesion molecule for proper neuronal migration (Litwack et al., 2004). In addition, in MDGA1 loss of function mice proper radial migration of superficial layer cortical neurons is blocked (Takeuchi and O’Leary, 2006), and MDGA-2 knockdown in chicken by RNA interference induced strong axon outgrowth phenotypes in MDGA expressing commissural interneurons (Jøset et al., 2011). Instead of turning rostral after crossing the ventral midline, commissural axons in MDGA2A knockdown animals stall at the contralateral side, unable to follow ipsilateral-projecting axon fascicles (Jøset et al., 2011).

In zebrafish embryos MDGA2A is expressed in motoneurons of the oculomotor (nIII) and trochlear (nIV) cranial nerve in the midbrain, and the branchiomotoneurons of the trigeminal, facial and vagal nerve in the hindbrain. In support of a role in axon outgrowth, MDGA2 is expressed in the spinal cord during neuronal migration and axon path finding in various species.

Only few MDGA binding partners have been discovered so far. We found earlier that chicken MDGA2 formed homophilic trans-interactions in multiple assays, while heterophilic interactions between MDGA2 and MDGA1 were not detected (Jøset et al., 2011). MDGA1 in contrast, did not form homophilic interactions but soluble recombinant protein was shown to bind to axon rich regions in chicken and this interaction was MAM domain dependent (Fujimura et al., 2006; Jøset et al., 2011). More recently, it was reported by two independent groups that both MDGAs interact via their Ig-repeats with neuroligin-2 in cis (Lee et al., 2013; Pettem et al., 2013). The affinity of MDGA1 for neuroligin-2 was in the low nanomolar range, while MDGA2 binding was weaker (Pettem et al., 2013). Taken together, these results suggest that MDGA1 and MDGA2 binding preferences clearly differ. MDGA1 may undergo strong heterophilic interactions in cis thereby regulating the function of its binding partners, while MDGA2 may preferentially form homophilic interactions in trans serving as an adhesion factor. Depending on the concentration of MDGA2 in trans and the presence of binding partners such as neuroligin-2 in cis, MDGA2s regulatory or adhesive function may dominate.

Recently, it has been shown that neuron to neuron as well as neuron to extracellular matrix contacts are important for facial motoneuron migration (Wanner et al., 2013). While contact to a specific pioneer neuron seems to be required to lead following facial neurons in the early phase of migration, interaction with axons of the medial longitudinal fascicle is required in a subsequent phase of migration (Wanner and Prince, 2013). In the case of facial branchiomotoneuron migration, cdh2 and Tag1 seem to play a crucial role (Wanner and Prince, 2013; Sittaramane et al., 2009); however, these molecules have no influence on trigeminal neuronal migration. In our case the knockdown of MDGA2A has no effect on neuron migration but enhances the mobility of trigeminal neurons, which relocated from their original place to settle at ectopic positions. This increased mobility might be due to the lack of MDGA2A mediated homophilic interactions in trans, weakening the cohesion of trigeminal neurons enabling them to migrate along their axonal fascicle. Interestingly, even though facial neuronal migration is unaffected in MDGA2A knockdown animals, axonal outgrowth of facial neurons is clearly impaired. In line with the fact that MDGA-2 is a homophilic cell adhesion molecule, the MDGA2A positive facial nerve is less compact and seems to contain fewer axons in MDGA2A morphants. In addition, increased axon defasciculation along the facial nerve can be seen and the entire nerve path deviates from patterns seen in wild type embryos, again suggesting that MDGA2A mediated homophilic adhesion is keeping the facial nerve compact. Moreover, at a well-defined choice point, where trigeminal axons in wild type animals make a characteristic 60° turn (Higashijima et al., 2000), axons in MDGA2A knockdown animals display many collagenals and large protrusions, as being unable to make the correct pathway decision. Consequently, the angle at which axons in MDGA2A knockdown animals are leaving this choice point is dramatically reduced, suggesting that some aspects of proper guidance are missing. A similar phenotype has already been observed in MDGA2A knockdown chicken embryos, where turning of commissural interneurons after midline crossing is impaired. Instead of turning rostral, commissural axons in MDGA2A deficient embryos stall after midline crossing being unable to interconnect with MDGA2A positive tracts on the contralateral site (Jøset et al., 2011). This similarity between the chicken and our zebrafish phenotype suggests that MDGA2A might confer adhesive interactions between different axonal tracts, thereby enabling follower tracts to use pioneer tracts as predetermined highways.

Interestingly, rare deletions in the MDGA2 gene were recently correlated with autism spectrum disorders (ASD) (Bucan et al., 2009). This puts MDGA2 in line with other neuronal cell adhesion molecules of the immunoglobulin family, such as
Identification and cloning of zebrafish MDGAs

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MATERIALS AND METHODS

Fish maintenance and breeding

Identification and cloning of zebrafish MDGAs

Whole mount in situ hybridization

Linearized MDGA containing plasmids were purified using the QiAquick PCR Purification Kit (Qiagen). In vitro transcription of DNA probes using the SP6 and T7 RNA polymerases was performed using the Roche DIG RNA Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland). Probes longer than 1000 bp were hydrolyzed prior to hybridization. Embryos predetermined for in situ hybridization were treated with 3 μM PTU [1- phenyl-2-hiourea (Sigma)] to suppress pigmentation. PTU-treated embryos were collected at different stages of development and staged by morphology. The embryos were dechorionated, fixed in paraformaldehyde (4% in PBS, pH 7.25) and incubated at 4°C overnight or at room temperature (RT) for 1–2 h. Subsequently, the embryos were dehydrated stepwise in methanol/PBT. To enhance penetration of the antisense RNA probe, embryos older than 24 hpf were treated with 10 μl/ml Proteinase K (Roche) at different durations, depending on their developmental stage. The reaction was stopped by rinsing in PBT (PBS pH 7.25, 0.1% Tween-20). Then, the embryos were post-fixed in paraformaldehyde (4% in PBS, pH 7.25) for 20 min and washed in PBT 5 times for 5 min. Embryos were prehybridized for 2–5 h between 62 to 68°C in hybridization buffer (50% formamide, 5×SSC, 5 mg/ml torula yeast RNA (type VI, Sigma), 50 μg/ml heparin (Sigma), 125 μg/ml fish sperm DNA (Roche), 0.1% Tween-20). Afterwards, the prehybridization buffer was replaced by pre-warmed hybridization buffer containing 1–2 ng/μl of DIG labeled antisense RNA. Hybridization occurred at 62°C–68°C overnight. The embryos were washed in a series of hybridization buffer/SSC steps for 15 min at 62 to 68°C. Embryos were subsequently washed in MABT (100 mM Maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 5 min at RT and blocked for 2 h in blocking solution (2% Bohrer & Blocking reagent in MABT). Antibody solution (anti-Digoxigenin-AP, Fab fragments from Roche diluted 1:4000 in blocking solution) was incubated ON at 4°C, followed by 3×15 min washes in blocking solution and 3×15 min in NTMT (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M MgCl, 1 mM Levamisol, 0.1% Tween-20). Staining solution (0.5 mg/ml NBT, 0.175 mg/ml BCIP (both from Roche) in NTMT) was applied for 1–4 h in darkness, and was replaced by PBT. Embryos were post-fixed in 4% PFA for 20 min, washed in PBT and brought into Glycerol for imaging and storage. For obtaining optimal pictures, larvae were mounted on an adapted glass slide in 100% glycerol (Sigma-Aldrich) and the DIC modus of a light microscope (Olympus BX61) and a colour camera (ColorView IIu, Soft Imaging System, Olympus) were used.

Antisense morpholino oligonucleotide injections

Antisense morpholino oligonucleotide injections

Production of anti-MDG2A peptide antibodies

For the generation of MDGA2 specific antibodies, a short peptide covering the amino acids 276–289 (LSWVRNTEELPKKS) of MDGA2 was synthesized (Eurogentec, Belgium). The used sequences were checked for infastrat familiar sequence homology using the MegaAlign program. Prior to immunization, pre-sera were tested for cross-reactivity by western blot analysis. Two rabbits were injected with the synthesized peptide. Rabbits were boosted after 14 days and 28 days, respectively. A first blood sample was taken at day 38, followed by an additional injection on day 56 and a second blood sample that was taken at day 66. The final bleeding was done after 87 days. Antibodies were delivered as sera and IgGs affinity purified against the corresponding MDGA2A peptide.

Confocal analysis

Fluorescent samples were mounted in G11 Glycerol Gelatin (Sigma) and viewed with a Leica SP2/SP8 Confocal Microscope. 20× and 63×
Glycerin objectives were used to picture confocal sections every approximate 0.6 μm with an average number of 150 steps.

**Light sheet microscopy**

We employed Digital Scanned Laser Sheet Microscopy (DLSM) for three-dimensional imaging, as previously described (Keller et al., 2008). The central thickness of the light sheet was set to 4 μm (FWHM). An Acroplan W 40×/0.8 water-dipping objective (Carl Zeiss) was used for fluorescence detection. Three-dimensional image stacks were recorded in 16 min time intervals with a CoolSnap 4K CCD camera (12 bit, 7.4 μm pixel pitch, 2048 × 2048 pixels; Roper Scientific). The spacing between single images in the three-dimensional stack was set to 2.2 μm and a total of 600 images were recorded per stack, covering a specimen volume of $379 \times 379 \times 1320 \mu m$.

**Image processing**

Images were deconvolved with the Lucy-Richardson algorithm and specimen drift was corrected by custom software developed in Matlab, as previously described (Keller et al., 2008). For visualization of the three-dimensional image data as a function of time, maximum-intensity projections were generated.

**Intensity measurement**

To measure the intensity of axons, a line was drawn manually along the axon, a region in a certain radius around that line was selected and the pixel intensities of that region in the maximum projection were averaged. An area at the edge of the image was used for background subtraction. This procedure was repeated for every maximum projection of a movie to obtain the time course of fluorescence intensity. The values from multiple embryos were averaged and plotted together with the standard error of the mean.

**Statistical analysis**

Significance of fluorescent intensity along the facial nerve was calculated by bootstrapping. For each time point the mean and confidence intervals at an alpha value of 0.05 were calculated from 1000 repeats. For determining the number of cells in nV clusters wt (n=11), control morpholino injected (n=6) and MDGA2A morphant (n=28) zebrafish in 3D reconstructions of confocal stacks using Bitplane Imaris software were analyzed. Statistical analysis was performed using two-tailed homoscedastic Student’s t-test. For the analysis of migrating cells a two-tailed heteroscedastic Student's t-test, significance was calculated by two-tailed homoscedastic Student’s t-test. Image J's angle tool on maximum intensity projections. Statistical significance was calculated for two-tailed homoscedastic Student’s t-test.

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**Competing interests**

The authors have no competing interests to declare.

**Author contributions**

MG conceived the study, EI, CMM, CJB, AL and PR designed and performed experiments. PKJ and EHS designed and built the DLSM microscope. EI, CMM, CJB and MG wrote the manuscript. UFG was involved in setting up the DLSM microscope and gave suggestions about the manuscript. CFN gave conceptual input to the project and provided fish and other important resources. All authors have read and approved the final manuscript.

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**References**


Fig. S1. Phylogenetic relationship and homology between MDGA proteins.
(A) Maximum likelihood phylogeny of members of the zebrafish (dr), fugu (tr), medaka (ol), stickleback (ga), mouse (mm), Xenopus (xt), chicken (gg) and human (hs) MDGA family. The phylogenetic tree was build using 722 representative amino acids determined by the program Gblocks after sequence alignment using MUSCLE. Bootstrap values above 50% (0.5) are shown. Fish MDGA proteins are shown in red and zebrafish proteins are highlighted in bold. As an outgroup to root the tree ciona (ci) MDGA was used. Note that in zebrafish and fugu MDGA-2 genes have retained their duplicates after the teleost specific whole genome duplication, resulting in an A and B paralog. The scale bar shows the percentage (0.5 equals 50%) of amino acid substitutions required to generate the corresponding tree.

(B) Homology comparison between individual domains of zebrafish MDGAs. Domain boundaries were identified using the smart program (http://smart.embl-heidelberg.de/) and subsequently compared pairwise using BLASTP. While the first number represents the percentage of identical residues, the second number indicates the number of conserved residues (eg. L vs. V; D vs. E; etc.). Note that the amount of conservation varies greatly between the different domains.

(C) Conservation between human, mouse and zebrafish MDGA1s.

(D) Conservation between mouse and zebrafish MDGA2s. Note that comparison between human and zebrafish MDGA2s gave similar results.
Fig. S2. MDGA-2A antibodies stain multiple tracts in the zebrafish hindbrain and spinal cord. (A) A ladder-like staining of MDGA-2A positive reticulospinal interneurons can be observed. Their MDGA-2A labeled axons project into one of the two major longitudinal fiber pathways through the CNS, the medial longitudinal fascicle (mlf) or the lateral longitudinal fascicle (llf). In the hindbrain, the mlf splits into dorsal and ventral components (mlfD and mlfV, respectively). (B) 3D reconstruction of the area above generated with the Imaris software. (C,D) MDGA-2A positive longitudinal tracts (dll and mlf) extend into the spinal cord. At each somite segment, the MDGA-2A antibody stains spinal motor axons (sma) emerging from the mlf. (E) A 3D reconstruction of the are depicted in C,D generated with the Imaris software. Scale bars equal 200 μm.

Fig. S3. Detailed quantification of the MDGA-2A phenotypes. (A) Fluorescence intensity in the region along the axon (yellow area) was measured in wt and MDGA-2A knockdown animals. Background fluorescence intensity in an area of identical size but outside the migration path of axons and neurons was subtracted to obtain the net intensity in the area of interest. (B) The fluorescence intensity along the trigeminal axon bundle was quantified in 8 control embryos and 5 MDGA-2A morpholino treated embryos. Shown are the mean and error of the mean (as dotted lines). Due to aberrant neuronal migration along the trigeminal axon bundle staining intensity in this area increases clearly in MDGA-2A knockdown animals. (C) Individual measurements of wt and MDGA-2A knockdown are shown. (D) Fluorescence intensity in an area below the axon bundle of the facial nerve is increased in MDGA-2A knockdown animals. Fluorescence intensity in a region underneath the facial axon bundle (yellow area) was measured in wt and MDGA-2A knockdown animals. Background fluorescence intensity in an area of identical size but outside the projection path of axons was subtracted to obtain the net intensity in the area of interest. (E) In MDGA-2A knockdown animals older than 30 hpf, the mean fluorescence intensity underneath the facial nerve was increased, representing increased branching and defasciculation. Note that in wt animals residual fluorescence intensity can also be detected, as transient branch formations also occur during regular development. (F) Individual measurements of wt and MDGA-2A knockdown are shown.
Fig. S4. Different MDGA2A morpholino oligonucleotides located around the ATG start codon cause identical phenotypes. (A) Morpholino induced MDGA2A knockdown increases mobility of trigeminal neurons. Representative images from wild type and zebrafish embryos treated with different MDGA2A morpholinos are shown. At 34 hpf trigeminal neurons in wild type embryos have formed a dense trigeminal cell cluster and have send out axons towards their targets (w1, w2). In animals treated with different MDGA2A start-site morpholinos the compactness of the trigeminal cell cluster is impaired (mA1/2, mB1/2, mC1/2). Trigeminal neurons migrate along their axon bundles leaving their place of origin (arrows). (B) MDGA2A knockdown influences axon turning and bundling of the facial nerve. Representative images from control (w3, w4) and MDGA2A morpholino treated embryos (mA3/4, mB3/4, and mC3/4) are shown. 34 h post fertilization facial neurons in wt embryos project axons along a predetermined path, displaying a 60° turn (pink dotted lines) at a well-documented turning point. In the different MDGA2A morphants this turning angle is strongly reduced and the formation of axon collaterals occurs much more frequently around this turning point. The scale bar represents 20 μm. (C) MDGA2A morpholinos efficiently reduce MDGA2A protein expression. wt and MDGA2A morpholinos treated embryos were stained with MDGA2A antibodies to visualize axonal tracts. In wt embryos tracts are clearly stained. Under identical staining and recording conditions axonal staining in MDGA2A knockdown animals is absent or very weak, demonstrating that the MDGA2A protein in these animals is strongly downregulated. As a landmark the otic vesicle (ov) is highlighted. The sequence of the morpholinos used (MDGA2A_MO-B/C) is given in the material and methods section. The scale bar represents 20 μm. (D) Statistical analysis of migration and axon guidance defects in MDGA2A knockdown animals. The number of analyzed trigeminal cell clusters and facial nerves for un.injected wild type, standard control and MDGA2A (MO-A, MO-B, MO-C) morpholino injected animals is given. The average cell number in the trigeminal cell cluster as well as the average turning angle of the facial nerve under the different experimental conditions are summarized. Note that the last two lines represent the pooled data of control (wt and standard control morpholino injected embryos) and MDGA2A (MO-A, MO-B, MO-C) morpholino injected animals.
**Movie 1.** In vivo development of cranial motoneurons observed by light sheet microscopy. Zebrafish islet-GFP wt larva were monitored between 24–36 hpf. Fluorescent images were taken every 16 min and processed as described in material and methods. Scale bare equals 20 µm.

**Movie 2.** Development and migration of trigeminal motoneurons in wild type and MDGA2A knockdown animals observed by light sheet microscopy. The left panel shows the development of trigeminal neurons during the time between 24 and 36 hpf in islet-GFP fish. Note that neurons within the trigeminal cell cluster remain tightly together, sending out axons into the trigeminal nerve. The right panel depicts the situation in MDGA2A knockdown animals. In the case of MDGA2A knockdown increased mobility and intense migration of trigeminal neurons along the trigeminal nerve can be observed. Scale bar represents 20 µm.

**Movie 3.** Development of the facial nerve in wild type and MDGA2A knockdown animals. Wild type and MDGA2A knockdown islet-GFP fish were monitored by light sheet microscopy between 24 and 36 hpf. The left panel illustrates the normal development of the facial nerve during this period of development. Around 30 hpf the facial nerve in wt larva displays a characteristic 60° turn. At this “choice point” temporary stalling and increased transient branching can be observed even in the wild type. However, most branches retract over time and the axon bundle stays fasciculated. The middle and the right panel depict facial nerve growth in MDGA2A knockdown animals. Note that the typically observed 60° turn is absent in MDGA2A morphants and that strongly increased branching and defasciculation along the facial nerve is seen in these larva. Scale bar equals 20 µm.