Sim1 Is a Novel Regulator in the Differentiation of Mouse Dorsal Raphe Serotonergic Neurons

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Abstract

Background: Mesencephalic dopaminergic neurons (mDA) and serotonergic (5-HT) neurons are clinically important ventral neuronal populations. Degeneration of mDA is associated with Parkinson’s disease; defects in the serotonergic system are related to depression, obsessive-compulsive disorder, and schizophrenia. Although these neuronal subpopulations reveal positional and developmental relationships, the developmental cascades that govern specification and differentiation of mDA or 5-HT neurons reveal missing determinants and are not yet understood.

Methodology: We investigated the impact of the transcription factor Sim1 in the differentiation of mDA and rostral 5-HT neurons in vivo using Sim1−/− mouse embryos and newborn pups, and in vitro by gain- and loss-of-function approaches.

Principal Findings: We show a selective significant reduction in the number of dorsal raphe nucleus (DRN) 5-HT neurons in Sim1−/− newborn mice. In contrast, 5-HT neurons of other raphe nuclei as well as dopaminergic neurons were not affected. Analysis of the underlying molecular mechanism revealed that tryptophan hydroxylase 2 (Tph2) and the transcription factor Pet1 are regulated by Sim1. Moreover, the transcription factor Lhx8 and the modulator of 5-HT1A−mediated neurotransmitter release, Rgs4, exhibit significant higher expression in ventral hindbrain, compared to midbrain and are target genes of Sim1.

Conclusions: The results demonstrate for the first time a selective transcription factor dependence of the 5-HT cell groups, and introduce Sim1 as a regulator of DRN specification acting upstream of Pet1 and Tph2. Moreover, Sim1 may act to modulate serotonin release via regulating RGS4. Our study underscores that subpopulations of a common neurotransmitter phenotype use distinct combinations of transcription factors to control the expression of shared properties.

Introduction

Cell and neuron subpopulations in the CNS differentiate in stereotypic, defined positions along the anterior-posterior and dorso-ventral axes of the neural tube and exhibit a cell-type-specific transcriptional code [1]. Among neuronal subpopulations, mesencephalic dopaminergic neurons (mDA) and serotonergic (5-HT) neurons are of particular interest, because of their involvement in neurological and degenerative diseases. Degeneration of mDA is associated with Parkinson’s disease [2], defects in the serotonergic system are related with depression, obsessive-compulsive disorder, and schizophrenia [3].

During development of the CNS, mDA and 5-HT neurons are among the first to emerge [4]. Because of their early onset 5-HT neurons are even regarded to play a role in several differentiation processes, acting as a growth and differentiation promoting factor in addition to being a neurotransmitter. 5-HT neurons are involved in the differentiation of precursor cells in their target regions. Both mDA and 5-HT neurons develop as distinct cell groups and have a close ontogenetic relationship: their respective Otx2- and Gbx2-expressing progenitors are located adjacent on either side of the midbrain-hindbrain boundary [5], their development critically depends on Shh and FGF8 [6], and the transcriptional code controlling differentiation of progenitors toward dopaminergic or serotonergic cell fate shares common determinants, such as Nkx2-2, Lmx1b, and Mash1 [7–9]. However, the transcriptional equipment of mDA additionally includes Lmx1a, Nurr1, Pitx3, and En1/2 [10], whereas differentiation of rostral 5-HT neurons involves Pet1 and Gata2 [11,12]. In spite of numerous studies, the transcriptional network and the developmental cascades that govern specification and early differentiation of dopaminergic or serotonergic neurons reveal missing determinants, and are not yet understood.

The Drosophila CNS midline cells derive from the mesectoderm that gives rise to CNS midline progenitor cells, and their development requires expression of the bHLH/PAS domain
transcription factor single-minded (sim) [13–16]. Thereby, sim activates expression of rho, en, and agf [17]. The two mammalian homologs, Sim1 and Sim2 [18], reveal differential expression patterns during mouse embryogenesis [19]. Sim1-/− and Sim2-/− mice die perinatally due to hypothalamic and respiratory defects, respectively [20,21]. Interestingly, Gata2, expressed in postmitotic serotonergic progenitors, and Gadd, a survival promoting factor for mDA [22], represent target genes of Sim1 [23]. Moreover, Sim1 has shown to be upregulated in mouse ventral regions of the CNS (M. Wiehle, N. Osterberg, and E. Roussa, unpublished observations). Here we demonstrate the impact of Sim1 in the differentiation of mDA and rostral 5-HT neurons using Sim1 gain- and loss-of-function experiments in vitro, and Sim1 deficient mice in vivo. The results show that development of mDA is Sim1-independent. Conversely, Sim1 represents an important regulator in the development of a subpopulation of rostral 5-HT neurons, the dorsal raphe nucleus, acting upstream of Petl and Tph2. Moreover, we have identified target genes of Sim1, namely Lhx8, RGS4, and Bm3.2 being upregulated in ventral hindbrain, compared to ventral midbrain, and Lhx8 and RGS4 as putative candidates involved in the specification of mouse rostral serotonergic neurons.

Results

Sim1 is expressed in mouse ventral midbrain and ventral hindbrain and co-localizes with TH and 5-HT

As the first step to investigate potential involvement of Sim1 in the differentiation of mDA and 5-HT neurons, we have determined that Sim1 is expressed in these brain areas at crucial developmental stages. Fig. 1A shows RT-PCR analysis of mouse E12 ventral midbrain, isthmus, and ventral hindbrain tissue using specific Sim1 primers. A transcript of the expected size of ~319 bp could be amplified from all probes examined (Fig. 1A, lanes 5–7). Sim1 expression was considerably stronger in mouse ventral midbrain, compared to hindbrain. Subsequently, cellular localization of Sim1 with the late markers for dopaminergic and serotonergic lineage, TH and 5-HT, respectively, has been performed in newborn mice. Fig. 1B illustrates double immunolabeling for TH and Sim1 on fixed paraffin sections from mouse midbrain at the area of substantia nigra pars compacta (SNC) and ventral tegmental area (VTA). Sim1 was exclusively expressed in cell nuclei, whereas TH showed an intracellular labeling pattern. At higher magnification, a co-localization of Sim1 could be observed with TH (Fig. 1C). However, whereas all TH positive neurons showed additionally immunoreactivity for Sim1 (Fig. 1C, arrows), many Sim1 immunopositive cells were devoid of TH immunoreactivity (Fig. 1C, asterisks), indicating a broader expression domain of Sim1. Similar results could be obtained in all rostral serotonergic neurons of newborn mice. Fig. 1D and 1E exemplarily illustrate double immunofluorescence for Sim1 (red) and 5-HT (green) in the area of dorsal raphe nucleus (DRN). Sim1 was found always co-localized with 5-HT neurons. Again, in several Sim1 positive cells 5-HT immunoreactivity could not be detected.

Sim1 is required for the differentiation of a subpopulation of mouse rostral serotonergic neurons, but not for mesencephalic dopaminergic neurons

In Drosophila melanogaster, Sim is required for development of all midline CNS cells [24]. We have investigated putative effects of the mouse homolog Sim1 in the induction and specification of ventral neuronal subpopulations, represented by mDA and rostral 5-HT neurons, in vivo.

Fig. 2A shows cell counts after immunohistochemistry of midbrain TH immunopositive cells of fixed wild type (wt) and Sim1−/− mutant mice embryos at E14.5. The results show no significant differences in the number of midbrain TH immunoreactive neurons between wt and Sim1−/− mutant embryos (p = 0.4023, n = 6). Similar results could be obtained in newborn mice (P0) for TH immunoreactive cells of the substantia nigra and ventral tegmental area (Fig. 2B; p = 0.3220, n = 6).

Analysis of the serotonergic system from wt (C) and Sim1−/− mutants (D) at E14.5 showed that the total number of 5-HT immunolabeled rostral serotonergic neurons was comparable between Sim1−/− embryos and their wt littermates (Fig. 2E; p = 0.15, n = 6). Similarly, in newborn mice (Fig. 2F), the total number of rostral 5-HT immunopositive cells (i.e. raphe nuclei B5-B9) showed no significant differences between wt (Fig. 2F) and Sim1−/− (Fig. 2H; p = 0.06, n = 6). However, when 5-HT immunoreactive neurons were quantified for each nucleus separately, a significant decrease in the number of serotonergic neurons in the dorsal raphe nucleus (DRN) was found in Sim1−/− (Figs. 2I), compared to the wt (Figs. 2G; **p = 0.009, n = 6). In contrast, the number of 5-HT immunopositive cells in the median and paramedian raphe nuclei were comparable between wt and Sim1 mutants (Fig. 2J; p = 0.61 and p = 0.27, respectively, n = 6).

These data implicate a selective role for Sim1 in the differentiation of a particular subset of rostral 5-HT neurons, whereas induction, specification, and maintenance of mDA are Sim1-independent.

MN9D cell line expresses dopaminergic and serotonergic markers

Elucidation for the role of a gene of interest in the development of mDA and 5-HT neurons can be complemented by an adequate in vitro system that allows gain of function and loss of function experiments. The MN9D cell line [25] expresses key dopaminergic markers and has been used in studies dealing with differentiation of dopaminergic neurons [26]. However, MN9D cells apparently express specific markers for the serotonergic lineage as well, as assessed by RT-PCR analysis, shown in Fig. 3A. Transcripts of the expected size for Gata2 (~110 bp, lane 3), expressed in mitotic and postmitotic serotonergic progenitors, and for Petl (~109 bp, lane 4), exclusively expressed in postmitotic 5-HT neurons, could be amplified from MN9D cells cDNA. In addition, expression of TPH2 (~99 bp, lane 5), a late specific marker for the serotonergic lineage, was also found. The gene of interest, Sim1, showed only weak expression level in MN9D cell line (~115 bp, lane 6). Moreover, besides genes involved in induction of 5-HT phenotype, expression of Nestin (~438 bp, lane 10), a marker for neural precursor cells, was also expressed, indicating that the MN9D cell line is comprised of a mixed cell population. Taken together, these results indicate that MN9D cells represent a suitable in vitro model system to study the molecular mechanisms underlying differentiation of mDA and 5-HT neurons.

Fig. 3B illustrates RT-PCR analysis after overexpression of Sim1 in MN9D cells 24 h, 48 h, and 72 h after transfection of the cells either with pcDNA3 expression vector (controls; ct) or with pcDNA3::Sim1 expression vector (Sim1), as described in Material and Methods. Sim1 was expressed at high levels 24 h after transfection with pcDNA3::Sim1, compared to the controls, and remained at this level up to 72 h after transfection. Consistently, western blot analysis using a specific anti-Sim1 antibody (Fig. 3C) showed a prominent immunoreactive band at ~85.5 kDa (arrow), corresponding to the molecular mass of full length Sim1. As shown in Fig. 3C, in pcDNA3::Sim1-transfected MN9D cells Sim1
protein is abundantly expressed 24 h (lane 2), and 48 h (lane 4) after transfection, compared to the MN9D cells that had been transfected with pcDNA3 (lanes 1 and 3) expression vector. Similar results were obtained 72 h after transfection (data not shown).

Expression of early and late dopaminergic markers is not affected by Sim1

We next examined whether Sim1 over-expression in the MN9D cells has an impact on the expression of genes associated with mDA or rostral 5-HT neurons.

Fig. 4A represents the western blot analysis of the MN9D homogenate from the controls, i.e. cells transfected with pcDNA3 (lanes 1 and 3) expression vector (ctrl) and from Sim1-overexpressing cells 24 h after transfection, using anti-GAPDH as a housekeeping gene and Pitx3 or TH antibodies. Protein abundance of the dopaminergic markers Pitx3 (~31.7 kDa, lanes 1 and 2) and TH (~41 kDa, lanes 3 and 4) was comparable between controls and Sim1-overexpressing cells (1.16±0.50 fold; p = 0.89, n = 3 and 1.27±0.35 fold p = 0.72, n = 5, for Pitx3 and TH, respectively). Similar results were obtained in probes collected 48 h and 72 h after transfection (data not shown).

Fig. 4B illustrates a representative immunoblot of homogenate from MN9D controls and MN9D overexpressing Sim1, probed with specific anti-Pet1 and anti-TPH2 antibodies. Using the anti-Pet1 and anti-TPH2 antibody respective bands at ~35 kDa and at
Figure 2. Phenotype analysis for midbrain dopaminergic and rostral serotonergic neurons of Sim1-/- mutants. Counting of TH positive cells after immunostaining in midbrain tissue sections revealed no differences in the number of midbrain dopaminergic neurons between wild type (wt) and Sim1-/- mutants at embryonic day (E)14.5 (A) and newborn (P0; B). ns: not significant. C-D: 5-HT immunofluorescence in frontal sections of wt (C) and Sim1-/- embryos (D) at E14.5 (DRN: dorsal raphe nucleus; Aq: aqueduct). E: Quantification of cell counts: numbers of 5-HT immunopositive
57 kDa could be detected in both samples, corresponding to the respective full length proteins. However, both Pet1 and TPH2 protein was significantly upregulated in MN9D cells overexpressing Sim1, compared to the MN9D cells that had been transfected with pcDNA3 vector (2.15 ± 0.32 fold, *p = 0.04 and 2.68 ± 0.41 fold, *p = 0.031, n = 4 for Pet1 and TPH2, respectively).

Figure 3. MN9D cells, a suitable in vitro model system. A: Expression of determinants of the serotonergic lineage in the MN9D cell line by RT-PCR. Gata2, Pet1, and Tph2 could be amplified from MN9D cells cDNA. The Sim1 primers used correspond to nucleotides (nt) 37-66 and nt:152-126 of the mouse Sim1. In addition, expression of the neural stem cell marker nestin was also detected. B: RT-PCR analysis of Sim1 expression in MN9D cells cDNA, 24 hours, 48 hours, and 72 hours after transfection of the cells with either pcDNA3::Sim1 or pcDNA3 expression vectors (ctl) and GAPDH as housekeeping gene. Sim1 expression was clearly increased in pcDNA3::Sim1-transfected MN9D cells, compared to the pcDNA3-transfected cells. C: Sim1 protein abundance by immunoblotting in homogenates of MN9D cells transfected either with pcDNA3::Sim1 or pcDNA3 expression vectors (ctl) 24 hours and 48 hours after transfection. The immunoblots were probed either with monoclonal antibody against GAPDH or with rabbit polyclonal antibody against Sim1. Sim1 protein was significantly upregulated in pcDNA3::Sim1-transfected MN9D cells, compared to the pcDNA3-transfected cells (**p<0.01 after densitometric analysis of the signal ratio Sim1: GAPDH and Student’s t-test, n = 3). Arrow points to the band of the expected size. The blots are representative for three different experiments. 30 μg protein was loaded per lane.
Expression of serotoninergic markers *Pet1* and *Tph2*, but not *Mash1* and *Gata2*, are controlled by *Sim1*

To further understand the mechanism underlying the effect of *Sim1* on serotoninergic neurons, we investigated whether expression of early and late determinants of the serotoninergic lineage might be influenced by *Sim1*. Fig. 5 illustrates quantitative real-time PCR (qRT-PCR) analysis of relative expression of *Gata2*, *Mash1*, *Pet1*, and *Tph2* in MN9D cells transfected either with control or *Sim1* expression vectors 24 h, 48 h, and 72 h. *Sim1* overexpression had no effect on *Gata2* (A) and *Mash1* (B) expression at all time points. By contrast, as shown in Fig. 5C, expression of *Pet1*, a transcription factor exclusively expressed in postmitotic 5-HT neurons, was found significantly upregulated in *Sim1* overexpressing MN9D cells, relative to the controls, at 48 h and 72 h after transfection (**p = 0.003** and **p = 0.01** for 48 h and 72 h, respectively; n = 3). Moreover, expression of *Tph2* (D), the rate-limiting enzyme in brain serotonin biosynthesis, was significantly upregulated in *Sim1* overexpressing cells, compared to the controls, at 48 h and 72 h as well (**p = 0.006** and **p = 0.0009** for 48 h and 72 h, respectively; n = 3). These data indicate that *Pet1* and *Tph2* expression is likely controlled by *Sim1*, either directly or indirectly.

Expression of *Sim1* target genes *RGS4*, *Lhx8*, and *Brn3.2*, is upregulated in ventral hindbrain

To investigate the difference between the transcriptional profiles between rostral 5-HT and mDA at their early stages of development we have screened for genes that are upregulated in mouse ventral hindbrain, compared to ventral midbrain tissue at E11.5 and therefore could be considered as potential candidates involved in the induction of 5-HT neurons. Fig. 6A shows qRT-PCR analysis and relative mRNA expression of the transcription factors *Bm3.2* and *Lhx8*, as well as for *RGS4*, a regulator of G protein signalling and modifier of 5-HT1A-mediated neurotransmitter release, in mouse E11.5 ventral hindbrain tissue, compared to ventral midbrain. The results showed significant upregulation of *Lhx8* by approximately 12.32 fold in mouse ventral hindbrain, compared to ventral midbrain. Expression of *Bm3.2* was found ~6.16 fold upregulated in ventral hindbrain. Finally, *RGS4* expression was upregulated by approximately 2.81 fold in the ventral hindbrain, thus considerably lower than *Lhx8* and *Bm3.2*, but still significantly higher than in mouse ventral midbrain.

Since *RGS4* and *Brn-2*, a homolog of *Bm3.2*, have been identified as target genes of *Sim1* [13,27], their expression has been investigated in MN9D cells transfected with pcDNA3::Sim1 expression vector and compared with the controls by qRT-PCR. As illustrated in Fig. 6B, *Sim1* overexpression in MN9D cells resulted in a significant increase of *RGS4* expression 24 h after transfection (**p = 0.003; n = 3**). This upregulation was however transient, since 48 h and 72 h after transfection *RGS4* expression was found comparable to the controls. Fig. 6C represents qRT-PCR analysis for *Bm3.2*, in controls and *Sim1* overexpressing cells. *Bm3.2* mRNA expression level showed no significant differences between the experimental groups 24 h after transfection. However, 48 h after transfection, *Bm3.2* was significantly upregulated in *Sim1*-overexpressing cells, compared with the MN9D cells transfected with pcDNA3 vector (**p = 0.0023; n = 3**). Regulation of *RGS4* and *Bm3.2* by *Sim1* has been further investigated at the protein level by immunoblotting (Fig. 6D) in homogenate of MN9D cells transfected with pcDNA3::Sim1 expression vector and compared to the controls, 48 h after transfection. The results show comparable protein levels of *RGS4* between controls and *Sim1* overexpressing cells, confirming the qRT-PCR data. In contrast, *Brn3.2* protein was found upregulated 48 h after transfecting MN9D cells with pcDNA3::Sim1 expression vector, compared to the controls.

To assess whether the upregulated genes *Bm3.2* and *RGS4* were expressed in rostral serotoninergic neurons, in situ hybridization was performed on mouse fixed cryosections. Figs. 6E-6G illustrate coronal sections from E14.5 mouse embryonic brain and Figs. 6H-6J, 6K, and 6M illustrate coronal sections from E18.5 mouse embryonic brain at the level of pontine dorsal raphe (Fig. 6L). Using a *Bm3.2* antisense probe, at both E14.5 (E) and E18.5 (H) *Bm3.2* was predominantly expressed in areas of hindbrain lacking serotoninergic neurons, thus showing no co-localization with 5-HT, illustrated by immunoho fluorescence in the respective consecutive sections (Figs. 6F and 6I). *RGS4* expression was detected in 5-HT neurons, as well as in areas of hindbrain lacking serotoninergic neurons, thus showing no co-localization with 5-HT. Using a *RGS4* sense probe, no labeling could be detected (Fig. 6M).

**RGS4**, *Bm3.2*, and *Pet1* are target genes of *Sim1* in vivo

The results obtained from the *Sim1* gain-of-function experiments were further verified by *Sim1* loss-of-function experiments.
Figure 5. Differential regulation of serotonergic lineage determinants by Sim1. Quantitative real-time PCR analysis of Gata2 (A), Mash1 (B), Pet1 (C) and Tph2 (D) mRNA levels in pcDNA3::Sim1-transfected MN9D cells and in pcDNA3-transfected cells. Expression of individual gene is shown as $2^{-\Delta\Delta Ct}$.

Gata2 and Mash1 expression remained unchanged in pcDNA3::Sim1-transfected MN9D cells, compared to pcDNA3-transfected cells 24 hours, 48 hours, and 72 hours after transfection.

In contrast, Pet1 and Tph2 expression was significantly up-regulated in pcDNA3::Sim1-transfected MN9D cells, compared to the controls 48 hours and 72 hours after transfection (*p<0.05; **p<0.01; and ***p<0.0001 using the Student’s t-test, n = 3).

doi:10.1371/journal.pone.0019239.g005
Therefore, MN9D cells were transiently transfected with Sim1 specific siRNA, coupled to CY5. 24 h after transfection and subsequent FACS isolation, cDNA synthesis and subsequent qRT-PCR using Pet1, Gata2, Brn3.2, Rgs4, and Tph2 specific primers. As shown in Fig. 7A, Pet1, Gata2, Brn3.2, and Tph2 expression was significantly downregulated 24 h after transfection of MN9D cells with Sim1 specific siRNA, compared to MN9D cells transfected with control siRNA. In contrast, Rgs4 expression 24 h after downregulation of Sim1 remained comparable with that of the controls. Together with the gain-of-function results, these data suggest Pet1, Brn3.2 and Tph2 as downstream targets of Sim1 (*p<0.05 for Pet1, Gata2, and Brn3.2, and *p<0.01 for Tph2, n = 5).

The biological significance of the in vitro data has been further validated in vivo. Therefore, we have used fixed brain tissue sections from wt and Sim1-/-- at P0 and performed in situ hybridization for Pet1 (Figs. 7B-7E), RGS4 (Figs. 7F-7L) and Brn3.2 (Figs. 7J-7M). In sections from wt mice at P0, Pet1 mRNA expression appeared distinct and of moderate intensity (Fig. 7B). At higher magnification (Fig. 7C) the area of the DRN is clearly positive for Pet1. In sections from Sim1-/-- brains at P0, labeling for Pet1 was decreased as in DRN; as shown in Figs. 7D and 7E, suggesting Pet1 as downstream target of Sim1. Similarly, as shown in Figs. 7F-7L, RGS4 expression was detected in the area of rostral serotonergic neurons in wt, whereas in Sim1-/-- mutants (Figs. 7H and 7L) RGS4 labeling was absent, demonstrating downregulation in vivo. Brn3.2 mRNA expression is shown in Figs. 7J-7M. Although Brn3.2 labeling of strong intensity could be observed in wt (Fig. 7J; asterisks), the area of rostral serotonergic neurons, including dorsal raphe nucleus (Figs. 7J, arrow, and 7K), and other raphe nuclei, were devoid of Brn3.2 expression. In Sim1-/-- (Figs. 7L and 7M) Brn3.2 expression was completely abolished, demonstrating that Brn3.2 is a target gene of Sim1 as well.

Finally, to validate the mRNA data at the protein level, protein extraction has been performed from 4%-fixed cryosections from hindbrain of wt and Sim1-/-- mutants, as described in Material and Methods, followed by western blot analysis. As illustrated in Fig. 7N, Pet1, RGS4 and Brn3.2 protein abundance was significantly downregulated in Sim1-/-- compared to the wt (0.57 ± 0.25 fold, *p = 0.032, 0.67 ± 0.12, *p = 0.048 and 0.70 ± 0.05, *p = 0.044, for Pet1, Rgs4 and Brn3.2, respectively, n = 4).

Discussion

In the present study we have addressed the question whether the mouse homologue of the Drosophila sim, nSim1, might be involved in the differentiation of two clinically important ventral neuronal populations, namely the mDA and rostral 5-HT neurons. These neuronal subpopulations reveal positional and developmental relationships and share common transcriptional determinants during their differentiation and specification.

After having shown that Sim1 is expressed in TH positive and 5-HT immunoreactive cells, we further investigated the biological significance of Sim1 during the development of mDA and 5-HT neurons in vivo, by examining Sim1 deficient mice at E14.5 and P0. Since Sim1 mutants die shortly after birth due hypothalamic developmental defects [27,28], an investigation in adult animals was not possible. Our results demonstrate comparable number of mDA in Sim1 mutants and wild type at both stages examined. In contrast, Sim1 has been shown to be involved in the differentiation of zebrafish diencephalic A11-related dopaminergic neurons [29]. Gene ablation of Sim1 had no effect on the total number of rostral 5-HT neurons at E14.5 as well, a time point in which individual rostral nuclei raphe cannot be distinguished. However, in Sim1-/-- newborn mice the number of 5-HT neurons in the dorsal raphe nucleus (DRN) was significantly decreased by 30% (Fig. 2J), but not in the median and paramedian raphe, demonstrating a selective effect of Sim1 on a rostral serotonergic subpopulation.

The serotonergic system is involved in the modulation and coordination of a variety of autonomic functions and behaviour, including the sleep-wake cycle, aggression, anxiety, mood, and stress. Besides, or even due to, its different functions the serotonergic system is characterized by extensive and complex cellular heterogeneity. First, 5-HT neurons consist of nine cell groups (B1-B9) that are separated into two clusters, the rostral (B5-B9) and caudal (B1-B4) nuclei raphe. Second, rostral and caudal 5-HT neurons are differentially regulated by developmentally potent signaling molecules, such as TGF-β [30], and also exhibit different electrophysiological properties, suggesting that they likely represent developmentally and functionally distinct neuronal subpopulations. Moreover, even within a given raphe nucleus individual cell subpopulations exist, characterized by different developmental background and topographically organized efferent projections. In particular, the neurons of DRN vary considerably with regard to their synaptic morphology and their electrophysiological properties from each other [31,32]. The DRN of the brainstem is the main source of serotonergic innervation of limbic structures fundamental in the regulation of emotionally influenced behaviour. Interestingly, serotonergic neurons that innervate the central nucleus of the amygdala, and subsequently exert a modulatory action on the vegetative centers of the hypothalamus, are restricted...
Figure 7. Sim1 loss-of-function in vitro and in vivo. A: Quantitative real-time PCR analysis for candidate genes in MN9D cells transfected either with control siRNA (ctl) or with specific Sim1 siRNA. Gene expression is shown as $2^{-\Delta\Delta C_{\text{t}}}$, Pet1, Gata2, Brn3.2, and Tph2, but not RGS4 expression was significantly down-regulated in Sim1 siRNA-transfected cells, compared to the controls, 24 hours after transfection (*p<0.05 and **p<0.01, using the Student’s t-test, n = 3). B-M: In situ hybridization for Pet1 (B-E), RGS4 (F-I) and Brn3.2 (J-M) in wild type (wt) and Sim1-/- mutants at P0. Pet1 expression was observed in the area of rostral 5-HT neurons (B) including the area of dorsal raphe in wt (C: magnification of the inset in B), but was considerably decreased in Sim1-/- (D). E: magnification of the inset illustrating the area of the dorsal raphe. RGS4 (F and magnification of the inset in G) was also expressed in the dorsal raphe in wt, and was considerably decreased in Sim1 mutants (H and magnification of the inset in I). In contrast, although Brn3.2 expression in wt was present (J, asterisks) the area of rostral 5-HT neurons was devoid of Brn3.2 expression (J, arrow and magnification of the inset in K). In Sim1-/- mutants Brn3.2 expression was absent (L, and magnification of the inset in M). N: Immunoblot analysis for Pet1, RGS4, and Brn3.2 in hindbrain of wt and Sim1-/- mutants. Pet1, RGS4, and Brn3.2 protein abundance was significantly downregulated in Sim1-/- hindbrain, compared to wt. (*p<0.05 after densitometric analysis of the signal ratio Pet1:GAPDH, RGS4:GAPDH or Brn3.2:GAPDH and Student’s t-test; n = 4). The blots are representative for four different experiments. 30 μg protein was loaded per lane.

doi:10.1371/journal.pone.0019239.g007
to the middle and caudal portions of the DRN [33]. Whether the observed loss of DRN 5-HT neurons in Sim1 mutants is restricted to a distinct neuronal subpopulation within this nucleus is not clear. Nevertheless, our results provide evidence that differential developmental transcriptional networks likely correspond to each neuronal subpopulation. Elucidation of the putative mechanism underlying Sim1 effects on rostral 5-HT neurons has been performed by gain- and loss-of-function experiments using the MN9D cells line as a culture system. The gene expression analysis performed in the present study (Fig. 5A) clearly demonstrates that these cells express determinants of the serotonergic lineage, represented by Gata2, Pet1, and TPH2. The transcription factor Pet1 together with Lmx1b, Nkx2.2, Mash1, Gat2a, Gat2c, and Phox2b form a transcriptional network, which specifies the differentiation of serotonergic neurons from hindbrain progenitors at E11 in the mouse. According to the current view for serotonergic neurons differentiation, Phox2b is switched off at E10.5. Together with Mash1 and Nkx2.2, the transcription factors Gat2a, Gat2c, Lmx1b, and Pet1 are activated, which subsequently define the cell fate by activating serotonergic lineage marker genes, such as TPH2. However, phenotype analyses from gene ablations studies have demonstrated that the suggested transcriptional network is still not complete and additional determinants are involved in the specification of rostral 5-HT neurons. Based on the results of the in vitro experiments, several lines of evidence propose Sim1 as a putative novel determinant in the transcriptional network orchestrating 5-HT neuron development. Sim1 overexpression did not affect expression of Gat2a though (Fig. 5A), a mitotic and postmitotic marker for rostral serotonergic neurons. This observation is quite surprising, since in a previous study in Neuro2A cells Gat2a has been identified as a downstream target of Sim1 [22]. Along this line, transfection of MN9D cells with specific Sim1 siRNA significantly downregulated Gat2a expression (Fig. 7A), demonstrating a role of Sim1 in maintenance of Gat2a expression. Sim1 overexpression had no effect on Mash1 as well, an early determinant of the transcriptional network underlying 5-HT development [9]. By contrast, Sim1 was able to activate Pet1 and Tph2, late markers specific for the serotonergic lineage. Both Pet1 and Tph2 were significantly upregulated after Sim1 overexpression, and significantly downregulated after transfection of MN9D cells with specific Sim1 siRNA, implicating Pet1 and Tph2 as putative downstream targets of Sim1. Moreover, conserved Pet1 binding sites are present in the promoter of several genes characteristic for the serotonergic phenotype, among them TPH2 [11], thus suggesting a putative alternative indirect cascade for Tph2 activation, by means that Tph2 regulation by Sim1 occurs secondarily through Pet1. In addition, in Sim1−/− only a subpopulation of 5-HT neurons was affected, and in Pet1−/− mutants 5-HT neurons were dramatically decreased but not completely lost, strongly suggesting that more than one pathway should be involved in the differentiation of 5-HT neurons. It is conceivable that Pet1 can be regulated by multiple transcription inputs at different subpopulation of 5-HT neurons. Along this line, we have revealed that for the dorsal raphe nucleus Sim1 is a crucial regulator. Notably, it has recently been shown that Pet1-dependent and Pet1-resistant 5-HT neurons exist, that have different morphological features, including targeting of different brain areas and formation of synaptic junctions [34].

We have also demonstrated that RGS4, Ddx5, and Bm3.2, all downstream targets of Sim1, are significantly upregulated in mouse E11.5 ventral hindbrain, compared to ventral midbrain (Fig. 6A). Sim1 overexpression in MN9D cells significantly upregulated RGS4 (Fig. 6B). Consistently, RGS4 expression (Figs. 7H, 7I) and protein abundance (Fig. 7N) was downregulated in hindbrain of Sim1−/− mutants. This result confirms and extends previous observations, in which expression of RGS4 is downregulated in hypothalamic domains in Sim1−/− mice [27]. Although the in vivo roles of RGS4 are not fully understood, it has been identified to have the capacity to modulate neurotransmitter receptors in the rat brain. Interestingly, RGS4 modulates 5-HT1A-mediated neurotransmitter release, in vitro and in vivo [35,36]. In the context of the present work, this observation is of particular interest, since 5-HT1A represent the autoreceptors in the DRN. Taken into consideration that 5-HT can also affect differentiation, RGS4 expression during early 5-HT neuron development could be critical for terminal differentiation of dorsal raphe 5-HT neurons. Indeed, as shown in Figs. 6G and 6H, a colocalization of 5-HT and RGS4 in vivo was apparent in mouse hindbrain.

In addition, after Sim1 overexpression, expression of a class IV POU domain transcription factors member, Brn3.2 (POU4F2), was found upregulated as well (Fig. 6C, 6D). Members of the class IV POU domain proteins are required for normal differentiation and survival of several neuronal populations [37]. The present study provides first evidence that Brn3.2 is downregulated in Sim1−/− mutants (Figs. 7L, 7M, 7N). However, Brn3.2 expression was restricted to areas of the hindbrain outside the serotonergic system, suggesting that differentiation of other neuronal subpopulations of the hindbrain, but not 5-HT neurons, might be controlled by Brn3.2. In contrast, Brn-2 (also known as POU3F2) has been identified to act downstream of Sim1 during anterior hypothalamus development [20], and has been shown to be expressed in rat adult DRN, where it colocalizes with TPH2. Importantly, Brn-2 activates the Tph2 promoter [38]. These observations, together with results of the present study, where Sim1 overexpression upregulated Tph2 expression (Fig. 5D), suggest a putative interaction of Sim1 with Tph2 via Brn-2 and provide an attractive scenario for putative mechanisms underlying the phenotype observed in Sim1−/− newborn mice.

Reduced expression of serotonergic markers such as TPH2 resembles observations obtained in the dopaminergic system of zebrafish, where in the absence of Arnt2/Sim1 function, the dopaminergic markers th, dat and ddc were reduced in posterior tuberculum and hypothalamus [29]. Taken into consideration that Sim1 has been described as a transcriptional repressor, further studies are needed to clarify the molecular interactions and pathways leading to activation of transcription factors involved in the terminal specification of serotonergic neurons. As an example, another possible scenario could imply that Sim1 may act indirectly by repressing inhibitors of serotonergic lineage.

In conclusion, the results of the present study strongly suggest Sim1 involvement in terminal differentiation of rostral 5-HT phenotype in at least two different processes. First, Sim1 acts on the development of the DRN through a developmental pathway leading to Tph2 activation via Brn-2, additionally or alternatively via Pet1. Second, Sim1 acts to modulate serotonin release of the B7 group of 5-HT neurons via regulating RGS4. As RGS4 is only transiently upregulated by Sim1, there likely exists a feedback loop to maintain a proper level of RGS4 and hence the homeostasis of serotonin release. Our study underscores that subpopulation of a common neurotransmitter phenotype use distinct combinations of transcription factors to control the expression of shared properties i.e. Pet1 and Tph2.

Materials and Methods

This study was carried out in strict accordance with the National Health and ethical regulations and care of animals was in accordance with institutional guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Freiburg ( Permit Number: X-07/27A).
Animals were sacrificed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

### RT-PCR

Total RNA was isolated from tissue of mouse ventral midbrain, ventral hindbrain, and isthmus, reverse transcribed and processed for PCR [39]. The forward and reverse primer sequences used are listed in Table 1.

For detection of cDNAs encoding the respective proteins, following protocol was used: denaturation at 95°C for 5 minutes, the optimum number of cycles -depending on primer pair- of PCR amplification were performed at following conditions: denaturation at 94°C for 1 minute, annealing at appropriate temperature for 1 minute, and elongation at 72°C for 1 minute. Final extension at 72°C for 10 minutes was terminated by rapid cooling at 4°C.

### Cell culture

The MN9D cell line, a hybridoma cell line established by fusing embryonic primary cells from mouse ventral midbrain with cells from the mouse neuroblastoma cell line N18TG2 [25] was used for in <i>vivo</i> experiments. Cells were cultured in DMEM/F-12 1:1, supplemented with 5% FCS, and 50 μg/ml gentamycin, passaged when confluent, and incubated in a 95% air /5% CO₂ atmosphere at 37°C. One day before transfection cells were plated at a density of 1 x 10⁶ cells in 25 cm² flasks. Cells were transfected with expression vectors pcDNA3 or pcDNA3::Sim1, 6 μg of plasmid DNA per flask. Transfection was performed with Lipofectamine (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol. After 4 h of incubation with DNA precipitate, complete medium containing 5% FCS was added. 24 h, 48 h, and 72 h post transfection cells were harvested and probes were processed for either RNA isolation and subsequent qRT-PCR or immunoblotting.

Alternatively, cells were seeded at 1 x 10⁶ cells in 25 cm² flask and transiently transfected with CY5-labeled siRNA targeting mouse <i>Sim1</i> mRNA (purchased from Qiagen). The siRNA was transiently transfected into MN9D cells with 750 ng siRNA in 30 μl HiPerFect reagent (Qiagen). A sequence that reveals no homology with any known mammalian genes has been labeled with Alexa 488 and was used as control siRNA. Cells were harvested and lysed 24 h after transfection, and gene expression was analyzed by qRT-PCR.

### FACS analysis of transfected MN9D cells with <i>Sim1</i> siRNA

MN9D cells successfully transfected either with CY5-labeled siRNA targeting mouse <i>Sim1</i> or with control siRNA labeled with Alexa 488 were analyzed by flow cytometry. Briefly, MN9D cells were sedimented (820 g, 10 minutes, 4°C), resuspended with HBSS containing 2% FCS and 10 mM HEPES. Cells were incubated at 37°C for 90 minutes, centrifuged, and resuspended with HBSS containing 2% FCS and 10 mM HEPES. Cells were then processed for RNA isolation and subsequent qRT-PCR.

### Real-Time PCR

Total RNA was isolated from MN9D cells transiently transfected with either pcDNA3 or pcDNA3::Sim1 expression vectors and subsequently 1.0 μg was reverse-transcribed. The primers used for qRT-PCR are listed in Table 1.

### Table 1. Primer sequences used for RT-PCR gene expression analysis.

| Primer | Accession | PCR-fragment |
|--------|-----------|--------------|
| Gapdh F: 5'-CGGCCGACCTCTCTTTGA-3' | NM008084 | nt 804-1210 |
| Gapdh R: 5'-TGACAGCAGCGGCAATA-3' | NM016701 | nt 1074-1512 |
| Nestin F: 5'-CAGGCTCTCTTGCTTCTTCG-3' | NM008090 | nt 37-147 |
| Nestin R: 5'-GGTGAGGTGGAATGTTTG-3' | NM008553 | nt 584-690 |
| Gata2 F: 5'-ACGTGAGTGGATGACTAC-3' | NM153111 | nt 484-593 |
| Gata2 R: 5'-AGCAAGCTGATGATGCTCC-3' | NM008084 | nt 804-1210 |
| Sim1 F: 5'-TGAGGTTTGGCAATCGC-3' | NM011376 | nt 37-152 |
| Sim1 R: 5'-GATGCGTGGATGATGCTCC-3' | NM010713 | nt 736-1052 |
| Tph2 F: 5'-AAGGCGTCAAGGTCACTA-3' | NM173391 | nt 432-531 |
| Tph2 R: 5'-GGGCGATCAGGTCACTA-3' | NM011376 | nt 37-152 |
| Bm3.2 F: 5'-CATCTGCTCCAGCATGACTG-3' | NM138944 | nt 607-703 |
| Bm3.2 R: 5'-CAGCGATGAGTCTCTTG-3' | NM010713 | nt 606-716 |
| Lhx8 F: 5'-ACAGCAGCTGACTGACATT-3' | NM009062 | nt 139-291 |
| Lhx8 R: 5'-CCAGCTGACAGGTGACATG-3' | NM011376 | nt 37-152 |
| RGS4 F: 5'-GAGTGCACAGGACATGAAAC-3' | NM008553 | nt 584-690 |
| RGS4 R: 5'-GTCTTTACAGGTCACTA-3' | NM011376 | nt 37-152 |

(nt: nucleotide).
doi:10.1371/journal.pone.0019239.t001
Real-time PCR was performed as described elsewhere [40]. Cycle conditions: denaturation at 95°C for 10 minutes, and 40 cycles of PCR amplification at 95°C for 30 seconds and 56°C for 30 seconds and elongation at 72°C for 1 minute. All PCRs were performed in triplicate on an ABI PRISM 7500 Sequence Detection System.

The mean ± SD of the Ct values for the genes of interest and GAPDH were determined and statistically analyzed using the Student’s t-test. *p<0.05 was considered as statistically significant. For documentation of the data, relative mRNA levels were calculated using the comparative Ct method (2^(-ΔΔCt)), as previously reported [39].

Immunoblotting

MN9D cells were harvested and homogenized, and protein concentration was determined according to Bradford [41]. For isolating proteins from hindbrain of 4%-PFA fixed brain cryosections from wildtype and Sim1-/- mutants, the method described by Scicchitano et al., [42] has been applied, using the Qproteome FFPE Tissue Kit from Qiagen, following the manufacturer’s instructions. After determination of protein concentration, electrophoresis and blotting procedures were performed as described [43]. Blots were incubated with primary antibody overnight at dilution 1:1,000 for Sim1 (rabbit polyclonal; Chemicon; raised against amino-acids 423-435 of human Sim1), 1:1,000 for TH (mouse monoclonal, Chemicon, Hofheim, Germany), 1:500 for Pitx3 (rabbit polyclonal; Chemicon), 1:1,000 for TPH2 (mouse monoclonal, Sigma, Tautkirchen, Germany), 1:1,000 for Pet1 (rabbit polyclonal, Abcam, Cambridge, UK), 1:500 for RGS4 and Brn3.2 (goat polyclonal, from Santa Cruz), and 1:10,000 for GAPDH (mouse monoclonal, Abcam). After incubation with secondary antibodies, blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray film. Subsequently, films were scanned and the signal ratio TH:GAPDH, Pitx3:GAPDH, TPH2:GAPDH, Pet1:GAPDH, RGS4:GAPDH and Brn3.2:GAPDH was quantified densitometrically. Differences in signal ratio were tested for significance using Student’s t-test. Results with levels of *p<0.05 were considered significant.

Animals

The generation of Sim1-/- mice has been described previously [20]. For morphological comparison wild type littermates were used as wild type controls. The morning of the day on which a vaginal plug was detected in mating was designated gestation day 0.5. Analysis was performed on 4% paraformaldehyde-fixed 14.5-day-old embryos (E14.5) and in newborn mice (P0).

Immunohistochemistry experiments embryos or heads of newborn mice were dehydrated, embedded in paraffin, and cut into 10 µm serial sections. For in situ hybridization, they were cryoprotected and cut into 10 µm cryosections.

Immunohistochemistry

Immunohistochemistry has been performed as described earlier [43]. Monoclonal mouse anti-TH (1:100; Chemicon), rabbit polyclonal anti-5HT antibody (1:1,000; Sigma) or rabbit polyclonal anti-Sim1 (1:400) were used as primary antibodies and goat anti-mouse or goat anti-rabbit IgG coupled to either horseradish peroxidase, or FITC as secondary antibodies.

Numbers of TH- and 5-HT-labeled neurons were counted on the complete series of 10 µm transverse sections after immunoperoxidase and immunofluorescence staining. A neuron was designated as TH or 5-HT positive if it revealed a darkly labeled cytoplasm and a clearly visible, unstained nucleus. Only cells fulfilling these criteria were included in the cell counts. To avoid double counting the same cell on two sequential sections, only every fifth section was counted.

In situ hybridization

Non-radioactive in situ hybridization on cryosections and preparation of digoxigenin-labelled probes for RGS4 [35], Pet1 [11] and Brn3.2 [44] were carried out as described by Ernsberger et al. [45].

Statistics

Data are presented as the mean ± SD. Statistical analysis was performed using the Student’s unpaired t-test. Differences were considered statistically significant at *p<0.05, **p<0.01 and ***p<0.001.

Acknowledgments

We thank Dr. Juha Partanen, Institute of Biotechnology, University of Helsinki, for providing us with the Pet1 probe and Dr. William H Klein, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX77030, for providing us with the Brn3.2 probe. The authors thank Melanie Feuerstein for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: ER. Performed the experiments: NO MW OO SH. Analyzed the data: NO ER. Contributed reagents/materials/analysis tools: CX CF. Wrote the paper: ER KK.

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