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<u>**REPORT**</u>

Regulation of neuronal specification in the ventral neural tube.

<u>**Background of the project**</u>

The construction of the nervous system consists of an integrated series of steps that begins with neural induction and the formation of the neural tube composed by multipotential progenitor cells. These progenitors generate functionally diverse neuronal and glial cell types at their correct positions and appropriate number. During development, the acquisition of specific neuronal identities is initiated by external signals, which trigger transcriptional networks in progenitors and newborn neurons to specify distinct neuronal fates (Briscoe & Novitch, 2008; Jessell, 2000). In the mammalian spinal cord, 14 cardinal populations of neurons have been identified based on their spatio-temporal transcriptional profiles, axonal projection and neurotransmitter phenotype (Briscoe & Novitch, 2008; Goulding, 2009; Jessell, 2000). The same general pattern was observed in the hindbrain (Gray, 2008), with the exception of neurons derived from the most ventral progenitor domain, termed p3. In the hindbrain, p3 progenitors give rise to serotonergic neurons, while in the spinal cord, topographical related progenitors produce glutamatergic V3 interneurons (Briscoe et al, 1999; Jacob et al, 2013; Zhang et al, 2008). These two classes of neurons are distinct in their physiological properties, connectivity and functions. Excitatory V3 interneurons are components of intraspinal networks that generate organized motor patterns (Borowska et al, 2013; Zhang et al, 2008). By contrast, serotonergic neurons innervate large regions of the brain and the spinal cord, where they modulate a variety of behaviors, and disturbances in the serotonin system have been linked to several neurodevelopmental disorders (Deneris & Wyler, 2012; Müller & Jacobs, 2010). We have asked how these two topographically related progenitor pools that share a common transcriptional code produce different classes of neurons. We have recently discovered that the proneural transcription factor Neurogenin3 (Neurog3) plays a pivotal role in controlling V3 *vs.* serotonergic fate (Carcagno et al, 2014). Gain-of-function experiments showed that Neurog3 in the ventral hindbrain respecifies serotonergic neurons into ectopic V3 Sim1^{+} interneurons. The mechanism underlying this fate conversion is the Neurog3-dependent repression of Ascl1, a serotonergic determinant, through a cascade involving Hes5 (Carcagno et al, 2014).

Conversely, we found that the spinal cord of Neurog3$^{-/-}$ embryos display augmented expression of Ascl1 in the p3 domain, which triggers ectopic serotonergic differentiation in the ventral spinal cord.

Hypothesis

The hypothesis of this work arises from the premise that progenitor cell identity is determined by the combined expression of transcription factors that instruct their differentiation, and finally consolidate cellular phenotype, and thus their functions. In this context, and based on preliminary data, we propose the following hypotheses: **I)** Changes in the spatial organization of the serotonergic system in the lineage of vertebrates is due to modificactions in Neurog3 expression pattern. **II)** Neurog3 exerts its action excusively by Ascl1 repression. **III)** Sox9 and/or Hox genes determine the onset of Neurog3 expression in the spinal cord.

Specific aims
    I)   Determine the association between Neurog3 expression and the spatial organization of the serotonergic system in vertebrates.
    II)   Analyze the mechanism of Neurog3 action.
   **III)**   Identify the mechanisms that control Neurog3 expression in the spinal cord.

Results

Association between Neurog3 expression and the spatial organization of the serotonergic system in vertebrates: *Neurog3 is not expressed in the spinal cord of zebrafish.*

In mammals, serotonergic neurons are found exclusively in the raphe nuclei. By contrast, aquatic vertebrates, including the lamprey, fish and amphibians, contain both the 5-HT brainstem raphe system and 5-HT cells embedded in the spinal cord motor networks (Branchereau et al, 2000; Harris-Warrick et al, 1985; Lillesaar, 2011). Interestingly, the display of serotonergic cells in these species resembles the phenotype we found in Neurog3 mutant mice (Carcagno et al, 2014). We analyzed the expression of Neurog3 in the spinal cord of zebrafish and found no expression of Neurog3 in the developing spinal cord (data not shown), in accordance with previous studies (Wang et al, 2001). This result indicates that changes on Neurog3 expression in the lineage of vertebrates could explain differences in the spatial organization of the serotonergic system.

Mechanism of Neurog3 action: *Double mutants Ascl1;Neurog3 do not exhibit serotonergic neurons in the spinal cord.*

In order to determine whether Neurog3 exerts its action exclusively through Ascl1 regulation or through Ascl1 independent mechanisms, we simultaneously affected the expression of Neurog3 and Ascl1 by double loss-of-function experiments (double mutants *Ascl1$^{-/-}$;Neurog3$^{-/-}$*).
We analyzed, serotonergic identity determinants Gata2, Pet1 and Lmx1b through *in situ* hybridizations, in the spinal cord of control and mutant mice. While single *Neurog3* mutants present ectopic serotonergic neurons in the spinal cord, these neurons were not observed in *Ascl1;Neurog3* double mutants (Figure 1). This result demonstrates that Ascl1 is responsible for the appearance of ectopic serotonergic neurons in the p3 domain of *Neurog3$^{-/-}$* mice. Therefore, we conclude that Neurog3 prevents serotonergic specification in the spinal cord exclusively through the suppression of Ascl1.
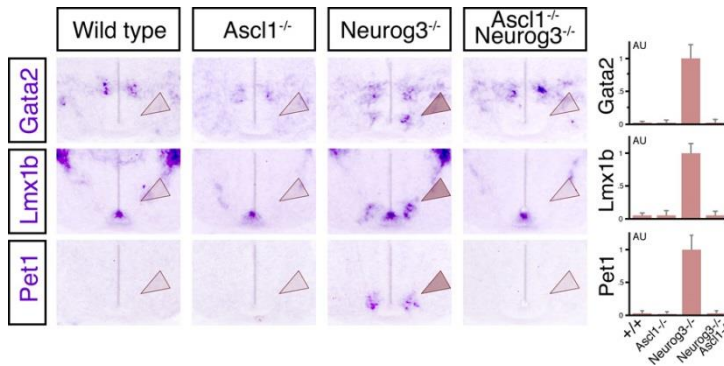
Figure 1 Heterotopic serotonergic specification in *Neurog3*^{-/-} spinal cord requires Ascl1. Cross sections of spinal cord of wild type mice, *Ascl1*^{-/-}, *Neurog3*^{-/-} and *Ascl1;Neurog3* double mutants. Analysis of the expression of Gata2, Pet1 and Lmx1b by *in situ* hybridization. The quantifications show that only *Neurog3*^{-/-} mice possess ectopic serotonergic differentiation (filled arrowheads), which is lost in the double mutants. Bars represent mean + SD.

Mechanisms that control Neurog3 expression in the spinal cord.

Sox9 does not regulate Neurog3 expression in the neural tube.

We found that Neurog3 induction in the ventral spinal progenitors is delayed compared to the expression of patterning genes. Neurog3 timing appears to be rather coincident with the transcription factor Sox9 (Stolt et al, 2003), with Sox9 preceding Neurog3 initiation by ~0.5 days. Importantly, in the developing pancreas, Sox9 has been shown to coordinate cell differentiation by directly activating Neurog3 expression (Lynn et al, 2007). In order to determine whether Sox9 regulates Neurog3 expression, we performed loss-of-function experiments. We generated conditional knockout mice *Sox9[flox/flox];Nestin:Cre*[+], in which Sox9 deletion is restricted to the central nervous system (Scott et al, 2010; Stolt et al, 2003). By immunohistochemistry, we found normal Neurog3 expression both in control (*Nestin:Cre*[-]*;Sox9[flox/+]* and *NestinCre*[-]*;Sox9[flox/flox]*) and Sox9 conditional knockout mice (*NestinCre*[+]*;Sox9[flox/flox]*) (Figure 2A). As shown, the number of Neurog3^{+} cells in Sox9 mutants is similar to controls (Figure 2B). This result indicates that Sox9 is not necessary for Neurog3 expression in the ventral spinal cord.
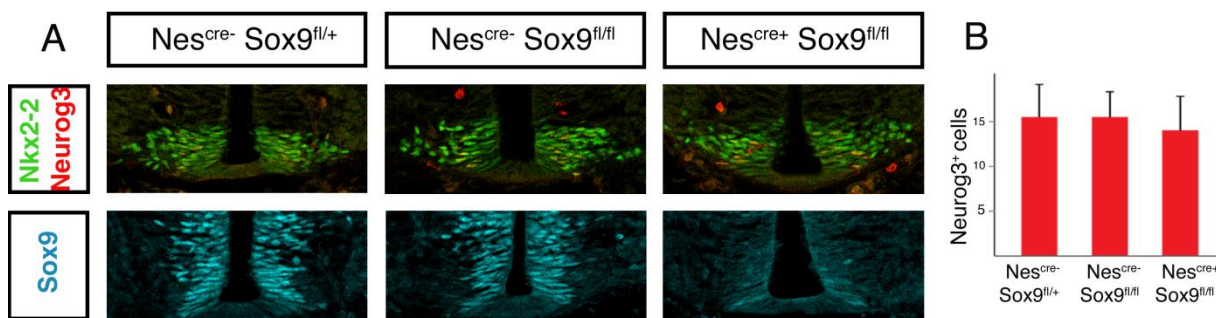


Figure 2 Expression of Neurog3 does not depend on Sox9
A) Cross sections of spinal cord at E11.5 stained with antibodies against Neurog3 and Nkx2-2 in control animals (*NestinCre*[-]*;Sox9[flox/+]* and *NestinCre*[-]*;Sox9[flox/flox]*) and Sox9 conditional knockouts (*NestinCre*[+]*; Sox9[flox/flox]*). B) Quantification of Neurog3^{+} cells. No differences in the number of Neurog3^{+} cells were detected among genotypes. The bars correspond to mean + SD.

Hox genes and the regulation of the serotonergic system

Hox cluster genes are essential in the establishment of the body plan and contribute to the establishment of differential cell identities along the rostro-caudal axis. Hox genes are found in all animal species in which they have conserved roles (McGinnis & Krumlauf, 1992). In most vertebrates, there are 39 genes distributed in 4 clusters (HoxA, HoxB, HoxC and HoxD). Each cluster consists of 13 genes (Hox1-Hox13) and, in the case of the central nervous system; each gene is expressed in specific coordinates (Arber et al, 2000). In general, Hox1-Hox5 genes are expressed in the hindbrain while Hox4-Hox11 are expressed in the spinal cord.

Given the different spatial distribution of these genes, Hox genes are candidates to regulate the spatial organization of the serotonergic system. Our hypothesis is that caudal Hox genes (Hox4-Hox11) control the expression of Neurog3 and suppress serotonergic differentiation in the spinal cord. In order to test this hypothesis, we ectopically expressed caudal Hox genes (HoxA5, HoxC6, HoxA7, and HoxC8) in the hindbrain by experiments of co-electroporation in chick embryos. After 4 days, embryos were collected for analysis and the presence of serotonergic neurons in the hindbrain was determined by immunohistochemistry with an antibody against 5-HT. We found no differences in the number of serotonergic cells in the electroporated side compared to the contralateral control side (Figure 3). These results indicate that this combination of caudal Hox genes is not sufficient to block the serotonergic differentiation in the hindbrain.

For this reason, experiments with different Hox genes combinations are necessary to determine the participation of these factors in controlling the expression of Neurog3 and the spatial restriction of the serotonergic system in the nervous system.
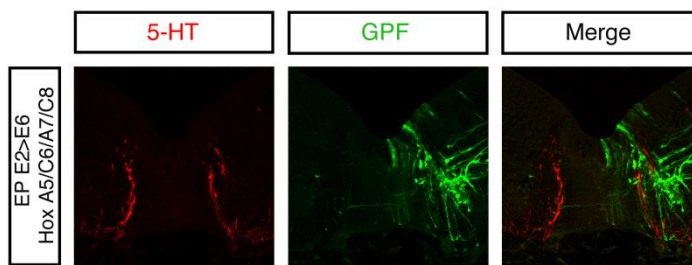
Figure 3 Overexpression of caudal Hox genes in the hindbrain does not affect serotonergic specification. Cross section of the chick ventral hindbrain co-electroporated with Hox genes A5/C6/A7 and C8, stained with antibodies against GFP and 5-HT. No differences were observed in the number of 5-HT$^+$ cells in the electroporated side relative to the contralateral control.

Purchases

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Acknowledgements

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