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BMP/SMAD Pathway and the Development of Dopamine Substantia Nigra Neurons

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Review of Jovanovic et al.

One of the major hallmarks of Parkinson's disease (PD) is the loss of dopamine release in the striatum resulting from progressive loss of mesodiencephalic dopamine-producing (mdDA) neurons. In PD, the most vulnerable neurons are in the ventral tier of the substantia nigra (SN); mdDA neurons in the adjacent ventral tegmental area (VTA) are mostly spared (Kalia and Lang, 2015). Studies that have characterized the embryonic pathways underlying mdDA neuron specification and differentiation, have shown that numerous signaling molecules, including sonic hedgehog, fibroblast growth factors, and Wnts influence the differentiation of mdDA neuronal subsets (Veenvliet and Smidt, 2014; Arenas et al., 2015). This knowledge has guided the design of protocols to differentiate stem cells into DA neurons, for both transplantation and *in vitro* PD models. Still, these protocols typically yield low numbers of fully differentiated DA neurons. Therefore, the search for more efficient protocols based on a greater understanding of mdDA neuron development *in vivo* continues (Arenas et al., 2015).

A recent paper published in *The Journal of Neuroscience* investigated whether the bone morphogenetic protein (BMP)/SMAD signaling pathway plays a role in the development of mdDA neurons (Jovanovic et al., 2018). BMPs are members of the transforming growth factor β (TGF-β) superfamily and binding to the BMP type I receptor results in phosphorylation of receptor-regulated SMADs, that is, SMAD1/5/8. The phosphorylated (p)-SMAD protein establishes a complex with a partner SMAD protein resulting in nuclear translocation and subsequent regulation of target genes (Katagiri and Watabe, 2016). BMPs play an important role in the development of the CNS, contributing to processes such as neurulation, neurogenesis, and neuronal differentiation (for review, see Bond et al., 2012).

Jovanovic et al. (2018) first demonstrated the presence of p-SMAD1/5/8 proteins together with transcript expression of Bmp5/6/7 and the BMP receptor Ib in the embryonic mesencephalic flexure (their Fig. 1). Next, they used several combinations of single and double BMP knockout (KO) animal strains (Bmp5, Bmp6, Bmp7, Bmp5/6, and Bmp6/7) to investigate the function of BMPs in mdDA development. They found no effect on the expression of the dopamine transporter suggesting no changes in mdDA neuronal formation in these mutants. In contrast, the Bmp5/7 double mutant, which was investigated at embryonic day (E)10 because these animals do not survive past this age (Solloway and Robertson, 1999), revealed that expression of Nurr1, a transcription factor that is required for the expression of tyrosine hydroxylase (Th), a pivotal marker of the DA phenotype (Zetterström et al., 1997; Smits et al., 2003), was absent (Jovanovic et al., 2018, their Fig. 1). Therefore, the authors concluded that postmitotic DA neurons failed to develop in the Bmp5/7 KO.

The authors next explored proliferation and neurogenesis, using the transcription factor Lmx1a/b to mark a mdDA progenitor region and phospho-HISTONE H3 to visualize mitotic cells. Fewer mdDA progenitors were present at E10.5 in Bmp5/7 KO embryos than in controls (Jovanovic et al., 2018, their Fig. 2). Importantly, the reduction of neuronal progenitors was restricted to the Lmx1a-positive subset, suggesting that Bmp5/7 specifically regulates the proliferation of mdDA progenitors. The authors subsequently showed that Msx1/2 and Ngn2, two proneuronal transcription factors that act sequentially to drive normal mdDA progenitor development (Andersson et al., 2006), were downregulated in Bmp5/7 mutants. These data suggest that neurogenesis is reduced in the Bmp5/7 mutant, and that

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Bmp5/7 promotes Msx1/2 expression. As such Bmp5/7 could be included in a previously proposed developmental model (Andersson et al., 2006), in which Bmp5/7 drives Msx1/2 expression, and consequently Ngn2 expression in the mdDA progenitor domain.

To test whether receptor-regulated SMAD signaling is required for mdDA neurogenesis, the Nestin-cre driver was used to conditionally delete Smad1 in neural precursor cells (Smad1^{Nes}). Because Smad1^{Nes} mutants survived past birth, the authors were able to investigate SMAD function in postmitotic mdDA neurons. This approach revealed a prolonged proliferation phase, which was accompanied by a reduction of postmitotic mdDA neurons (Jovanovic et al., 2018, their Fig. 5). To test whether the SN and VTA were equally affected by the absence of Smad1, the authors examined the expression of subset-specific markers within Th-positive neurons, specifically Sox6 and Girk2 to identify SN neurons and Calb to visualize VTA neurons (Poulin et al., 2014). The number of Th+/Sox6+ and Th+/Girk2+ double-positive neurons were reduced in Smad1^{Nes} mutants, whereas the ratio of Th+/Calb+ double-positive neurons remained unchanged, suggesting that the loss of Smad1 affects SN neurons more than VTA neurons (Jovanovic et al., 2018, their Fig. 6).

These results led Jovanovic et al. (2018) to hypothesize that enhancing BMP/SMAD signaling would increase purity when generating DA neurons from induced neural stem cells (iNSCs). Importantly, BMP/SMAD signaling is generally inhibited in early phases to convert stem cells into neural stem cells (Chambers et al., 2009). Consistent with their hypothesis, adding BMP5 and BMP7 to the final maturation phase of a standard protocol for the induction of DA neurons, but without changing the intermediate differentiation phase toward dopamine precursors (Reinhardt et al., 2013), the authors accomplished a 2- to 3-fold increase of Th+ neurons as a portion of total (TuJ+) neurons. Furthermore, they observed a higher percentage of Th+ cells coexpressing SN-marker Girk2 (~8%) than Th+ neurons that coexpressed VTA-marker Calb (~3%; Jovanovic et al., 2018, their Fig. 7). Whether this represented a significant difference was not indicated however. Nonetheless, the authors concluded that the BMP/SMAD signaling pathway could be used to program neuronal stem cells into DA neurons more efficiently.

How does the BMP/SMAD pathway promote SN neuronal identity?

Jovanovic et al. (2018) provide compelling data suggesting the importance of BMP/SMAD signaling in mdDA neuronal development. Moreover, they reveal that this signaling pathway might be involved in mdDA subset specification, as they report a significant loss of Th+ neurons that express SN markers (i.e., Sox6+, Girk2+), when BMP/SMAD signaling is disrupted. In contrast, the percentage of Th+/Calb+ neurons, a VTA marker, remained unchanged in Smad^{Nes1} mutants (Jovanovic et al., 2018, their Fig. 6). What might explain the reduction of SN neurons in Smad1^{Nes} mutants?

One clue is the reduced number of Th+ neurons that also express the transcription factor Pitx3+ at E14.5 and P0 in Smad1^{Nes} mutants (Jovanovic et al., 2018, their Fig. 6). Indeed, several papers have shown that Pitx3 is essential for the development of SN neurons (Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004). Whether the SMAD/BMP pathway regulates Pitx3 expression directly or indirectly in mdDA progenitors is unclear. However, a candidate to mediate indirect regulation is the transcription factor En1, because the En1+ fraction of TH-neurons was reduced in the experiments by Jovanovic et al. (2018) (their Fig. 6), and En1 is a known regulator of Pitx3 (Veenvliet et al., 2013; Kouwenhoven et al., 2017).

Another clue is the relationship between the temporal expression of Bmp5/7 in the mesencephalon and the different time points at which SN and VTA neurons are born. Previous work suggests that the majority of SN neurons are born at E10, whereas VTA neurons are predominantly born between E10 and E12 (Bye et al., 2012). From the Jovanovic et al. (2018) study, it is clear that Bmp7 transcript, BMP5, and p-SMAD1/5/8 proteins are already present within or very close to the mdDA progenitor domain at E10 (their Fig. 1). This means that at the peak of SN neurogenesis, several components of the BMP/SMAD pathway are present and could thus shape the specific developmental profile of these cells. In contrast, a large percentage of VTA neurons are born after E10 and might therefore be exposed to a lesser degree to BMP/SMAD signaling. More detailed information on the temporal expression of the BMP/SMAD components in the mesencephalon could provide insight regarding this hypothesis.

Improved iNSC protocol by incorporating BMP/SMAD pathway

In the second part of their paper, Jovanovic et al. (2018) focus on optimizing the *in vitro* generation of DA neurons by adding BMP5/7 to the maturation stage of a typical protocol. Notably, this altered protocol seems to generate a larger number of Th+ neurons (as a portion of Tuj1+ cells). The reason for this improvement is unfortunately not easily explained. The *in vivo* data (Jovanovic et al., 2018, their Figs. 1–5) strongly support a role for BMPs in developmental regulation of early mdDA progenitors. However, the supplementation of BMPs to the *in vitro* protocol occurs during the (later) maturation phase. It is possible that the increased Th+ yield results from a neurotrophic effect of BMPs, because it has been shown that BMP7 can protect DA neurons against toxins *in vivo* (Harvey et al., 2004) and can be neuroprotective for primary neurons as well (Cox et al., 2004; Chou et al., 2008).

In addition to increasing Th+ yield, the supplementation of BMP5 and BMP7 could possibly form a new lead to a protocol that also produces Th+ cells that more often coexpress the SN-specific marker Girk2 than the VTA-specific marker Calb. Two recent studies described another effective method to promote the production of differentiated DA neurons: the use of the cell surface marker Corin (Doi et al., 2014; Kikuchi et al., 2017). These are all exciting results in terms of the search for a protocol that generates *in vitro* DA neurons with a SN character that could replace the SN neurons that have been lost in PD. Notably however, are the results of another recent paper (Kirkeby et al., 2017), showing that striatal transplantation of DA progenitors with a caudal-mesencephalic phenotype, i.e., expressing markers generally more associated with a VTA phenotype, represented more successful grafts, than DA progenitors with a diencephalic phenotype. Combined, these studies suggest that it is essential to carefully choose appropriate DA neuron progenitors for effective transplantation. In light of this, the conclusion that BMP/SMAD signaling influences mdDA progenitors *in vivo* and may potentially regulate SN and VTA subsets through transcription factors such as En1 or Pitx3, is valuable for the optimization of protocols that reprogram neuronal stem cells.

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