LETTER TO THE EDITOR

Reply: Quantitative evaluation of the human subventricular zone

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Sir, We would like to thank Höglinger and colleagues for their interest in our recent publication in Brain (van den Berge et al., 2011). In this study, we showed that the number of adult neural stem cells and precursors in the main neurogenic niche of the human brain, the subventricular zone, is not significantly diminished in patients with Parkinson’s disease compared to age- and sex-matched controls. In addition, we provided evidence that two different human neural stem cell lines did not respond with an increase in cell proliferation following exposure to dopamine or dopamine agonists. We also examined the number of proliferating neural stem cells and precursors in the subventricular zone of mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mouse model for Parkinson’s disease, and these results supported our findings in the human brain. Our data, however, are inconsistent with three previous studies (Höglinger et al., 2004; O’Keeffe et al., 2009; O’Sullivan et al., 2011).

In a letter to the editor, Höglinger and colleagues argue that our data should be interpreted with caution, and they state that our anatomical definition of the region of interest, the sampling procedure, some of the immunostaining procedures, and the quantification methods were of limited precision. Here, we would like to extend on the used methodologies as described in the methods section and supplementary material of our article (van den Berge et al., 2011) and we would like to take the opportunity to clarify the issues raised by Höglinger and colleagues. We agree that the human subventricular zone is highly variable in width, as we have shown before (van den Berge et al., 2010), and that anatomical matching between donors is necessary for obtaining reproducible quantitative data. In our recent publication (van den Berge et al., 2011) we observed again a significant variability in the number of glial fibrillary acidic protein-δ (GFAPδ) expressing cells and cells expressing different cell proliferation markers between donors, both in aged individuals and in cases with Parkinson’s disease. Such variability in the human subventricular zone can also be observed in the data presented in previous studies by other groups, although it is not always explicitly described (Bernier et al., 2000; Curtis et al., 2005; Quinones-Hinojosa et al., 2006; Marti-Fabregas et al., 2010).

To establish cell proliferation in human studies, one has to rely on the use of endogenous cell proliferation markers, as opposed to animal models, where bromodeoxyuridine (BrdU) can be administered. We have tried to overcome this limitation in our studies by using multiple markers to study cell proliferation and the number of neural stem cells in the subventricular zone, i.e. proliferating cell

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nuclear antigen (PCNA), phospho-histone H3 and GFAP. Each of these markers showed a similar highly variable pattern within and between donors. We have also shown in earlier work (van den Berge et al., 2010) that PCNA and GFAP expression largely overlap thereby confirming that these two proteins are expressed by the same cell type, which is highly likely the B cell (which is the adult neural stem cell in the subventricular zone). PCNA is also expressed by precursor C- and A cells and we also found a high PCNA expression in ependymal cells, implying that PCNA expression is not limited to neural stem cells and precursor cells, which has been suggested previously (Funato et al., 1996). PCNA expression in ependymal cells is unexpected, as these cells are assumed to be post-mitotic (Spassky et al., 2005), although this view has been contended, and ependymal cells may actually function as neural stem cells (reviewed in Chojnacki et al., 2009). In previous autopsy studies, either the number of PCNA cells (a marker of proliferating cells), epidermal growth factor-receptor (EGFR; expressed in precursors), or Musashi1 (expressed in neural stem cells and precursors) immunopositive cells were quantified in the subventricular zone of patients with Parkinson’s disease and controls to investigate the effect of dopamine depletion on the neural stem cell and precursor pool in humans (Höglinger et al., 2004; O’Keeffe et al., 2009; O’Sullivan et al., 2011).

We agree with Höglinger and colleagues that a systematic randomized sampling using stereological principles is the best approach. Design-based stereology is the gold standard for accurately and efficiently estimating the total number of cells in a region of interest (West et al., 1991; Schmitz and Hof, 2005). Due to the retrospective nature of our study, stereological analysis of the entire subventricular zone was, however, not possible. Therefore we decided to quantify the immunostaining of several markers for neural stem cells and cell proliferation within an anatomically-defined standardized region at the level anterior to the commisura anterior (Fig. 1), an area that was present in all donors from the Netherlands Brain Bank. As we observed a large variability in the number of immunostained cells in the subventricular zone and the thickness of the subventricular zone between donors, we decided to quantify the area of PNCA-immunostained cells within the area of interest applying a systematic randomized sampling procedure using ImagePro image analysis software, although we are well aware of the limitations of this approach.

For our study we obtained post-mortem brain material from the Netherlands Brain Bank, of which the complete clinical history and neurohistopathological information of the donors was made available (www.brainbank.nl). The brain material is processed according to a highly standardized protocol. One hemisphere is directly fixed in 4% formalin, after 4 weeks this hemisphere is sliced in 1-cm thick coronal sections (Fig. 1) and various anatomical regions are dissected out using standardized protocols by experts. Paraffin embedded tissue blocks containing the nucleus caudatus and the putamen aligned by a large area of the lateral ventricular wall (~2 cm in length) were further processed for the immunostainings described in our study. Paraffin sections (8-μm thick) were cut and immunostained for the quantification of PCNA in the human subventricular zone. We first made an overview with a ×5 objective (Fig. 2A), the subventricular zone was outlined and 20% of this area was selected by systematic randomized sampling (Fig. 2B). Subsequently, five to six images were taken with a Sony black-and-white camera at a higher magnification and area of interest was drawn including the subventricular zone, but excluding the ependymal layer, as this was intensely positive for PCNA (Fig. 2C). All images were taken with the same camera settings. The background was measured and subtracted from the image. Then, a threshold, at which specific staining was identified, was set to calculate the surface area of the PCNA expression (Fig. 2C). We calculated the average percentage of subventricular zone area that was occupied by PCNA staining in 20% of the area of interest as selected by systematic randomized sampling. The pictures in Fig. 2 of our manuscript (van den Berge et al., 2011) were selected to illustrate the considerable variation.
in PCNA-immunostained area in the subventricular zone between donors. Based on these pictures, Högglinger and colleagues now estimate that there is a 30% decrease in the subventricular zone of the Parkinson’s disease compared to the control, which in our opinion cannot be concluded from analysis of a single picture of a single donor. In contrast to the Högglinger et al. (2004) and O’Keeffe et al. (2009) studies, our study includes a well-described donor population, which is matched for a broad range of variables. Högglinger and colleagues express their concern about the staining intensity and the effect on the percentage of the immunostained area. We agree that it is difficult to say anything about the quantity of PCNA per cell, but it is certainly possible with our technique to quantify the surface area that is immunopositive for PCNA. They agree with the approach described in the O’Sullivan et al. (2011) study, in which a highly similar quantification technique is applied to measure Musashi1 immunostaining.

Triggered by the remarks in the letter to the editor by Högglinger and colleagues, we carefully examined the papers they referred to, in which the methodology to quantify the neural stem cells and precursors in the human subventricular zone was described. Högglinger et al. (2004) were the first to report on the number of PCNA expressing cells in the subventricular zone of controls and patients with Parkinson’s disease. At that time we had strong indications that we had found a novel marker for neural stem cells in the subventricular zone (Roelofs et al., 2005; Middeldorp et al., 2010). Therefore, we aimed at repeating this study in a larger, neuropathologically well-described selection of controls and patients with Parkinson’s disease, to prove that patients with Parkinson’s disease had a decreased number of neural stem cells in the subventricular zone. However, we were not able to find a significant decrease in patients with Parkinson’s disease. This discrepancy might be clarified by several differences between the studies. In our view the human data presented in the study by Högglinger et al. (2004) have to be interpreted with some caution for a number of reasons. First, PCNA expression was quantified in only four cases with Parkinson’s disease and four control cases, which is too few, considering the large variation we see in GFAP/PCNA expression in the subventricular zone (van den Berge et al., 2010, 2011). Furthermore, the authors have performed parametric statistical tests, while it is highly unlikely that the data were normally distributed.

**Figure 2** Example of our systematic randomized sampling method on a tissue section of a 79-year-old female control case (Netherlands Brain Bank 99-052). In (A) a digital image is shown of the complete striatal tissue section immunostained for PCNA. In red the area of interest is indicated. In (B) a higher magnification of the area of interest is shown (in green), and this area was divided by the image analysis software in sub-areas (in red). Twenty per cent of the total surface in red was chosen for further analysis by the software with a systematic randomized approach. These areas were indicated with a blue dot in the middle. If a blood vessel was in the area indicated by the software, we chose the field to the left of this area. Higher magnification images were made (C), the area of the subventricular zone is indicated in blue, the background was subtracted and the PCNA immunostained cells were outlined by a thresholding procedure (in red). The length of the subventricular zone was measured and the surface percentage of the PCNA immunostained area (red) within the subventricular zone area (blue) was calculated.
distributed. The individual patients used in this study are also not well-described with respect to their pathological Braak staging for Parkinson’s disease and Alzheimer’s disease (Braak and Braak, 1991; Thal et al., 2000; Braak et al., 2003), medication and cause of death, except that they were clinically and pathologically diagnosed with Parkinson’s disease, were treated with levodopa and were matched for sex, age (controls 68.5 ± 3.2; Parkinson’s disease 66.0 ± 11.1 years) and post-mortem delay (controls 26.5 ± 4.8 and Parkinson’s disease 27.8 ± 10.5 h). The control cases were without neurological and neuropsychiatric disease. A discrepancy between our study and the Höglinger et al. (2004) study is that our patient cohort of 10 controls, five patients with incidental Lewy body disease (iLBD) and 10 patients with Parkinson’s disease had a higher mean age (controls 79.5 ± 6.6; iLBD 89.4 ± 7.2; Parkinson’s disease 79.3 ± 5.0 years) and a significantly lower post-mortem delay (controls 5.1 ± 1.25; iLBD 7.5 ± 2.5; Parkinson’s disease 6.0 ± 1.5 h). All this and additional information about the cohort was provided in our article (van den Berge et al., 2011). Lastly, the exact quantification method is not extensively described in the materials and methods, except that the PCNA-positive cells were counted in the subventricular zone from the corpus callosum to the vena thalamostriata.

The second study investigated epidermal growth factor receptor (EGFR)-positive cells in the human subventricular zone (O’Keeffe et al., 2009), which are presumed to represent neural precursor cells. However, there is currently no evidence that EGFR expression is limited to precursors, and it is known that EGF and EGFR levels are decreased in other brain areas in Parkinson’s disease (Iwakura et al., 2005). We have attempted to reproduce the EGFR expression study in the human subventricular zone, but we did not observe the staining pattern that was described in this study (O’Keeffe et al., 2009). The discrepancy might be caused by the antibody we used, as we were unable to obtain the same antibody that was used in the O’Keeffe et al. (2009) study. The immunostaining presented by O’Keeffe et al. (2009) convincingly shows an intensity difference in EGFR staining between a control and a Parkinson’s disease case. However, it needs to be noted that strong immunostaining was also seen in the ependymal layer. Again in this study no extensive details were given on the used quantification method and it is unclear whether the ependymal cell layer is excluded from quantification. As the EGFR is mainly membrane localized, it is extremely difficult to identify individual cells for counting; it would have been helpful, therefore, if the exact quantification technique was explained. In this study, six controls and six patients with Parkinson’s disease were studied and information about their age and clinical score was presented. Unfortunately, no extensive clinicopathological data of each individual patient were provided and the statistics were again parametric.

The most recent study describing an effect of Parkinson’s disease on neurogenesis in the subventricular zone in humans shows a negative correlation of the disease duration and a positive effect of levodopa use on the number of neural stem cells in the subventricular zone (O’Sullivan et al., 2011). In this study the authors used Musashi1 expression as a measure for neural stem cell activity, while Musashi1 is actually a marker for both neural stem cells and precursors, as well as a population of mature astrocytes (Sakakibara and Okano, 1997). This makes it difficult to draw a definitive conclusion about the effect of the Parkinson’s disease disease progression and levodopa therapy on neural stem cells. In this study a large cohort of Parkinson’s disease patient material (n = 32) was studied and paraffin embedded tissue was used at the coronal level of the anterior commissure rostral to the globus pallidus. This is a highly similar area to the brain area we used in our study. Furthermore, in this study they quantified the percentage area of immunostaining in five digital images taken from the subventricular zone and ependymal layer, which was again very similar to our study. The statistical analysis of the clinical progression and neuropathological analysis of the patient population has been performed thoroughly in the O’Sullivan et al. (2011) study. Although a negative correlation was found between Musashi staining density and Parkinson’s disease duration, this effect was not directly coupled to nigrostriatal dopaminergic loss.

With regard to our mouse work, Höglinger and colleagues also express their concern about potential technical problems that might have contributed to our lack of effect of dopaminergic denervation on neural stem cell proliferation in the mouse subventricular zone. We investigated cell proliferation in the subventricular zone of a chronic mouse model for Parkinson’s disease, induced by the toxin MPTP. In these mice, we could show dopaminergic denervation of the striatum, but again no difference in subventricular zone cell proliferation. We agree that BrdU labelling is a reliable method to quantify cell proliferation in the subventricular zone. In analogy with our human data we decided, however, to perform PCNA and phospho-histone H3 immunostaining on the MPTP mouse model to quantify cell proliferation. It has been reported before that PCNA staining in mice can give consistent results in comparison with BrdU incorporation (Mokry et al., 2003), as we also discuss in our article (van den Berge et al., 2011). The immunostainings on the mouse tissue have been tested for specificity and omission of the primary antibodies resulted in no staining. There are a number of factors that can explain the discrepancy between our data and those in other published studies, which did show an effect of Parkinson’s disease on the cell proliferation in the subventricular zone. We will discuss these below. Previous studies in animal models of Parkinson’s disease have yielded contradictory results. A number of papers showed an increase in subventricular zone cell proliferation (Liu et al., 2006; Aponso et al., 2008; Peng et al., 2008; Peng and Andersen, 2011), others a decrease (Baker et al., 2004; Höglinger et al., 2004; Freundlieb et al., 2006; Winner et al., 2006), using BrdU labelling and/or immunostaining for Ki-67 or PCNA. Two previous studies showed that there was no change in precursor proliferation using PCNA staining (Winner et al., 2004; Marxreiter et al., 2009). Thus, our data are in accordance with these studies. Furthermore, it has also been shown that subventricular zone cell proliferation is decreased in transgenic Parkinson’s disease models, expressing human mutant a-synuclein and LRRK2, where there is no dopaminergic denervation (Winner et al., 2008, 2011). This implies that the decrease in subventricular zone cell proliferation may not be mediated by a lack of dopamine, but is caused by other experimental factors, such as acute neuronal damage caused by Parkinson’s disease-inducing genes and toxins. Neurogenesis is affected by many physiological and
pathological factors and processes (reviewed in Zhao et al., 2008), and it is conceivable that the neuronal damage induced by the used toxins or mutated genes affects the results in the involved studies (reviewed in Kim and Szele, 2008).

Finally, we would like to respond to the issues raised regarding the cell culture work in our article. Culturing primary adult neurospheres from human post-mortem brains does currently not yield enough cells for an extensive analysis of the effect of dopamine agonists and antagonists on neural stem cell proliferation. We agree with Höglinger and colleagues, that these experiments are important to perform; therefore we are now putting effort into immortalizing the human adult neural stem cells derived from post-mortem brains from controls and patients with Parkinson’s disease. In our article (van den Berge et al., 2011) we described that we do find the messenger RNA of the D1–D5 receptors in the human subventricular zone, therefore we expect that the human adult neural stem cells also express these receptors. But of course this has to be verified by quantitative polymerase chain reaction on primary cells. We decided to test whether human neural stem cells react to dopamine agonists and antagonists. As it was unfeasible to perform these experiments on the primary neural stem cells isolated from post-mortem human brains, we chose to use two different human neural stem cell lines. We are aware of potential limitations, as these cells originate from foetal brains. We treated the cells with dopamine, different dopamine agonists and antagonists. We measured the stability of dopamine in our culture system with HPLC, as described in our article, and since we observed that dopamine levels were decreased up to 60% after 8 h, we supplemented our cell cultures daily with fresh dopamine. The D2 receptor agonist bromocryptine and the D2 receptor antagonist sulphide are known to be stable. SKF-38393 is likely to be oxidized, and only in this treatment condition we might have treated the cells suboptimally. Höglinger and colleagues misinterpreted our BrdU incorporation results in Fig. 6G of the original article. We have indicated that the control condition without dopamine displays a 100% BrdU labelling. This might indeed be a little misleading, as we have normalized the BrdU incorporation in the control condition with and without growth factors to 100%, meaning that not all cells are BrdU positive. The raw data show a clear difference in BrdU labelling in cultures with and without growth factors.

In conclusion, the concerns that were raised by Höglinger and colleagues are valid regarding the difficulty of interpreting quantitative data on human tissue. Our study, however, provides, in comparison with earlier published data, an extensive analysis of the human tissue from a large cohort of well-characterized controls, incidental cases and Parkinson’s disease cases. The tissue we used was of high quality, with a very short post-mortem delay. Extensive neuropathological and clinical data are available of the individual cases used in our study. Next to markers for adult neural stem cells, GFAP, we also examined two cell proliferation markers, PCNA and phospho-histone H3 and quantified the PCNA-immunopositive area in the subventricular zone using a systematic randomized sampling design. We quantified the number of dopaminergic neurons in the substantia nigra using stereological principles and quantified the tyrosine hydroxylase expression in the subventricular zone, which is a measure for dopaminergic innervation. Furthermore, we have complemented our study with studying a potential direct proliferative effect of dopamine on human neural stem cell cultures and we have analysed, in analogy with our human data, the subventricular zone proliferation and tyrosine hydroxylase expression in the subventricular zone of the chronic MPTP mouse model. We are aware that many studies have shown that dopaminergic innervation is important in regulating subventricular zone neural stem cell proliferation, although other studies have been published that do not support this hypothesis. We have discussed the discrepancies and similarities between these studies and our data extensively in our article (van den Berge et al., 2011) and in this response. Taken together, we stick to our earlier conclusion that our data do not support the dopaminergic control hypothesis of the adult subventricular zone neural stem cells.

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