Short title:

PGC guidance in marmoset and mouse embryos

Title:

Primordial germ cells do not migrate along nerve fibres in marmoset monkey and mouse embryos

Authors:

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Abstract:

Primordial germ cells (PGCs) are the embryonic precursors of spermatozoa and eggs. In mammals, PGCs arise early in embryonic development and migrate from their tissue of specification over a significant distance to reach their destinations, the genital ridges. However, the exact mechanism of translocation is still debated. A study on human embryos demonstrated a very close spatial association between migrating PGCs and developing peripheral nerves. Thus, it was proposed that peripheral nerves act as guiding structures for migrating PGCs. The goal of the present study is to test whether the association between nerves and PGCs may be a human-specific finding or whether this represents a general strategy to guide PGCs in mammals. Therefore, we investigated embryos of different developmental stages from the mouse and a non-human primate, the marmoset monkey (*Callithrix jacchus*), covering the phase from PGC emergence to their arrival in the gonadal ridge. Embryo sections were immunohistochemically co-stained for tubulin beta-3 chain (TUBB3) to visualize neurons and Octamer-binding protein 4 (OCT4) as marker for PGCs. The distance between PGCs and the nearest detectable neuron was measured. We discovered that in all embryos analysed of both species, the majority of PGCs (> 94 %) was found at a minimum distance of 50 µm to the closest neuron and, more importantly, that the PGCs had reached the gonads before any TUBB3 signal could be detected in the vicinity of the gonads. In conclusion, our data indicate that PGC migration along peripheral nerves is not a general mechanism in mammals.

Key words: embryonic development / marmoset monkey / primordial germ cell migration / gonad
Introduction:

Primordial germ cells (PGCs) are the undifferentiated embryonic precursors of sperm and egg cells and are fundamental in reproduction since they are the only cells during embryonic development able to relay their genetic information to the next generation. In mice, PGCs are specified via inducing signals from cells of the proximal posterior epiblast starting at embryonic day (E) 6.0 (Lawson et al., 1999), and an initial population of 30 – 50 PGCs expressing distinctive markers has formed in the extra-embryonic mesoderm by E 7.25 (Ginsburg et al., 1990). From there, they have to transit via the hindgut endoderm and the dorsal mesentery towards their destination, the urogenital ridges. Between E 9.5 and E 10.5 the PGCs reach the genital ridges developing laterally to the aorta where they form the embryonic gonads (McLaren, 2003). In primate embryos, PGCs seem to arise in the amnion (Sasaki et al., 2016) and they, too, translocate to the genital ridges via the dorsal wall of the yolk sac, the hindgut endoderm and the dorsal mesentery.

The exact mechanism of this translocation from their ventral extraembryonic tissue of specification towards the dorsal body wall of the developing embryo is still debated. Passive movement of the cells via morphogenetic changes of the surrounding tissues may play an important role in transporting the PGCs closer to their destination (Aeckerle et al., 2015, Freeman, 2003). However, in order to move from the hind- and midgut to the gonadal ridges, active PGC migration has to be involved. Images of human PGCs published by Politzer as early as 1933 show cytoplasmic protrusions on the cells, suggesting an amoeboid migratory movement (Politzer, 1933). (Molyneaux et al., 2001) tagged Oct4 with GFP in genetically modified mice and thus were able to visualise and take time-lapse videos of PGCs during the stages of their migration. They showed that the PGCs exhibit active locomotion; however this does not seem to be an intrinsically directed movement. Rather, it was proposed that the PGCs have to follow contact guidance clues or chemotactic signals in order to find the way to their
site of function. Factors proposed to be involved in chemotaxis of PGCs include tumour
growth factor-β (TGFβ) (Godin and Wylie, 1991), stromal-derived factor 1 (SDF1 or
CXCL12) (Bucay et al., 2009, Doitsidou et al., 2002) and stem cell factor (SCF; or kit ligand,
KL) (Farini et al., 2007, Hoyer et al., 2005).

It was first suggested by (Hoyer et al., 2005) that PGCs might be guided by nerve fibres, since
they found an association of human PGCs with autonomic nerve fibres of the dorsal
mesentery in a study on c-Kit and SCF distribution in human embryos. This hypothesis was
further investigated by (Mollgard et al., 2010). In 4 – 8 weeks post conception (pc) human
embryos they identified migrating PGCs and neurons and found that indeed, a large
proportion of PGCs were located within bundles of autonomic nerve fibres on their route from
the dorsal mesentery to the gonadal ridges. They also observed by immunohistochemical
marker staining that the innervation of the human gonadal ridges starts between 29 and 33
days pc, the same time that the first PGCs arrive at their destination. They concluded from
their data that in human embryos the PGCs preferentially follow peripheral autonomic nerve
fibres during their translocation from the dorsal mesentery to the gonads.

The aim of the present study was to investigate whether this method of PGC guidance and
transition could be observed also in other mammalian species and whether it might represent
an evolutionary conserved, i.e. general strategy of mammalian germ cell development. For
this purpose, we investigated 8 mouse embryos and 8 embryos of a non-human primate, the
common marmoset monkey (Callithrix jacchus), covering different developmental stages
from the emergence of the PGCs to their arrival at the genital ridges. Tissue sections were
immunohistochemically double-stained and morphometrically analysed with regard to the
spatial and temporal relationship of migrating PGCs to surrounding neurons.
Material and Methods:

Marmoset monkey embryos and post-natal testes:

All animal studies were performed in accordance with the German Animal Protection Law. The animals were obtained from the common marmoset (*Callithrix jacchus*) breeding colony of the German Primate Center (Deutsches Primatenzentrum, DPZ). Non-human primate husbandry at the German Primate Center including its institutional guidelines on housing and care of marmosets were approved by the local and regional veterinary governmental authorities (reference number 122910.3311900, PK Landkreis Göttingen). All animal work was performed by experienced veterinarians and trained staff in agreement with the requirements of the German animal protection law (Deutsches Tierschutzgesetz, §7). Animal experiments to obtain the marmoset embryo material used in this study were approved by the ethics committee of the animal welfare office of the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES), which is in charge of this approval, under license number 42502-04-12/0708. All parts of this study were conducted in full compliance with the German legislation requirements. Marmoset embryos of defined stages were obtained by hysterotomy as described previously (Aeckerle *et al.*, 2015). All surgical procedures on the animals including anaesthesia and analgesia were performed by a specialised and experienced veterinarian. Sections of embryos ranging from gestational day (GD) 65 to GD 90 were chosen, approximately representing the Carnegie stages 15-23 (Merker *et al.*, 1988, Phillips, 1976). Additionally, sections of three common marmoset foetuses of GD 100 and GD 117 from the tissue bank of the Platform Degenerative Diseases of the DPZ were analysed. A list of the specimens used in this study is given in Table I.

Post-natal common marmoset testes were also taken from the tissue bank of the Platform Degenerative Diseases of the DPZ. All fixed postnatal tissue samples were pre-existing and
not obtained specifically for this study, but were collected from animals that had to be sacrificed for unrelated purposes.

Mouse embryos:

Female CD1 mice were obtained from the animal facility of the European Neuroscience Institute (ENI; Göttingen, Germany), at a defined number of days after vaginal plug detection. The mice were sacrificed by cervical dislocation and the uterus removed for embryo collection. Embryos for this study were obtained at embryonic day (E) 8.5, E 9.5, E 10.5 and E 11.5, respectively. The list of samples can be found in Table II.

Tissue processing and immunohistochemical double-staining:

The embryos were fixed directly after retrieval in Bouin’s solution for 4 – 24 hours depending on the embryo size. Fixation was followed by several washing steps with 70 % EtOH over at least 2 days. Then tissues were embedded in paraffin and sectioned at 5 µm. The embryos were positioned in order to obtain either transversal or sagittal sections. Immunohistochemical double-staining was performed in a three-day process using the EnVision™ Doublestain System Kit by Dako (Code #K5361). Briefly, the slides were deparaffinised, rehydrated and an antigen retrieval was performed by heating the slides in 10 µM Na-Citrate buffer in the microwave for 10 minutes. Subsequently, endogenous phosphatase and peroxidase enzymes were blocked using the blocking agent provided in the kit. The first primary antibody directed against OCT4 in mice (Santa Cruz #sc-9081 (H-134), polyclonal rabbit IgG, diluted 1:150) and OCT4A in marmoset (Cell Signaling Technology #2890 (C52G3), monoclonal rabbit IgG, diluted 1:300) was incubated over night at 4°C and stained the next day using the 3,3’-diaminobenzidine (DAB+) chromogen. After washing and blocking with an agent to
prevent double staining of the same antigen, the slides were incubated with the second primary antibody against TUBB3 (Sigma-Aldrich #T8660, monoclonal mouse IgG, diluted 1:2000) over night at 4°C. All incubation steps were performed using a humidified chamber. On the third day, the second antibody-signal was visualised using PermanentRed chromogen. The slides were counter-stained for 10 – 15 seconds in Mayer’s Hematoxylin Solution. Control stainings using IgG antibodies at the same protein concentration as the primary antibodies were performed in order to exclude unspecific antibody binding. Images were taken using the Leica Aperio CS2 Digital Slide Scanner and morphometrically analysed with the Aperio ImageScope® software.
Results:

PGC localisation in the common marmoset monkey (*Callithrix jacchus*):

Eight common marmoset embryos of different developmental stages (Gestational day (GD) 65, 68, 72, 75, 90) were analysed in this study, and a total of 853 PGCs were counted on 30 histological sections.

The PGCs were first grouped according to the anatomical compartment they were found in (Figure 1). Looking at the total cells, approx. 3% of PGCs were detected in the epithelium of the gut, 12% either in the gut mesenchyme, the dorsal mesentery or the peri-aortic region and 14% of PGCs were attributed to the dorsal body wall. With 71% the majority of PGCs was located in the developing genital ridges, or the mesonephros and gonads in more developed embryos. Separating the sections according to the gestational day of the embryos, the proportion of PGCs that have reached the gonad expectedly increased from 13.7% on GD 65 over 47.7% on GD 72 to 93.3% on GD 90.

From each detected PGC the distance to the closest neuron was measured and the results divided into three groups: (I) Cells more than 50 µm away, (II) cells that were found between 50 – 20 µm from the nearest neuron and (III) cells with a distance of less than 20 µm.

The main finding of our examination was that for over 96% of the total detected PGCs no nerve cells could be detected within a distance of 50 µm. 1.64% of the total PGCs were found in the distance range of 50 – 20 µm and only 1.75% (15 cells total) less than 20 µm away. Of these 15 cells only two were found in direct contact with a neuron (Figure 2). Sub-dividing the sections according to gestational day does not reveal any differences in the PGC-neuron distance: at least 95.3% of PGCs are found at a distance > 50 µm (GD 72), 0.8 – 2.8% are found in the 50 – 20 µm distance range, and only max. 3.9% of PGCs could be detected less than 20 µm from the closest neuron (GD 72).
PGCs in the mouse embryo:

In the mouse, a total of 3482 PGCs were counted on 64 sections of 8 embryos of consecutive embryonic days (E 8.5 – E 11.5). Looking at mouse embryos provided the advantage of a more systematic investigation of PGC migration, as migration happens over a shorter time than in the marmoset. The developmental span of E 8.5 – E 11.5 covers almost the entire range of PGC migration. Additionally, it allowed us to better follow the neuronal development and draw conclusions on potential interactions with PGC migration. This observation could not be made in the marmoset embryos since the neuronal development (but not the PGC translocation!) in the earliest investigated stage (GD 65) had already progressed further than that in the oldest investigated mouse embryo (E 11.5).

Again, PGCs were first classified according to their tissue location (Figure 3). On E 8.5 98.25 % of PGCs were found either in the gut epithelium or the mesenchyme surrounding the gut. On E 9.5 the majority of PGCs (84.77 %) were migrating through the gut mesenchyme and the mesentery. 7.28 % were found in the gut epithelium and 7.95 % had already reached the location where the genital ridges started to form. By E 10.5 the genital ridges were clearly distinguishable from the surrounding tissue, and while they now harboured most of the detected PGCs (84.67 %), 9.17 % of PGCs were found in the gut mesenchyme, the mesentery or the region surrounding the aorta. By E 11.5 by far the most of the PGCs (> 90 %) had reached the developing gonads. The remaining cells were detected mostly in the peri-aortic region or the mesentery. These findings reflect the PGC migration / translocation process.

Approximately 2 % of total PGCs were also found at ectopic regions (regions that deviate from the “normal” migration route) such as the neural tube.
Measuring the distance of each PGC to the closest neuron revealed results similar to those obtained in the marmoset embryos (Figure 4): On E 8.5 only 0.88 % of cells were found at a distance of less than 20 µm from a neuron and 0.44 % in the distance range of 50 – 20 µm (2 and 1 cell in total, respectively). 98.68 % of cells were more than 50 µm away. This percentage even increased in the data obtained on E 9.5, where not a single PGC was detected within a 50 µm distance of a neuron. On E 10.5 and E 11.5 the results remained similar, with 98.12 % and 95.02 % of cells detected in the > 50 µm distance category, respectively. On E 11.5 the proportion of PGCs that were found closer to a neuron increased slightly, with 2.8 % and 2.9 % per category, respectively.

In the mouse embryos we observed a strong increase in PGC number between E 10.5 and E 11.5 (for comparison see Figure 3 C/D), which indicates the onset of intense PGC proliferation. Clusters of several PGCs can be found outside the gonad on E 11.5, whereas only single cells were observed on the days before.

Comparing neuronal development between mouse and marmoset embryos:

Figure 5 A-C exemplarily shows an E 10.5 mouse embryo double stained for TUBB3 and OCT4 as well as single stainings of the two markers. While OCT4-positive PGCs were clearly detectable in the gonad, no TUBB3 signal was visible in neither the gonad nor the adjacent tissue. Figure 5 D-G shows the neuronal development observed in mouse embryos by TUBB3-staining: On E 8.5, neurons were clearly visible in the developing brain and started to appear in the neural tube (Figure 5 D). The intensity of these signals increased on the E 9.5 embryos (Figure 5 E). Interestingly, a clear gradient of TUBB3-positive neurons in the neural tube from cranial to caudal was observed between those two embryonic days (data not shown). On E 9.5, first ganglia started to appear in the mesenchyme around the gut, which did
not show any histological signs of smooth muscle differentiation at this stage. While the signals in the aforementioned tissues became more pronounced on E 10.5, neurons also started to appear in the region around the aorta (Figure 5 F). This was best recognisable on the transversal embryo sections. Presence of neurons in the mesentery was not observed but cannot be excluded. Most important for this study was the finding that by E 11.5 there was still no innervation of the gonad detectable (Figure 5 C+G), although 90% of all PGCs were present in the gonad (Figure 3 E).

These findings regarding the spatio-temporal development of the peripheral nervous system in the mouse are different from the marmoset embryos, where at the earliest investigated stage (GD 65) the neuronal development had already progressed further than that in the latest mouse embryo. In the GD 65 marmoset embryos, TUBB3-staining gave a clear signal in the brain, neural tube, ganglia, ganglia of the gut, the gut epithelium and the gut mesenchyme (Figure 1 A). There was also a strong signal in the epithelia of the mesonephros. However, importantly, also in the marmoset embryos, there was no signal detectable in the gonads of even the oldest investigated embryo (GD 90, Figure 1 D), confirming the mouse data that PGCs are present in the developing gonad before any nerve fibres can be detected in the vicinity of the embryonic gonad.

Presence of neurons in the common marmoset monkey testis:

In order to answer the question at which point neurons do appear in the gonad, sections of common marmoset monkey testes of different foetal and post-natal stages were stained for OCT4A or TUBB3. The results are shown in Figure 6. At GD 100, the seminiferous tubules had already formed and the OCT4A-positive germ cells could be found enclosed within these tubules (Figure 6 A). While TUBB3 staining resulted in signals in the epithelium of the
developing epididymis, no TUBB3 was detected within the actual testis. Only some light pink background staining was seen (Figure 6 B). At GD 117, TUBB3 signal could be detected in the epithelium of the epididymis as well as in the enteric ganglia in the outer (muscle) layer of the developing gut (Figure 6 C), which was also observed in the younger embryonic stages (see Figure 1D for comparison). Additionally, at this stage nerves were detected in the forming Tunica albuginea, but again not within the testicular mesenchyme (Figure 6 D). In the neonatal and juvenile marmoset monkey testis, TUBB3 signal could be detected in the testis capsule and also sporadically within the testis itself, then often in close proximity to blood vessels forming a neurovascular pathway (Figure 6 E+F).
Discussion:

The current comparative analysis of PGC translocation in the marmoset embryo extends and quantitates previous observations in the marmoset embryo by (Aeckerle et al., 2015), which described a wide spatio-temporal (diffuse) distribution of PGCs over a large portion of the migration period. Furthermore, we included mouse embryos in this comparative analysis as a non-primate reference species. In contrast to the marmoset, in the mouse embryos the PGCs can be followed during their translocation almost like a regular wave or homogenous cohort of cells with predictable locations at different embryonic days.

As described in the results, the onset of intense PGC proliferation in the mouse embryo can be determined histologically by the appearance of clusters of multiple PGCs around the gonad on E 11.5, where at the same location only single cells could be observed at E 10.5. At the same time the proportion of PGCs found less than 50 µm away from the closest neuron increases from < 2 % on E 10.5 to approx. 5 % on E 11.5. This can be explained by the appearance of neurons in the peri-aortic space close to the gonads. Importantly, our data indicate that the neurons only appear in the vicinity of the gonad when the PGCs are already present in the gonad rather than the other way round.

Neither in the mouse nor in the marmoset embryos we detected any innervation of the gonad before the PGCs arrived there, which is in contrast to the observations in human embryos. This is probably the most important finding in this study, since it clearly speaks against the hypothesis of peripheral nerves acting as guiding structures for migrating PGCs – as it has been described for humans. Published data (Hoyer et al., 2005, Mollgard et al., 2010) and those presented here rather suggest that nerve fibre-mediated guidance of PGCs is not a conserved phenomenon in mammals and may have developed only very recently in primate evolution. In this context it is important to mention a study performed by (Sasaki et al., 2016) on cynomolgus monkey (Macaca fascicularis) embryos, which belong to the group of Old-
World monkeys and are therefore evolutionarily even closer related to humans. They investigated this question as well by double-staining of migratory PGCs and neurons and came to the same conclusion that no structural associations were detectable.

The question remained at which point, and if at all, neurons can be detected in the developing common marmoset monkey gonad. Since data on the innervation of the gonad is relatively scarce, we stained sections of common marmoset testes at different foetal and post-natal stages. We found that before birth, no neurons were detectable in the testis mesenchyme, which is in contrast to the reports in human embryos, and that the germ cells became incorporated into the developing seminiferous tubules around GD 100 or earlier. From this point onward, the spatial separation makes any direct physical influence of neurons on germ cell development very unlikely. But even in the post-natal testes up to the post-pubertal adult, nerves within the testis mesenchyme were rare.

The markers for the visualisation of PGCs (OCT4) and neurons (TUBB3) were chosen carefully. Most importantly, the same markers were used in the human embryo study by (Mollgard et al., 2010) as well as the study in cynomolgus monkey embryos (Sasaki et al., 2016), facilitating comparison between the different species. Even if it is not shown again in this manuscript, a previous study in marmoset embryos tested several potential germ cell markers and found OCT4A to reliably identify PGCs (Aeckerle et al., 2015). In the study on human embryos by (Mollgard et al., 2010), TUBB3 was shown to reliably detect neurons even in the earliest developmental stages. In our study, using TUBB3 resulted in high-quality immunohistochemical stainings with very sensitive and clear signals in both investigated species. In fact, the TUBB3 protein sequence between human and marmoset TUBB3 is 99.8% (449/450 amino acids) identical (BLASTP alignment of human TUBB3 sequence (Transcript ID ENST00000315491.11) with marmoset sequence (Transcript ID ENSCJAT0000009328.3) using Ensembl database.
(https://www.ensembl.org/Multi/Tools/Blast?db=core)), further substantiating the comparability of our data and the published data (Mollgard et al., 2010). We therefore feel confident that our selected markers and antibodies are well suited to answer the study question.

One could argue that inclusion of the PGCs that are already found in the gonads into the analysis may bias the results, since they make up a considerable percentage of total cells but are no longer migratory. However, looking at the individual developmental days in both marmoset and mouse embryos, we see no difference in the number of cells that are closely associated with neurons, independent of the PGCs’ localisation. For example, on mouse embryonic day 9.5, ~ 92% of PGCs are still migratory outside the gonad, and not a single PGC was detected close to a neuron (see figures 3E and 4C). On marmoset gestational day 65, only approx. 14% of PGCs have reached the gonads, but more than 96% of total cells were found in the > 50 µm-distance category (see figures 1E and 2B). From this, we can only draw the conclusion that our data set suitably answers the study question, and that PGC migration and translocation in the marmoset and mouse embryo are not dependent on peripheral nerve guidance to reach the gonad.

We are aware of the limitations of histological sections as they only allow two-dimensional analysis. A closer association between PGCs and nerve cells in the third dimension cannot be completely excluded. In order to minimize this problem, consecutive sections of the same embryos were analysed to obtain a better spatial resolution. However, to analyse in detail and finally prove the spatial relationships between peripheral nerves and PGCs it would be favourable to have a three-dimensional representation of the tissues of interest.

Using this visualisation method, the possibility that the TUBB3 signal intensity lies under the detection threshold, and present neurons might therefore not be visible, can also not be excluded. However, we think this is unlikely since, again, the findings in the human embryos
were obtained by the same method and Møllgard and colleagues describe clearly detectable nerve fibres (Mollgard et al., 2010).

In summary, no spatial association between PGC migration and nerve fibers could be observed, neither in mice nor in two non-human primate species. This finding falsifies our hypothesis that PGC guidance by developing peripheral nerves is a conserved mechanism in mammalian embryonic development. When we compare our findings to those observed in the human embryos, we conclude that the observations by Møllgard et al. – other than representing a general mammalian strategy – rather reflect a species-specific trait of human PGC development.
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Author contributions:

EW: conduction of experiments and analysis of results, graphical representation of data, writing of manuscript.

MS: conduction of experiments and analysis of results. Final approval of manuscript.

RB: study design, financial support, correction and approval of manuscript.

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All authors declare no conflict of interest.
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Figure Legends:

**Figure 1** PGC locations in the common marmoset monkey (*Callithrix jacchus*) embryo.

Immunohistochemical double staining of OCT4A (brown) and TUBB3 (pink) (A-D). 

- **A)** Representative sagittal overview section of a GD 65 embryo. 
- **B)** Higher magnification of area of prospective gonad in A. 
- **C)** Sagittal section of a GD 65 embryo (different than shown in A). Black arrows highlight migrating PGCs in the gut mesenchyme. 
- **D)** Developing gonad in a GD 90 embryo containing post-migratory PGCs. 


**E)** Graphical representation of tissue locations of PGCs in all investigated slides, separately analysed for each gestational day, $n =$ number of cells available for analysis.

**Figure 2** PGC distance to closest neuron in the common marmoset monkey (*Callithrix jacchus*) embryo. 

- **A)** Sagittal section of a GD 72 marmoset monkey embryo. 
- **B)** Graphical representation of the distance between the observed PGCs and the respectively closest detectable neuron, separately analysed for each gestational day, $n =$ number of cells available for analysis.

**Figure 3** PGC tissue locations in the mouse embryo. 

- **A-D)** Sections of different embryonic stages immunohistochemically stained for OCT4. 
- **A)** Representative transversal section of an E 8.5 embryo. 
- **B)** Representative sagittal section of an E 9.5 embryo. 

*Downloaded from Bioscientifica.com at 12/02/2018 04:41:40AM via free access*
transversal section of an E 10.5 embryo \textbf{D}) Representative transversal section of an E 11.5 embryo. A: dorsal aorta, Gl: gut lumen, M: mesenchyme, Mes: mesentery, Nt: neural tube. The asterisks mark the developing genital ridge. PGCs are highlighted and colour-coded according to the tissue they were detected in. Scale bar \( \pm \) 90 \( \mu \)m (B), 200 \( \mu \)m (A, C, D). \textbf{E}) Graphical representation of tissue locations of PGCs in all investigated slides, separately analysed for each embryonic day, \( n = \) number of cells available for analysis.

\textbf{Figure 4} PGC distance to closest neuron in the mouse embryo. \textbf{A+B}) Sagittal and transversal section of E11.5 mouse embryos immunohistochemically double stained for OCT4 (brown) and TUBB3 (pink). Exemplary distance measurements between representative PGCs and the respectively closest detectable neuron. Scale bar \( \pm \) 80 \( \mu \)m (A), 60 \( \mu \)m (B). Asterisk marks the prospective gonad. PGCs are highlighted and colour-coded according to the tissue they were detected in. \textbf{C}) Graphical representation of the distance between the observed PGCs and the respectively closest detectable neuron, separately analysed for each embryonic day, \( n = \) number of cells available for analysis.

\textbf{Figure 5} Neuronal development observed in the mouse embryo. \textbf{A}) Representative sagittal overview section of an E 10.5 embryo immunohistochemically double stained for OCT4 (brown) and TUBB3 (pink). Scale bar \( \pm \) 2 mm. \textbf{B+C}) Higher magnification of the area highlighted in A), immunohistochemically stained for OCT4 (B) or TUBB3 (C). Scale bar \( \pm \) 300 \( \mu \)m. \textbf{D-G}) Sections of different embryonic stages immunohistochemically stained for TUBB3. Scale bar \( \pm \) 200 \( \mu \)m. \textbf{D}) Representative transversal section of an E 8.5 embryo. \textbf{E}) Representative transversal section of an E 9.5 embryo. \textbf{F}) Representative transversal section of an E 10.5 embryo. \textbf{G}) Representative transversal section of an E 11.5 embryo. A: dorsal aorta, Bv: brain vesicle, Gl: gut lumen, H: heart, L: liver, Nl: neural lumen, Nt: neural tube.
The asterisks mark the developing genital ridge. Pink circles highlight appearing neurons. **H)**

Table shows the observed TUBB3 signal intensity in different tissues at different developmental stages in the mouse embryo.

**Figure 6** Innervation of the common marmoset monkey (*Callithrix jacchus*) testis.

Immunohistochemical staining for OCT4A (brown, A) or TUBB3 (pink, B-F). **A+B)**

Transversal section of testis and epididymis of a GD 100 marmoset foetus. **C)** Transversal section of a GD 117 marmoset foetus. **D)** Higher magnification of a GD 117 marmoset foetal testis. Erythrocytes present in the blood vessels unspecifically bind the antibody thereby producing a false-positive signal. **E)** Section of a marmoset monkey neonatal testis. **F)**

Section of a marmoset monkey juvenile testis. T: testis, Epi: Epididymis, Gt: gut. The asterisks exemplarily mark seminiferous tubules. Pink arrows highlight neurons. Scale bar $\equiv 50 \, \mu m$. 
148x248mm (150 x 150 DPI)
A) GD 100

B) GD 100

C) GD 117

D) GD 117

E) Neonatal

F) Juvenile

189x238mm (150 x 150 DPI)
Table I – Marmoset monkey (*Callithrix jacchus*) embryos / foetuses used in this study.

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<th>Embryo</th>
<th>GD</th>
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<th>BPD (mm)</th>
<th>FROD (mm)</th>
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<td>Sagittal</td>
</tr>
<tr>
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<td>N/A</td>
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</tr>
<tr>
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<td>11.9</td>
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</tr>
<tr>
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<td>100*</td>
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</tr>
<tr>
<td>10</td>
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<td>N/A</td>
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<td>Transversal</td>
</tr>
<tr>
<td>11</td>
<td>117*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Male</td>
<td>Transversal</td>
</tr>
</tbody>
</table>

GD: Gestational day; CRL: Crown-rump-length; BPD: biparietal diameter; FROD: fronto-occipital diameter; N/A: Not available, *Gestational day calculated from delivery of the previous litter.
Table II – Mouse embryos used in this study.

<table>
<thead>
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<th>Embryo</th>
<th>E</th>
<th>CRL (mm)</th>
<th>Sex</th>
<th>Sectioning plane</th>
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<td>N/A</td>
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<td>2.9</td>
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<td>11.5</td>
<td>N/A</td>
<td>Male</td>
<td>Transversal</td>
</tr>
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</table>

E: Embryonic day; CRL: Crown-rump-length; N/A: Not available.