Neurog2 Deficiency Uncovers a Critical Period of Cell Fate Plasticity and Vulnerability among Neural-Crest-Derived Somatosensory Progenitors

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Neurog2 Deficiency Uncovers a Critical Period of Cell Fate Plasticity and Vulnerability among Neural-Crest-Derived Somatosensory Progenitors

Highlights

- *Neurog2*^−/−^ mutant trunk DRGs contain reduced numbers of all sensory neuron subtypes
- *Neurog2* influences the onset and accuracy of the successive sensory neurogenic waves
- Pools of *Neurog2*-deficient sensory progenitors either die or become melanoblasts
- *Neurog2* deficiency illustrates cell fate plasticity and vulnerability among NCCs

Authors

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In Brief

Ventéo et al. report that in contrast to cervical levels, trunk dorsal root ganglia of *Neurog2*^−/−^ mutants contain reduced numbers of all somatosensory neuron subtypes due to multiple defects in neural-crest-derived progenitors—including sensory-to-melanocyte fate switch, apoptosis, and delayed differentiation—that affect the accuracy of the successive waves of neurogenesis.
Neurog2 Deficiency Uncovers a Critical Period of Cell Fate Plasticity and Vulnerability among Neural-Crest-Derived Somatosensory Progenitors

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SUMMARY

Functionally distinct classes of dorsal root ganglia (DRG) somatosensory neurons arise from neural crest cells (NCCs) in two successive phases of differentiation assumed to be respectively and independently controlled by the proneural genes Neurog2 and Neurog1. However, the precise role of Neurog2 during this process remains unclear, notably because no neuronal loss has been reported hitherto in Neurog2−/− mutants. Here, we show that at trunk levels, Neurog2 deficiency impairs the production of subsets of all DRG neuron subtypes. We establish that this phenotype is highly dynamic and reflects multiple defects in NCC-derived progenitors, including somatosensory-to-melanocyte fate switch, apoptosis, and delayed differentiation which alters neuronal identity, all occurring during a narrow time window when Neurog2 temporarily controls onset of Neurog1 expression and neurogenesis. Collectively, these findings uncover a critical period of cell fate plasticity and vulnerability among somatosensory progenitors and establish that Neurog2 function in the developing DRG is broader than initially envisaged.

INTRODUCTION

Our ability to detect and process mechanical, proprioceptive, thermal, or noxious information relies on the somatosensory nervous system. At trunk levels, the primary receptors of this system, located in the dorsal root ganglia (DRG), form a heterogeneous population of functionally specialized neurons whose diversity is largely established early during embryogenesis (Lallemand and Ernfors, 2012). DRG neurons arise from neural crest cells (NCCs) that also contribute at this axial level to sympathetic neurons, glial cells, smooth muscles, or melanocytes. Lineage segregation among NCCs involves time- and environment-dependent changes in their developmental competence, notably through the induction of specific transcriptional regulators (Pavan and Raible, 2012; Dupin and Sommer, 2012; Bhatt et al., 2013). The bHLH transcription factors Neurog2 and Neurog1 are early markers of the somatosensory lineage (Sommer et al., 1996) in which they orchestrate neuronal differentiation presumably by respectively and independently controlling two successive waves of neurogenesis. It is indeed assumed that a first group of progenitors differentiates between embryonic day 9.5 (E9.5) and E11.5 under the control of Neurog2 to generate mecano/proprioceptors as well as few large-diameter nociceptors, whereas a second group arising slightly later from NCCs, either directly or indirectly by the boundary cap cells, differentiates between E10.5 and E13.5 under the control of Neurog1 to mainly produce small-diameter nociceptors (Figure S1A; Lawson and Biscoe, 1979; Frank and Sanes, 1991; Ma et al., 1999; Maro et al., 2004; George et al., 2010; Bachy et al., 2011; Ohayon et al., 2015). This model is supported by the phenotypes of Neurog1−/−;Neurog2−/− double knockouts (dKOs) in which no DRG neuron is ever produced and of Neurog1−/− mutants in which late-born small nociceptors are specifically absent. In contrast, direct evidence for a specific role of Neurog2 during the first neurogenic wave remains elusive, notably because no neuronal loss has been reported in Neurog2−/− mutants at cervical levels (Ma et al., 1999). Here, we show that at trunk levels, Neurog2 deficiency affects the genesis of subsets of all DRG neuron subtypes as the result of multiple defects in pools of first- and second-wave somatosensory progenitors.

RESULTS AND DISCUSSION

First-Wave Somatosensory Progenitors Mainly Form Nociceptors in the Absence of Neurog2 Function

We previously characterized DBZEB;Wnt1Cre;Egr2Δ2f/+ (DWE) transgenic mice in which concomitant overexpression of a dominant-negative form of ZEB1/2 proteins in NCCs and genetic ablation of boundary cap cells triggered the early complete elimination of second-wave somatosensory progenitors at thoracic levels, while largely sparing those of the first wave (Figures S1A and S1B; Ohayon et al., 2015). In these animals, DRG neurogenesis, assessed by transient NeuroD expression, started on time but prematurely ended at E11.5 (Figures 1A, 1B, and 1D), consistent with the entire depletion of Neurog1-positive (+) second-wave progenitors from this stage (Figures 1A, 1B, and 1E). In
Absence of DWE in the absence of good opportunity to assess the behavior of first-wave progenitors S1A, S1B, S1D, and S1E). This model, thus, represented a DWE to numbers increased until E13.5 to reach a plateau comparable to E11.5, and their numbers appeared in a delayed manner from E11.5, and their neurogenesis eventually started in these animals and continued until E13.5, which correlated with a second, Neurog2-independent, phase of Neurog1 expression (Figures 1C–1E). In turn, SCG10+ neurons appeared in a delayed manner from E11.5, and their numbers increased until E13.5 to reach a plateau comparable to DWE embryos, albeit shifted in time, representing 18.4% (±2.85%) of the normal DRG contingent at E14.5 (Figures 1C and 1F). Furthermore, an analysis of the neuronal subtypes generated in 2–DWE embryos revealed a striking increase of TrkA+ nociceptors and a concomitant decrease of TrkB+ and TrkC+ mechano/proprioceptors compared to DWE animals (Figures 1G, 1I, and 1O). This was evocative of an identity switch also observable at the level of the dorsal root entry zone (Figures 1M–1O). In line with this, although expression of the late-born nociceptors determinant Runx1 (Chen et al., 2006) was virtually absent in DWE embryos, it was recovered in 2–DWE animals (Figures 1H, 1J, and 1L). Therefore, without excluding that few first-wave progenitors might be depleted in the absence of Neurog2 function (see Ma et al., 1999 and Figure 4L), these data show that a large proportion is temporarily maintained and subsequently differentiates in a delayed manner through the compensatory action of Neurog1 to produce mainly nociceptors instead of mechano/proprioceptors (Figure S1C). This "intra-lineage" switch combined with the loss of small nociceptors in Neurog1−/− mutants (Ma et al., 1999) suggests that beyond its proneural role, Neurog1 might also participate in specifying the nociceptive identity. Alternatively, although not exclusively, this switch may be the consequence...
showed that both classes were reduced by 29% (± respectively TrkA+/Ret- and TrkA+/Ret+ at this stage (Chen et al., 2006; Liu and Ma, 2011; Lallemend and Ernfors, 2012), segregating into ‘peptidergic’ and ‘non-peptidergic’ classes, affected. Analyses at E18.5, when the latter population is being affected, suggesting that late-born small-diameter nociceptors were affected. Analyses at E18.5, when the latter population is being segregated into ‘peptidergic’ and ‘non-peptidergic’ classes, respectively TrkA+/Ret- and TrkA+/Ret+ at this stage (Chen et al., 2006; Liu and Ma, 2011; Lallemend and Ernfors, 2012), showed that both classes were reduced by 29% (±15.3%) and 32.5% (±13.3%), respectively, in the mutants (Figures 2C and 2D). In line with this, numbers of nociceptor subtypes expressing MrgprD, CGRP, or TrpM8 (Liu and Ma, 2011) were all decreased by 26.4% (±5.6%; n = 8), 12% (±6.4%), and 26.8% (±6.4%), respectively (Figures 2C and 2D). These data, thus, show that at thoracic levels, Neurog2 deficiency affects subsets of all somatosensory neuron subtypes, including populations of late-born nociceptors. It is of note that although similar results were found at abdominal levels (not shown), they contrast with previous data at cervical levels (Ma et al., 1999; see below).

**Thoracic DRG of Neurog2−/− Mutants Contain Reduced Numbers of All Neuronal Subtypes**

These observations raised the possibility that thoracic DRG of Neurog2−/− mutants may contain fewer mechano/propr ioceptors but supernumerary nociceptors. Analyses of Neurog2−/− embryos at E13.5 revealed that numbers of TrkB+ and TrkC+ mechano/propr ioceptors were indeed reduced by 25% (±9.3%) and 17% (±11.3%), respectively (Figures 2A and 2B). Unexpectedly however, we also found a drastic 29.6% (±3.8%) reduction of TrkA+ neurons (Figures 2A and 2B). This could not simply reflect a deficit of early-born large-diameter nociceptors, which represent only 10% of the entire TrkA+ population (Chen et al., 2006; Lallemend and Ernfors, 2012), suggesting that late-born small-diameter nociceptors were affected. Analyses at E18.5, when the latter population is being segregated into “peptidergic” and “non-peptidergic” classes, respectively TrkA+/Ret- and TrkA+/Ret+ at this stage (Chen et al., 2006; Liu and Ma, 2011; Lallemend and Ernfors, 2012), showed that both classes were reduced by 29% (±15.3%) and 32.5% (±13.3%), respectively, in the mutants (Figures 2C and 2D). In line with this, numbers of nociceptor subtypes expressing MrgprD, CGRP, or TrpM8 (Liu and Ma, 2011) were all decreased by 26.4% (±5.6%; n = 8), 12% (±6.4%), and 26.8% (±6.4%), respectively (Figures 2C and 2D). These data, thus, show that at thoracic levels, Neurog2 deficiency affects subsets of all somatosensory neuron subtypes, including populations of late-born nociceptors. It is of note that although similar results were found at abdominal levels (not shown), they contrast with previous data at cervical levels (Ma et al., 1999; see below).

**Neurog2 Deficiency Generally Impairs Neurogenesis in Trunk DRG**

Reduced numbers of mechano/propr ioceptors in the trunk DRG of Neurog2−/− mutants could logically be explained by the developmental switch undergone by first-wave progenitors. In contrast, deficiency of late-born nociceptors was a priori counterintuitive, except if the second neurogenic wave was also altered. To test this, we performed a time-course analysis of DRG neurons formation. At E10.5, when the first neurogenic wave is ongoing and the second has started (Lawson and Biscoe, 1979; Ma et al., 1999), Neurog2−/− mutants could be considered as Neurog1−/−; Neurog2−/− dKOs because Neurog1 expression was undetectable and neuronal differentiation was impaired, despite the presence of numerous Sox10+ NCCs in the coalescing DRG (Figures 3A–3D and 3F). This shows that Neurog2 generally controls an initial phase of Neurog1 expression and onset of neurogenesis also in second-wave progenitors. It also indicates that invasion of the DRG primordia by NCCs is largely Neurog1/2 independent (see also Figure 4N). By E11.5, however, Neurog1 was eventually induced in the mutants and neurogenesis was initiated and continued until E13.5, as in WT (Figures 3A–3E). Nevertheless, at

**Figure 2. Thoracic DRG of Neurog2−/− Mutants Contain Reduced Numbers of All Neuronal Subtypes**

(A) Representative images of immunofluorescence staining for TrkA and TrkB or for TrkC at E13.5 on thoracic DRG transverse sections of WT and Neurog2−/− mutants. (B) Quantification of Trk+ neurons in WT (blue bars; n = 7) and Neurog2−/− (green bars; n = 7) embryos at E13.5. Data are represented as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. (C) Representative images of immunofluorescence staining for TrkA and Ret or for CGRP and MrgprD and of in situ hybridizations for MrgprD and TrpM8 on thoracic DRG transverse sections of WT and Neurog2−/− mutants at E18.5. Scale bar, 50 μm. (D) Relative quantification of the peptidergic TrkA+/Ret- and non-peptidergic TrkA+/Ret+ populations and of MrgprD+, CGRP+, and TrpM8+ nociceptor subtypes in WT (blue bars; n = 6) and Neurog2−/− (green bars; n = 8) embryos at E18.5. Data are represented as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
all stages, numbers of \textit{NeuroD}+ precursors and of \textit{SCG10}+ neurons were reduced compared to WT (Figures 3C and 3D), leading to a 34.48% (±6.17%) decrease of the whole DRG contingent at E14.5 (Figure 3D). This was notably evident at E12.5 when the production of late-born nociceptors normally peaks (Figures 3C; Lawson and Biscoe, 1979; Ma et al., 1999). Therefore, and also taking into account that most mutant first-wave progenitors atypically differentiate concomitantly to second-wave progenitors (Figure 1), these data indicate that \textit{Neurog2} deficiency perturbs the normal course of the second \textit{Neurog1}-dependent neurogenic wave, an issue further supported by extrapolated dissociation of the two neurogenic waves in WT versus \textit{Neurog2}−/− embryos (Figures S2A and S2B).

In addition, we found that these neurogenic defects were correlated to a deficit of \textit{Neurog1}+ progenitors at all stages (Figures 3A, 3B, and 3E), particularly affecting those of the second-wave (Figure S2C). However, because \textit{Neurog2} can influence \textit{Neurog1} expression (see above), we also assessed \textit{Sox10} expression. At E10.5, we found a slight increase, albeit not significant, of the \textit{Sox10}+ population in the DRG primordia of \textit{Neurog2}−/− embryos (Figures 3A, 3B, 3F, and 4N), further supporting that most mutant progenitors were temporarily maintained undifferentiated at this stage. In contrast, by E11.5, their numbers were significantly reduced by 29.2% (±7%) compared to WT (Figures 3A, 3B, 3F, and 4N), which expectedly mainly concerned second-wave progenitors (Figure S2D). Altogether, these data show that \textit{Neurog2} deficiency generally impairs DRG neurogenesis, not only from the first wave whose timing and accuracy are altered but also from the second wave which is affected in two ways: (1) its onset is also delayed due to postponed induction of \textit{Neurog1}, and (2) it is eventually initiated from a smaller progenitor reservoir that appears specifically depleted between E10.5 and E11.5.

In parallel, lineage analyses based on the great stability of the GFP protein expressed from the \textit{Neurog2} locus (Figure S2E; Andersson et al., 2006) supported that \textit{Neurog2} was not only expressed in first-wave progenitors but also transiently in a significant proportion of second-wave progenitors (Figure S2F),
Neurog2 appears able to trigger neuronal differentiation in first-wave progenitors by itself, whereas in second-wave progenitors it is required to ensure integrity and on-time induction of Neurog1 that, in turn, has the proneural role (Ma et al., 1999; Figure S4A).

We next aimed at determining the causes of the progenitor depletion observed in Neurog2−/− mutants. Prompted by previous observations made at lumbar levels at E11.5 (Ma et al., 1999), we assessed cell death. At thoracic levels, although we could not detect any dying cells in mutant embryos at E10.5 and E11.5, we did so at an intermediate stage referred to as E10.75, indicating that apoptosis indeed contributes to this defect (Figure 4A). In parallel, we also noticed that the spatial distribution of the mutant Sox10+ NCCs in the region of the coalescing DRG was atypical. Indeed, in E11.5 Neurog2−/− embryos, we systematically detected supernumerary Sox10+ cells at positions reminiscent of the “dorsolateral pathway” normally invaded by melanoblasts (Figures 4B and 4C; Hari et al., 2002, 2003).

Cell Fate Switch toward the Melanocyte Lineage and/or Apoptosis Underlie the Depletion of Somatosensory Progenitor Pools in Neurog1/2 Mutants

In situ hybridizations for Sox10 on thoracic DRG transverse sections from WT (H and J) and Neurog2−/− (I and K) embryos on thoracic DRG transverse sections (H and I) or on whole-mount preparations (J and K) at E11.5. (L) Quantification of Mitf+ melanoblasts in WT (blue column; n = 10), DWE (brown column; n = 5), Neurog2−/− (green column; n = 10), Neurog1−/−;Neurog2−/− (dKO, purple column; n = 5), or Neurog1−/− (orange column; n = 4) embryos at E11.5. Data are represented as means ± SEM, n.s., not significant; **p < 0.01; ***p < 0.001. (M) In situ hybridization for Sox10 on thoracic DRG transverse sections from Neurog1−/−;Neurog2−/− at E10.5, E11.5, and E12.5. (N) Quantitative time-course analyses of Sox10+ progenitors in the DRG of WT (blue curve), Neurog2−/− (green curve), and Neurog1−/−;Neurog2−/− (dKO, purple curve) between E10.5 and E12.5. Data are represented as means ± SEM. **p < 0.01; ***p < 0.001. (O–Q) In situ hybridizations for Sox10 (O) or Mitf (Q) and immunofluorescence staining for GFP (P) on thoracic DRG transverse sections of Neurog1−/−;Neurog2−/− embryos at E11.5.

Figure 4. Cell Fate Switch toward the Melanocyte Lineage and/or Apoptosis Underlie the Depletion of Somatosensory Progenitor Pools in Neurog1/2 Mutants

(A) Immunofluorescence staining for activated-Caspase3 (aCasp3) on thoracic DRG transverse sections from WT and Neurog2−/− embryos at E10.5, E10.75, and E11.5. (B and C) Representative images of in situ hybridizations for Sox10 on thoracic DRG transverse sections from WT (B) and Neurog2−/− (C) embryos at E11.5. Red frames indicate positions of the close-ups shown on the right.

(R and S) Immunofluorescence staining for activated-Caspase3 (aCasp3) on thoracic DRG transverse sections from Neurog1−/−;Neurog2−/− (R) and Neurog1−/− (S) embryos at E10.5 and E11.5. Red arrows in (B–I) and (O–Q) point to the dorsolateral migratory path. Red dotted lines in (A), (H), (I), (R) and (S) delimit the DRG.

Scale bars, 5 μm.

See also Figures S3 and S4.
At this stage, many ectopic GFP+ cells were also observed in this region, occasionally already at E10.5 (Figures 4D–4G), prompting us to assess the melanocyte determinant Mitf (Opdecamp et al., 1997). At E11.5, although only few Mitf+ melanoblasts were detected in the trunk region of WT embryos, either on sections (Figure 4H) or on whole-mount preparations (Figure 4J), their numbers were massively increased in Neurog2−/− mutants (Figures 4I, 4K, and 4L). These data, thus, show that in the absence of Neurog2, a population of NCCs normally dedicated to the DRG adopts a melanocyte fate. Moreover, analyses on E11.5 2−/−DWE animals revealed only a minor increase of the Mitf+ population (Figure 4L; Figures S3A and S3B), supporting that this cell fate conversion mainly concerned second-wave progenitors. It is of note that we did not observe any obvious ectopic GFP+ or supernumerary Sox10+ cells in other regions of Neurog2−/− embryos, including in the sympathetic ganglia (Figures S3D and S3E; data not shown). Taken together, these data establish that the somatosensory progenitor depletion observed in the trunk of Neurog2−/− embryos involves at least two processes, namely, apoptosis and cell fate conversion toward the melanocytic lineage (Figures S4A and S4B), both occurring during the narrow time window when Neurog1 is undetectable and neurogenesis is blocked. It is of note that during the same period at cervical levels, Neurog1 expression and neurogenesis have already started in the mutants (Ma et al., 1999). Therefore, temporal divergences in the “Neurog2-independent” upregulation of Neurog1 expression may explain why no neuronal loss has been observed at this axial level. Nevertheless, because DRG development normally follows a temporal antero/posterior (A/P) gradient, this may rather reflect the existence of distinct spatial patterning modalities along this axis. Interestingly, such phenotypic divergences along the A/P axis have been also reported in the sympathetic chain of mutants (Figures 4I, 4K, and 4L). These data, thus, show that cell death was greatly exacerbated in dKOs at E11.5 compared to WT, as in Neurog2−/− embryos (Figures 4M and 4N). By E12.5, in contrast to Neurog2−/− mutants, all Sox10+ NCCs were depleted in dKO (Figures 4M and 4N). As anticipated, this reflected a partial cell fate conversion toward the melanocytic lineage (Figures 4L and 4O–Q; see also Figure S3E). However, numbers of Mitf+ cells in dKOs were not statistically different from Neurog2−/− mutants (Figure 4L). Instead, we found that cell death was greatly exacerbated in dKOs at E11.5 (Figure 4R). Consistent with this, in Neurog1−/− single mutants we did not detect any supernumerary melanoblasts (Figure 4L; Figure S3C) but found that second-wave progenitors were progressively eliminated from E11.5 through apoptosis (Figure 4S; summarized in Figure S4C). Taken together, these data show that the additional loss of Neurog1 function in a Neurog2−/− mutant background triggers the rapid complete elimination of all NCC-derived somatosensory progenitors by an extended period of apoptosis, not an exacerbated fate switch phenotype (Figures S4D). This certainly illustrates heterogeneity among NCCs and suggests that some are early committed to the somatosensory lineage (Lo et al., 2005) and/or have lost the competence to interpret signals instructive for alternative fates (Figures 4D–4G). Prompting us to assess their destiny in the melanocytic lineage (Figures 4L and 4O–Q; see also Figure S3E). However, numbers of Mitf+ cells in dKOs were not statistically different from Neurog2−/− mutants (Figure 4L). Instead, we found that cell death was greatly exacerbated in dKOs at E11.5 (Figure 4R). Consistent with this, in Neurog1−/− single mutants we did not detect any supernumerary melanoblasts (Figure 4L; Figure S3C) but found that second-wave progenitors were progressively eliminated from E11.5 through apoptosis (Figure 4S; summarized in Figure S4C). Taken together, these data show that the additional loss of Neurog1 function in a Neurog2−/− mutant background triggers the rapid complete elimination of all NCC-derived somatosensory progenitors by an extended period of apoptosis, not an exacerbated fate switch phenotype (Figures S4D). This certainly illustrates heterogeneity among NCCs and suggests that some are early committed to the somatosensory lineage (Lo et al., 2005) and/or have lost the competence to interpret signals instructive for alternative fates and eventually die in case of abortive neurogenesis.

In conclusion, these findings illustrate heterogeneity among NCC-derived somatosensory progenitors and uncover a critical period of cell fate plasticity and vulnerability for this population. They also establish that the individual requirement of Neurog2 in the forming DRG is more complex and broader than initially envisaged.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.002.
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AUTHOR CONTRIBUTIONS

S.V., S.D., P.Cabochette, A.D., and A.P. performed the experiments. S.V., S.D., P.Cabochette, A.P., and P.Carroll designed the experiments and interpreted the data. A.P. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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**LEAD CONTACT AND MATERIALS AVAILIBILITY**

Further information and request should be directed to and will be fulfilled by the Lead Contact, Alexandre Pattyn (alexandre.pattyn@inserm.fr). This study did not generate new unique materials or reagents.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Experiments described in this study were performed on mouse embryos from embryonic day (E) 9.5 to 18.5. Embryos were allocated to each experiment depending on their genotype which resulted in the comparison of genetically modified models with control littermates. 2-8 months old females and males of the DBZEB (Ohayon et al., 2015), Egr2<sup>fl/fl-DT</sup> (Maro et al., 2004), Neurog1<sup>−/−</sup> (Ma et al., 1999), Neurog2<sup>−/−</sup> (Andersson et al., 2006), and Wnt1Cre (Danielian et al., 1998) strains were used for the breeding and were maintained and genotyped as previously described. They were provided <i>ad libitum</i> with standard mouse pellet food and water and housed at room temperature with a 12h light/dark cycle. All procedures were conducted according to the French Ministry of Agriculture and the European Community Council Directive no. 86/609/EEC, OJL 358. Protocols were validated by the Direction Départementale des Services Vétérinaires de l’Hérault (Certificate of Animal Experimentation no. 34-376).

**METHOD DETAILS**

**In Situ Hybridization (ISH)**

Antisense RNA probes for DBH, gfp, Mitf, MrgrprD, NeuroD, Neurog1, Neurog2, Runx1, SCG10, Sox10 and TrpM8 were labeled using the Dig RNA labeling kit (Roche) and purified on G50 columns (GE Healthcare). For staining on cryosections, staged embryos were dissected out in cold Phosphate Buffer Saline (PBS), fixed for 2 hours at 4°C in 4% Paraformaldehyde(PFA)-PBS, cryoprotected in 20% Sucrose-PBS overnight (o.n.) at 4°C, embedded in OCT-compound (Tissue-Tek) and stored at −80°C until use. 12-14μm
transverse sections were done using a cryostat (Microm), placed on glass slides and stored at −20°C until use. For the staining procedure, slides were dried at room temperature (RT) for 20 minutes (min) and incubated o.n. at 70°C with the appropriate probes diluted 1/100-1/200 in the hybridization buffer (50% Formamide-10% Dextran Sulfate-1X Salt Solution-1mg/ml yeast tRNA-1X Denhardt’s). They were washed twice 1 h at 70°C in 50% Formamide-1X Sodium/Sodium Citrate buffer-0.1% Tween 20. They were then washed 3 times 10 min at RT in MABT (1X Maleic acid buffer-0.1% Tween20), blocked in 20% Sheep Serum-2% Blocking Reagent (Roche)-MABT and incubated o.n. at 4°C with the anti-DIG antibody (Roche) diluted 1/2000 in the same buffer. Slides were washed 3 times for 10 min at RT in MABT, incubated twice for 15 min in buffer B3 (100mM Tris pH 9.5, 100mM NaCl, 50mM MgCl2, 0.1% Tween 20) and incubated with the NBT and BCIP substrates (Roche) diluted in B3. Slides were washed several times in water for 2 hours, dried at RT and mounted in Mowiol under coverslips.

For whole-mount in situ hybridization, staged embryos were dissected out in PBS, fixed o.n. at 4°C in 4% PFA-PBS, washed in PBS several times, progressively dehydrated in 100% EtOH and stored at −20°C until use. Embryos were progressively rehydrated in PBS-0.1% Tween20 (PBT), bleached in 2% H2O2-PBT for 1 h, washed 3 times in PBT, treated with 10μg/ml Proteinase K-PBT for 10 min, rinsed in PBT and post-fixed in 4% PFA-PBS for 20 min at 4°C. They were then washed in PBT, pre-hybridized in the hybridization buffer (50% Formamide-1.3xSSC-5mM EDTA-50μg/ml yeast tRNA-0.5% CHAPS-2% Tween 20) for 1 h at 70°C, and hybridized o.n. at 70°C with the Dig-labeled Mitf antisense-RNA probe diluted 1/100 in the hybridization buffer. Embryos were then washed twice 30 min in hybridization buffer, rinsed in TST (10mM Tris pH 7.5, 0.5M NaCl, 0.1% Tween 20) and incubated twice 30 min at 37°C with 10μg/ml RNase A-TST. They were then washed in TST at RT and then twice in hybridization buffer for 30 min at 65°C. Embryos were then washed twice 10 min and the 1 h in MABT at RT, incubated in 20% Serum-MABT at RT for 1 h and incubated o.n. at 4°C with the anti-DIG antibody (Roche) diluted 1/2000 in 2% Serum-MABT. They were then washed in MABT for 3 days. They were rinsed several times in B3 and stained with the NBT/BCIP-B3 substrate solution (Roche). The staining was stopped by washing several times in PBT. Embryos were post-fixed in 4% PFA-PBT, washed in PBT, progressively transferred in 80% Glycerol-PBT and stored in the dark at 4°C.

**Immunofluorescence Staining on Cryosections**

Primary antibodies used in this study were as follows: Chicken anti-GFP (1/2000; Abcam); Goat anti-Ret (1/100; R and D Systems), TrkB (1/2000; R and D Systems) and TrkC (1/1000; R and D Systems); Rabbit anti-activated Caspase3 (1/2000; Cell Signaling), CGRP (1/500; Sigma-Aldrich) and TrkA (1/500; Millipore); Sheep anti-TH (1/1000; Thermo Scientific). Secondary antibodies (Molecular Probes) used were as follows; Donkey anti-rabbit IgG Alexa Fluor 594-conjugated (1/2000); Donkey anti-goat IgG Alexa Fluor 488-conjugated (1/2000); Donkey anti-sheep IgG Alexa Fluor 568-conjugated (1/2000) and Goat anti-chicken IgG Alexa Fluor 488-conjugated (1/1000).

For the staining procedure, slides were dried 20 min at RT, rinsed with PBS, blocked for 30 min at RT in 4% Serum-PBS-0.1% Triton X-100 and incubated o.n. at 4°C with the appropriate primary antibody(ies) diluted in the same buffer. Slides were then washed 3 times 10 min in PBS-0.1% Triton X-100 and incubated 1 h at RT with secondary antibody(ies) diluted in the same buffer. They were then washed 3 times 10 min at RT, mounted under coverslips in Mowiol and rapidly imaged. For Ret immunofluorescence staining, an antigen retrieval step was added at the beginning of the protocol. Slides were incubated 15 min at 68°C in 10mM Sodium Citrate Buffer-0.5% Tween-20 pH6 and were then cooled down at RT for 15 min. They were washed 3 times in PBS-0.1% Triton X-100 for 5 min and incubated in the blocking buffer.

Note that pictures showing ISH or immunofluorescence staining are representative of at least three independent animals.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Quantitative analyses were carried out on at least 3 independent animals of each genotype, except at E14.5 for which each side of 2 distinct embryos were counted. Thoracic DRG sections were processed either for ISH or immunofluorescence and imaged using a Zeiss microscope. Cell counts were performed on 6-8 sections for each animal depending on the stage using the ImageJ software. Only cells with a clearly identifiable nucleus were counted. Statistical analyses were performed using the Microsoft Excel software and expressed as mean ± standard error to the mean (s.e.m). They were analyzed by two-sided Student t tests: p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered as statistically significant. Note that no statistical methods were used to predetermine sample sizes, but numbers of animals and sections analyzed for each genotype and for each stage are consistent with those typically used in the field.

In Figures 3F, 3G, 3H, and 3J are presented extrapolated numbers of NeuroD+, SCG10+, Neurog1+ and Sox10+ cells from the first or the second neurogenic waves in WT or Neurog2+/− mutants. “First-wave numbers” were respectively determined by analyzing and quantifying DWE and 2−/−DWE animals, in which only first wave progenitors are present (see also Figure 1) (Ohayon et al., 2015). “Second-wave numbers” were respectively deduced by subtracting the average numbers of cells counted in WT versus DWE animals on one hand, and in Neurog2−/− versus 2−/−DWE embryos on the other hand.

**DATA AND CODE AVAILABILITY**

This study did not generate any dataset or code.