Response by Nilima Prakash to "Studying Mesodiencephalic Dopaminergic Neuron Development In Vivo to Improve Stem Cell Therapy in Parkinson's Disease" by Jesse V. Veenvliet

We thank Veenvliet for the positive comments on our article and would like to address some issues raised in this Journal Club.

The experimental variance between independent PSC in vitro differentiation experiments is indeed a major concern of the clinical applicability of such differentiation strategies. We controlled the plating density (same cell number was plated at the beginning of each differentiation experiment) and timing (factors were always added within the same 24-hour-period) in our protocol. However, cells had to be passaged at least once for each differentiation experiment, and passage number therefore differed in our experiments. The last differentiation experiment (Con3/5_19) rendered mdDA neurons resembling more the endogenous (*in vivo*) neurons probably because iPSCs used as starting material approached a senescent status after repeated passages and might have become more prone to neuronal differentiation.

As long as this experimental variance cannot be controlled completely, such differentiation paradigms are not suitable for conducting detailed analyses of signaling pathways during the differentiation of PSCs into mdDA neurons, because the probability of measuring artifacts is relatively high. Moreover, such experiments require transcriptome analyses of dynamic time-points during the differentiation procedure and not "end-point" measurements as performed in our study.

The in vitro-differentiated, iPSC-derived mdDA neurons (including both the WNT1/DKK3-untreated (control) and the WNT1/DKK3-treated cells) generally were not *bona fide* mdDA neurons, as clearly demonstrated by our comparative transcriptome analysis using E13.5/E14.5 mdDA neurons.

Kriks et al., 2011 used only human PSCs, a very similar dual SMAD inhibition protocol, and the potent GSK3beta inhibitor CHIR99021 (CHIR99). We used exactly the same CHIR99 concentration as Kriks et al. for mouse PSC differentiation, but observed the opposite effect: mouse PSCs/NSCs tended to die prematurely, leading to an overall reduced number of differentiated mdDA neurons in these cultures. This clearly indicates, in our opinion, that species-specific differences may also be relevant in this context.

We don't think that WNT1 is a canonical (WNT/beta-catenin) signaling factor that increases merely mdDA progenitor proliferation. If this were true, we should have observed overall increased numbers of TH+/PITX3+ double-positive cell clusters and TH+ and/or PITX3+ cells in the WNT1-treated cultures, which was clearly not the case (Fig. 6H, I). Moreover, DKK3 might act via a WNT-unrelated pathway, such as TGFbeta/SMAD signaling, in this context.

The use of another pan-mdDA reporter (TH) for transcriptome analysis may not add much more information: The "end-point" analysis meant that a DKK3-responsive cell population was most likely present at the beginning of the differentiation procedure under both conditions, and removed in the control condition (without DKK3/WNT1) but promoted to differentiate into PITX3-GFP+ cells in the +DKK3/WNT1 condition. DKK3-non-responsive cells ("background") were included in both conditions as long as they differentiated into PITX3-GFP+ cells. Our experiments thus depended on the relative proportion of DKK3-responsive to non-responsive PITX3-GFP+ mdDA neurons in the cultures. As clearly shown in Fig. 6J, this proportion among all TH+ cells was rather small (approx. 13% (+DKK3) and 21% (+DKK3/WNT1)). To resolve this conclusively, a specific reporter for DKK3-responsive PITX3+ cells is needed.