Stem Cell Reports

Supplemental Information

Monosynaptic Tracing using Modified Rabies Virus Reveals Early and Extensive Circuit Integration of Human Embryonic Stem Cell-Derived Neurons

Shane Grealish, Andreas Heuer, Tiago Cardoso, Agnete Kirkeby, Marie Jönsson, Jenny Johansson, Anders Björklund, Johan Jakobsson, and Malin Parmar

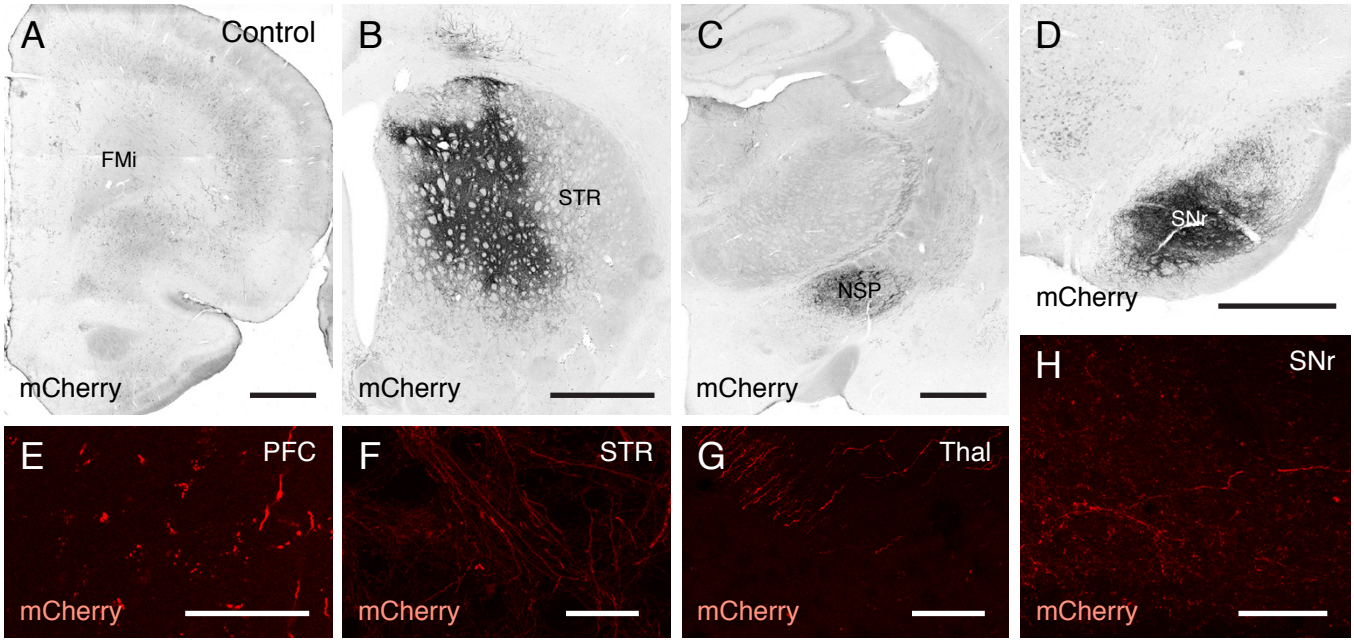

Figure S1. ***In vivo*** **testing of control lentiviral constructs.**

Related to Figure 1

(A-B) DAB immunohistochemistry for mCherry revealed that host neurons of the infected striatum (STR) had mCherry$^+$ fibres extending rostrally to the prefrontal cortex (PFC) (n=6). **(C, D)** At sites distal to the injection, only mCherry$^+$ fibres and no cell bodies, could be observed. The fibres observed included a bundle of mCherry$^+$ fibres extended along the nigrostriatal pathway (NSP), giving rise to a terminal network in the substantia nigra pars reticulata (SNr). **(E-H)** At no anatomical level throughout the brain was a single traced neuron observed in any animal, confirming the specificity of the ΔG-rabies and also that the presence of rabies glycoprotein is necessary for monosynaptic tracing events to occur.

Scale bars: **(A-D)** = 1 mm; **(E-H)** = 100 μm

Grealish et al. **Figure S2.**

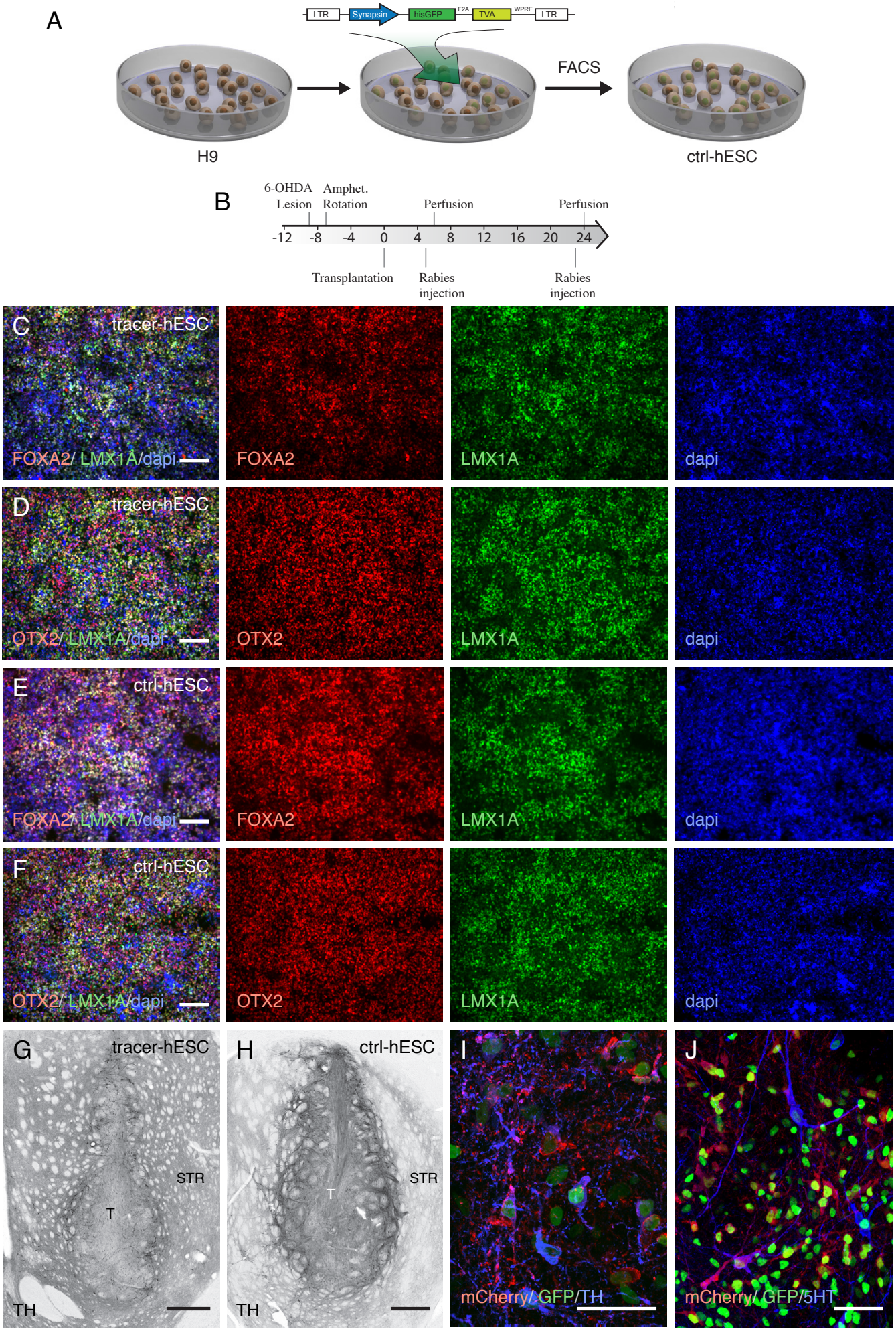

Figure S2. *In vitro* and *in vivo* validation of transgenic tracer- and control-hESCs.

Related to Figure 2

(A) Schematic illustration of generation of control hESCs, and **(B)** time-line of the host-to-graft experiment. **(C-F)** Both tracer and control hESCs could efficiently be patterned into midbrain identity as detected by co-expression of FOXA2, LMX1a and OTX2. **(G)** Grafts of tracer (n=6) and control (n=6) hESCs gave rise to a transplant (T) rich in TH^+ dopaminergic neurons. **(I)** Many of the GFP/mCherry expressing starter neurons expressed TH **(J)** but very few expressed 5HT.

Scale bars: **(B-E)** = 100 μm; **(F-I)** = 500 μm.

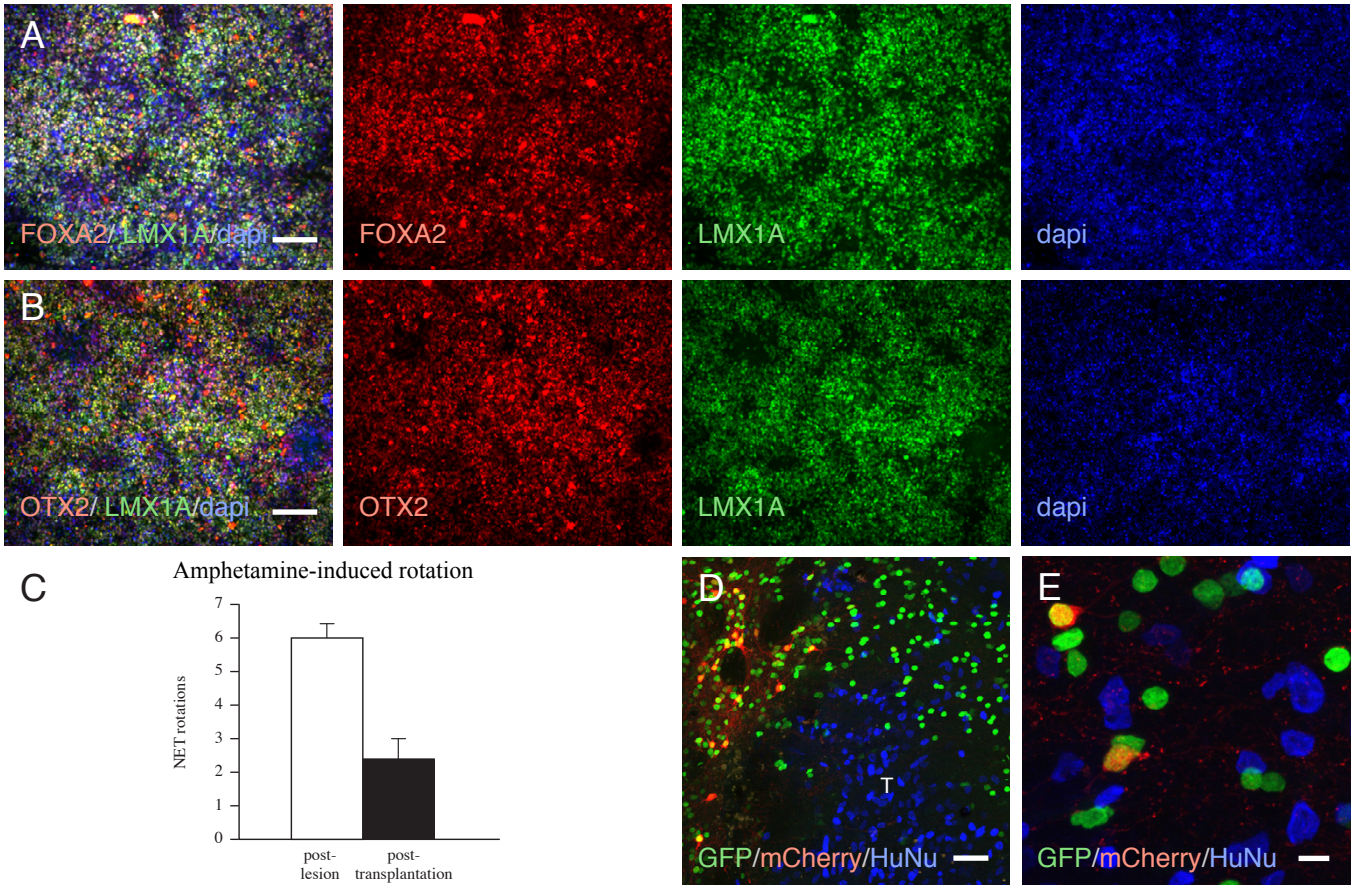

Figure S3. ***In vitro*** **midbrain patterning of wildtype hESCs used in graft to host experiments.**

Related to Figure 4

(A, B) Wild type hESCs were efficiently patterned towards midbrain identity as detected by co-expression of FOXA2, LMX1A and OTX2. **(C)** Amphetamine-induced rotation showed that the transplants analyzed in this study were functional, when assessed 6 months after transplantation (n= 5 rats; mean ± SEM; $p < 0.01$ compared to post-lesion; two-tailed paired t-test). **(D)** In animals receiving the control lentiviral vector in the striatum (n=4), no tracing events within the transplant could be observed. **(E)** Any mCherry^{+} neurons present were at the perimeter of the HuNu+ transplant, and none of the HuNu^{+} cells co-expressed GFP or mCherry.

Scale bars: **(A-B)** = 1 mm; **(D)** = 100 µm; **(E)** = 10 µm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

hESC culture, transgenesis and differentiation

Human ESCs H9 (WA09, passage 31-40 from WiCell) were maintained on γ-irradiated mouse embryonic fibroblasts in DMEM/F12, 20% KSR, 0.05 mM 2-mercaptoethanol, 0.5% Pen/Strep and 10 ng/mL FGF-2 (R&D Systems), and passaged using EDTA. For production of tracer and control cell lines, cells were transduced at a multiplicity of infection (MOI) of 30 with lentiviral particles of the Syn_HisGFP_TVA and the Syn_HisGFP_TVA_Gp constructs, respectively. We enriched transgenic cells in the population based on weak leakage of GFP from the synapsin promoter in undifferentiated cells. Cells with the strongest fluorescence in the GFP channel (gating 30-40% of total population) were sorted out and used for continued experiments. Cells were differentiated towards a VM phenotype using a previously described protocol (Kirkeby et al., 2012). Briefly, for patterning towards a VM fate 10 μM SB431542, 100 ng/ml rhNoggin, 200 ng/ml Shh-C24II and 0.7 μM CHIR99021 was added to the culture medium from day 0-9 of differentiation. From day 11 of differentiation, BDNF (20 ng/ml), GDNF (10 ng/ml) and ascorbic acid (200 μM) was added to the medium, and on day 16, cells were dissociated with accutase and resuspended in HBSS + 0.05% DNAse to a concentration of 75,000 cells/μl for transplantation.

Surgical Procedures

All surgical procedures were done under general anaesthesia using a fentanyl and medetomidine (20:1) solution injected intraperitoneally. All rats received a unilateral 6-hydroxydopamine lesion of the right medial forebrain bundle with a volume of 4 µl, at a freebase concentration of 3.5µg/µl, to the following co-ordinates relative to bregma: A/P -4.4; M/L -1.2; D/V (from dura) -7.8; tooth bar -2.4. Two weeks later animals were screened for lesion success using amphetamine-induced rotations. Briefly, the rats were injected with 2.5 mg/kg, i.p. (Apoteksbolaget, Sweden) and recorded using an automated rotometer system (Omnitech Electronic Inc., USA). The animals were recorded for 90 minutes, and only full body turns were counted and then expressed as net turns per minute, with rotations towards the side of the lesion given a positive value. All rats were stratified across the experimental groups.

For the host to graft experiment the host rats were grafted with a total of 300 000 modified hESCs at day 16 of differentiation towards a ventral midbrain fate of either control or tracing lines to the striatum in a volume of 2 µl, at a concentration of 75 000 cells/µl, to each of the following co-ordinates relative to bregma: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. One week prior to termination, the rats underwent injection of ΔG-rabies at a dilution of 5% of the stock at two sites (one within and one adjacent to each graft site) with 2 deposits at each site. Each deposit consisted of a volume of 1µl injected at a rate of 0.5 µl per minute and a diffusion time of 2 minutes, delivered at the following co-ordinates: (1) A/P +1.2; M/L -2.6 and -3.0; and (2) A/P +0.5; M/L -3.0 and -2.6; D/V (from dura) -4.0 and -5.0; tooth bar -2.4.

For the graft to host experiment the rats were allocated to receive either control or tracing lenitvector into the striatum or PFC 4 weeks prior to transplantation. To target the striatum 2 µl per site of lentivector was injected into the following co-ordinates: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. To target the PFC 1 µl per deposit was injected at: (1) A/P +3.5; M/L -0.8; D/V -2.5; and (2) A/P +2.5; M/L -0.8; D/V -3.7; tooth bar -4. After 4 weeks, all hosts received and intra-striatal transplants of wild-type hESCs at day 16 of differentiation patterned towards a midbrain fate. 300 000 cells were grafted in a volume of 2 µl, at a concentration of 75 000 cells/µl, to each of the following co-ordinates relative to bregma: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. One week prior to perfusion the rats underwent injection of ΔG-rabies at a dilution of 5% of the stock at sites flanking the original deposits of lenitvector. For the striatum, each deposit consisted of a volume of 0.75 µl injected at a rate of 0.2 µl per minute and a diffusion time of 2 minutes, delivered at the following co-ordinates: (1) A/P +1.5; M/L -2.6; (2 and 3) A/P +1.2; M/L -2.2 and -3.0; (4 and 5) A/P +0.5; M/L -3.3 and -2.6; (6) A/P 0.0; M/L -2.6; (for all sites) D/V (from dura) -4.0 and -5.0; tooth bar -2.4.For the PFC each deposit consisted of a volume of 0.5 µl injected at a rate of 0.2 µl per minute and a diffusion time of 2 minutes at the following co-ordinates: (1) A/P +3.5; M/L -0.8; D/V -3.7 and -2.5; (2) A/P +3.0; M/L -0.8; D/V -3.7 and -2.5; (3) A/P +2.5; M/L -0.8; D/V -3.7 and -2.5; tooth bar -4.

Immunohistochemistry

All procedures and protocols were performed as detailed in (Grealish et al., 2014). All animals were transcardially exsanguinated with physiological saline solution before perfusion with fresh, ice-cold 4% paraformaldehyde. The brains were extracted and post-fixed for 2 hours, before cryopreservation in 25% sucrose solution prepared using PBS. The brains were sectioned on a freezing microtome in a 1:8 series at a thickness of 35 μm.

For this study all primary antibodies were used as follows: rabbit anti-5-HT (1:10 000; Incastar 20080); rabbit anti-DARPP32 (1:4 000; Santa Cruz SC11365); chicken anti-GFP (1:1 000; Abcam ab13970); goat anti-FOXA2 (1:500; Santa Cruz M-20); mouse anti-human NCAM (1:1 000; Santa Cruz Eric-1); mouse anti-HuNu (1:200; Millipore MAB1281); rabbit anti-LMX1A (1:5 000; Millipore ab10533); mouse anti-mCherry (1:1 000; Abcam ab65856); goat anti-OTX2 (1:1 000; R&D AF1979); chicken anti-TBR-1 (1:1 000; Millipore AB2261); mouse anti-tyrosine hydroxylase (1:5 000; Immunostar); rabbit anti-tyrosine hydroxylase (1:1 000; Millipore ab152).

Quantifications

To assess the relative proportion of dopaminergic and serotonergic starter neurons one anterior section and one section of the graft core was stained for mCherry, GFP and TH or mCherry, respectively. For each rat (n=7) these two sections were imaged at x20 magnification using a Leica DMI6000B microscope and all single mCherry, all single TH, as well as all co-expressing mCherry/TH expressing cells were counted off-line using Adobe Photoshop. To quantify the number of traced neurons in different host structures, all coronal sections (1:8 series) stained for mCherry were imaged at x20 magnification using a Leica DMI6000B microscope. Using Adobe Photoshop all sections were scanned visually for tracing events, which were recorded and registered. Coronal sections with tracing events were overlaid with the appropriate coronal image from the Paxinos and Watson rat brain atlas and the location of the traced cells was registered in the respective structure.

REFERENCES

Kirkeby, A., Nelander, J., and Parmar, M. (2012). Generating regionalized neuronal cells from pluripotency, a step-by-step protocol. Frontiers in cellular neuroscience *6*, 64.