**Transplanted embryonic neurons integrate into adult neocortical circuits**

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**Summary**

The capacity of the adult mammalian brain to compensate for neuronal loss caused by injury or disease is very limited. Transplantation aims to replace lost neurons, but it is not known to which extent new neurons can integrate into existing circuits. Using chronic *in vivo* two-photon imaging we show here that embryonic neurons transplanted into the visual cortex of adult mice mature into bona-fide pyramidal cells with selective pruning of basal dendrites achieving adult-like densities of dendritic spines and axonal boutons within 4-8 weeks. Monosynaptic tracing reveals that grafted neurons receive area-specific, afferent projections matching those of pyramidal neurons in the normal visual cortex. We further show that stimulus selective responses refine over the course of many weeks and finally become indistinguishable from those of lost neurons. Thus, grafted neurons can integrate with great specificity into neocortical circuits, which normally never harbor new neurons in the adult brain.

**Main Text**

Neuronal cell loss after brain injury or in the course of neurodegenerative disease cannot be repaired, despite some promising results involving the transplantation of cells from various sources1,2. However, it is not known to which extent transplanted neurons can replace lost neurons by adequate integration into the existing circuits. Transplantations have been successful in improving clinical symptoms e.g. in patients with Parkinson’s Disease, but cells, typically derived from human fetal ventral mesencephalic tissue, are transplanted into an ectopic location, the striatum 3. Accordingly, the crucial question of functional replacement of lost neurons by adequate integration could not be answered in this paradigm where transplanted neurons receive connections appropriate for their new location4. True functional replacement of lost neurons demands the proper integration of new neurons into pre-existing circuits at homotopic sites, a process which is still largely unexplored. It has previously been shown that transplanted neurons send out correct efferent projections and form synapses with host neurons5-7, but very little is known about the inputs onto transplanted neurons4,8 and their participation in the specific processing tasks that the respective brain regions perform.

We therefore investigated the integration of transplanted neurons into the mammalian cerebral cortex, a brain region in which the neural circuitry is relatively well understood. Specifically, we chose to study layer 2/3 of the primary visual cortex (V1), where pyramidal cells are a main integration site of visual inputs. These neurons are characterized by a set of key receptive field properties9, making them ideally suited to study the proper functional integration of transplanted neurons on a cellular level. To ablate specifically upper layer pyramidal neurons we used a previously established selective lesion method10 and transplanted neurons from the late embryonic cortex into the affected area. We show that these cells not only survive but differentiate into upper layer pyramidal neurons, receive appropriate synaptic input and develop receptive field properties indistinguishable from those of lost neurons. Thus, the adult mammalian brain retains the capacity to replace lost neurons, and new neurons supplied to the brain are able to functionally integrate into existing neuronal circuits.

*Grafted neurons have the layer identity of lost neurons*

In order to investigate the integration of neurons into injured brain circuits we ablated upper layer projection neurons in V1 by retrogradely labeling callosal projection neurons with chlorine e6 (Ce6) coupled latex beads (Fig. 1a, b). Subsequent laser-photoactivation induced apoptotic cell death10 in Ce6+ upper layer neurons11 (Fig. 1a, c; see Methods). Seven to ten days after photoactivation we transplanted donor cells from the mouse embryonic neocortex genetically labeled with a fluorescent protein (see Methods and Extended Data Fig. 1) into the area depleted of projection neurons (Fig. 1a, d; Extended Data Fig. 2a). By four weeks post transplantation (wpt) the overwhelming majority of new neurons had acquired morphologies typical of mature pyramidal cells in L2/312,13 (Fig. 2a-b; Extended Data Fig. 2b, c). Most cells remained restricted to the site of transplantation (Fig. 1d) and the majority (77%, 200 cells, n=2 mice) of fluorescent, transplanted neurons, displayed upper layer identity as demonstrated by Cux1 immunoreactivity (Fig. 1e). To test for cell fusion between transplanted and host neurons14,15, Emx1-Cre driven GFP+ donor cells16 were grafted into a tdTomato reporter mouse line17 (Extended Data Fig. 3a). The complete absence of GFP+/tdTomato+ cells (n=10 mice, Extended Data Fig. 3b) and the intricate morphological development of transplanted neurons over time (see below) shows that cell fusion does not occur in our experimental paradigm and is thus not a confounding factor.

*Structural maturation of transplanted neurons*

In order to follow the development and integration of individual transplanted neurons we used long-term *in vivo* two-photon imaging through a cranial glass window implanted on top of V118 (Extended Data Fig. 2a, b). We examined how neurite morphogenesis of transplanted neurons proceeds in the cerebral cortex, a region of the adult brain that normally does not generate and integrate new neurons. The early development of individual transplanted neurons was followed *in vivo* at short imaging intervals (2-5 d) within the first 4 wpt and at weekly intervals thereafter (Fig. 1a, 2c-f, see Methods). Already at 4 wpt, many transplanted neurons reached a fully mature pyramidal cell-like morphology (Fig. 2a, b; Extended Data Fig. 2c). Notably, neurons that had survived the initial phase of transplantation and had extended dendrites by 12 dpt were present until the end of the imaging period (97% after 2 months, 8 mice; 94% after 11 months, 2 mice; Extended Data Fig. 4d).

Already by 3 to 4 dpt neurons extended hundreds of µm of branched neurites, some clearly identifiable as axons, with growth cones at their tips (Fig. 2c, d). As early as 5 to 6 dpt a short, main apical dendrite featured primary and secondary branch points, outlining the subsequent, more sophisticated structure of the apical dendritic tree (Fig. 2c, f). Thus, neuronal polarity is unequivocally established by 6 dpt. Until the end of the second week, apical dendrites grew to their full length and formed only few additional secondary or tertiary branches (Fig. 2f). In contrast, the initial neurites that would later form the basal dendrites underwent massive rearrangements that lasted until the fourth wpt (Fig. 2e). Structural development of the basal dendrites included pruning as well as *de novo* growth of processes. Thus, also for transplanted neurons the maturation of the apical dendritic tree precedes the formation of basal dendrites, similar to the normal development of pyramidal cells12. Dendritic structural development is completed by 4 wpt, and the dendritic tree remained stable until the end of the imaging period.

*Formation of dendritic spines and axonal boutons*

The elaborate morphology of dendritic arbors as well as the long-term survival of transplanted neurons suggests their stable integration at the synaptic level. As dendritic spines and axonal boutons are generally regarded as reliable structural correlates of synapses, we selected distinct dendrites and axons to follow the formation, development and dynamics of individual synaptic structures within days after transplantation and up to 11 months thereafter (Fig. 1a; Fig. 3; see Methods). Axonal boutons were first detected as early as 3 dpt (Extended Data Fig. 4b) and were able to form within a few µm of the growth cone (Extended Data Fig. 4c). In contrast, dendritic spines were not detected before 6 dpt and usually formed in the second week on pre-grown, arborized dendrites (Extended Data Fig. 4a). Spine and bouton density increased massively up to 4 wpt, and subsequently reached a plateau (16 dendrites, n=5 mice: 1.38 ±0.17 µm-1; 33 axons, n=6 mice: 0.23 ±0.016 µm-1 at 4 wpt; Fig. 3d, e). Accordingly, the high initial turnover rates, largely based on high fractional gain of new structures and an elevated, but less pronounced loss, decreased up to 4 wpt (Fig. 3f, g). Early-formed spines and boutons have a considerably higher probability of elimination compared to synaptic structures formed at 4 to 9 wpt (spines: 1.5 - 2.4 times; boutons: 2 - 8 times increased chance of elimination; Fig. 3h-i; Extended Data Fig. 5). Turnover rates only stabilized after a subsequent period of transient increase between 5 and 8 wpt (one-way RM-ANOVA 4-10 wpt, p<0.0001; Tukey posttests; 2493 individual spines, 1600 individual boutons) at a level of <15% (spines: 0.125 ±0.013; boutons 0.139 ±0.018; both 10 wpt; Fig. 3f, g). Altogether, by 8-10 wpt transplanted neurons have developed largely stable and persistent pre- and postsynaptic structures. Notably, spine densities and turnover rates at later stages (>8 wpt) are comparable to the previously reported numbers for L2/3 pyramidal cells in the visual cortex of young adult and adult mice19.

*Formation of appropriate afferent connectivity*

As the above results show that transplanted neurons acquire upper layer pyramidal cell morphology with largely stable synaptic structures over time, we next examined their brain-wide synaptic inputs using modified rabies virus (RABV)-based monosynaptic tracing20. Cells from E18 neocortex or E14/E15 primary cortex cultures (Extended Data Fig. 1) were transduced with a receptor that renders them to be selectively infected by the (pseudotyped) rabies virus and a glycoprotein that allows its propagation20,21 (see construct depicted in Fig. 4a, left; red fluorescent labeling) and then transplanted into lesioned V1. Rabies virus (RABV; construct Fig. 4a, right; green fluorescent labeling) was injected into the transplantation site 4 or 12 weeks later, and labeled neurons were examined after 1 week. Starter neurons (RFP+/GFP+) were located exclusively within the transplant (Fig. 4c, d) and were surrounded by a large number of GFP-only, monosynaptic input neurons (Fig. 4c).

Brain-wide analysis of RABV-traced circuits at 4 wpt revealed 20 anatomical regions containing neurons that formed afferent connections with transplanted neurons (Fig. 4e; Extended Data Table 1 for abbreviations). The visual cortex showed the highest number of input neurons and thus the highest connectivity ratio (see Methods; 21.49 ±5.08, n=6 mice; Fig. 4f; see also Extended Data Fig. 6a, b). Thus, transplanted neurons receive their strongest input from neighboring neurons (Extended Data Fig. 6c) as is the case also in the endogenous circuitry.

Interareal connectivity included afferents from ipsilateral cortical areas like other sensory, motor and association areas, as well as long-range connections from subcortical nuclei and contralateral cortical areas (Fig. 4e-g). Importantly, all regions providing input to transplanted neurons are also known to project to V1 normally22-24, and no aberrant input could be detected in the brain-wide analysis of monosynaptic input to neurons transplanted into lesioned V1. For instance, we observed strong innervation from the dLGN, which relays information from the retina, and from higher-order integrative cortical areas, such as the retrosplenial cortex (RS) and the posterior parietal association area (PtPa). Connectivity ratios for these areas were several orders lower than local connectivity, but well comparable to endogenous neurons, whose monosynaptic input was determined by transducing them during development with the vector required for later monosynaptic tracing (Fig. 4f, Extended Data Fig. 8). Remarkably, the connectivity ratio calculated by dividing the number of monosynaptically connected neurons in a given region by the number of starter neurons amongst the transplanted neurons in the respective brain was found to be overall rather similar between transplanted and endogenous neurons. This was also the case for areas with rather weak input (Fig. 4f), many of which could only be revealed in mice with a sufficient number of starter neurons (typically more than 20 starter neurons, see Extended Data Fig. 7a). Overall, we found similar brain-wide patterns of connectivity at 4 and 12 wpt, with only a few areas revealed solely at 12 wpt (n=6 mice; Fig. 4g; Extended Data Fig. 7). This was the case for the claustrum, which is known to project to the visual cortex25, the pons, previously shown to innervate the visual cortex26, and the dorsal striatum, a connection reported before27 and in 3 different experiments in the Allen Mouse Brain Connectivity Atlas (see Fig.4, legend). Taken together, the monosynaptic input to transplanted neurons in adult injured V1 closely resembles the input to endogenous neurons generated during development in this area, even at a quantitative level. The presence of virtually all expected inputs to transplanted neurons is a prerequisite for their engagement in the processing of visual information.

*Transplanted neurons process visual inputs*

V1 is the first major integration site for visual information. Specific stimulus features, for instance the orientation and movement direction of edges and bars, are encoded at the level of single cells9,28. Thus, specific and selective responses of transplanted neurons to such stimulus attributes would be highly indicative of the correct functional integration and, ultimately, the ability of transplanted neurons to replace lost ones in an adult network.

In order to investigate whether transplanted neurons take part in visual information processing, we selectively labeled embryonic neurons with a red fluorescent structural marker (tdTomato) and a genetically encoded calcium indicator (GECI, GCaMP6s29 or Twitch2B30 in a Cre-dependent manner (Fig. 5a; see Methods). At 4 to 15 wpt, we presented full field gratings moving in 8 different directions to the eye contralateral of the imaged cortical region in anaesthetized mice. We recorded the responses of individual neurons using *in vivo* two-photon imaging (Fig. 5b; see Methods) by measuring the average change in GECI fluorescence relative to baseline during visual stimulation of both, somata and axons, and, in some instances, also dendritic spines (Fig. 5c-g; Extended Data Fig. 9; see Methods).

Almost all observed neurons with significant changes in fluorescence (ANOVA p<0.05; Fig. 5d) exhibited stimulus-evoked responses (dF/F >3**σ** or dR/R >0.05, 27/28 cells, n=5 mice; Fig. 5e) and, approximately half of the visually responsive neurons showed strong orientation and/or direction preference (13/27 cells, n=5 mice; Fig. 5f-g). Notably, many neurons displayed relatively sharp tuning properties typical for excitatory projection neurons in L2/3 of adult mouse V19 (Fig. 5f-g). In some instances we were able to record specific, stimulus-driven input on the level of single dendritic spines (Fig. 5c-g, bottom row). Consistent with transplantations into the binocular region of V1, we found individual neurons that were responsive to visual stimulation of both, the ipsi- and contralateral eye (Extended Data Fig. 10a).

Transplanted neurons responsive to visual stimulation exhibited some degree of orientation and/or direction preference as early as 5 wpt (Fig. 6a). However, the individual preferred direction and tuning profile of many repeatedly imaged neurons varied markedly over subsequent time points (9/13 cells, n=4 mice; Fig. 6a, d), indicating ongoing functional integration. It is not before 10 wpt that the tuning of transplanted neurons finally stabilized, with almost no changes in preferred orientation (Fig. 6a, b, d). Notably, stable tuning closely correlates to the final stabilization of synaptic turnover rates (>8 wpt; Fig. 3f, g). In addition to the changes in individual tuning preference, the overall orientation and/or direction tuning of new neurons sharpened between 6-10 wpt (Fig. 6c, e-g) and response properties became increasingly selective (Fig. 6e-g). Both, average orientation and direction tuning stabilized 10-15 wpt at values previously reported for neurons imaged in L2/3 of adult mice V131 (Fig. 6c). In summary, transplanted neurons undergo a period of functional development, before assuming stable, selective and persistent tuning properties indistinguishable from endogenous layer 2/3 pyramidal neurons in adult V1.

**Discussion**

Our *in vivo* imaging data show how embryonic neurons, transplanted into the lesioned, adult visual cortex, develop normal morphology and stable synaptic structures within 4-9 weeks. At this time, they display tuning to stimulus orientation and/or direction of motion similar to endogenous neurons. Brain wide mapping of inputs onto transplanted neurons reveals highly specific and quantitatively comparable monosynaptic input from the appropriate regions throughout the brain. Together, these data demonstrate that adult neural circuits, such as the cerebral cortex, which normally never integrate new neurons, are in principle readily able to incorporate new neurons in a meaningful manner.

Beyond the crucial implications for neural repair, these data allow addressing fundamental questions of how integration into preexisting circuits may be similar or different from that during early development. The morphological maturation of transplanted neurons appears comparable or if at all slightly faster than reported for normal development32,33. Also the differences in apical and basal dendrite growth resemble the ones found during development, where distinct molecular regulators have been implicated in apical and basal dendrite outgrowth of pyramidal neurons34, including specific pruning mechanisms for basal dendrites35. This suggests that the distinct mode of growth and pruning of apical and basal dendrites of transplanted neurons recapitulates selective mechanisms governing outgrowth and branching of these dendritic compartments during development.

Likewise the appearance and turn-over of dendritic spines in transplanted neurons follows the timing observed during neocortex development, reaching final densities 3 weeks after the birth of pyramidal neurons in mouse or rat sensory cortex36,37. This seems different from adult generated neurons integrating into the olfactory bulb or dentate gyrus which reach their final spine density considerably later (only after 8 weeks38,39). Thus, maturation and integration of transplanted neurons resembles the timing observed during normal development, demonstrating that embryonic neurons integrating into the adult cerebral cortex do not require prolonged periods for maturation or excessive pruning or re-wiring to get incorporated into the circuitry.

Similarly, we also observed remarkably little exuberance or pruning in the input to transplanted neurons, as most of the brain regions monosynaptic connected to transplanted neurons at 4 wpt were also found to be connected 2 months later. This suggests that there is no major rearrangement of regional connectivity after 4 weeks. The inputs to the transplanted neurons in the visual cortex, derive from a total of 23 anatomical regions that have all been described to project to the visual cortex22,23 and some have also been previously observed by RABV-retrograde tracings in intact V124. Importantly, previous analysis of connectivity of neurons transplanted into the cerebral cortex not only found a more limited number of ipsilateral cortex regions connected, but also observed input from contralateral subcortical areas that are not known to normally target the visual cortex40. Notably, transplanted ESC-derived cortical neurons receive only 18% of the normal dLGN innervation to host neurons8, while cortical neurons transplanted here achieve quantitatively comparable levels in most regions or higher levels of innervation from the dLGN. Other quantitative differences in the innervation index of transplanted and endogenous neurons were observed in the ipsilateral cortex (SS and MO) and other thalamic nuclei. These may be due to the presence of transplanted neurons also in other layers. For example, some transplanted neurons were located in layer 4, where innervation from the dLGN is denser than in layer 2/341. Since the targeted cell death procedure employed here requires multiple bead injections into the contralateral cortex, innervation from this partially damaged region might be expected to be lower.

The high density of dendritic spines and the presence of monosynaptic inputs from virtually all brain regions known to project to V1 already after 4 weeks suggest that at this time point transplanted neurons are largely integrated into cortical circuits. Yet, functional imaging commencing shortly thereafter shows that tuning for stimulus orientation and direction is initially relatively broad, indicating that the first connections formed onto grafted neurons are stochastic, not conveying a high degree of functional specificity. Tuning sharpens substantially over the next weeks, which might reflect activity-dependent functional refinement, possibly driven by visual input42. Indeed, circuit remodeling is known to be correlated with elevated spine dynamics43, as we also observe here during the respective period.

The slow, gradual sharpening of tuning observed in transplanted neurons differs from the postnatal development of mouse visual cortex, where highly selective responses have been found very early, immediately after eye opening44, and adult like values are reached within only one week45. This initial, sharp tuning is thought to depend largely on feed-forward inputs conveying shared feature selectivity to clonally related neurons, which earlier in development were electrically coupled by gap junctions46,47. Subsequently, gap junctions are replaced by conventional chemical synapses46 during a process of activity dependent refinement of intracortical connections. Transplanted neurons might skip the first step of this developmental sequence, and the formation of connections onto these cells might be dominated by activity-dependent remodeling, starting from an initial state of low selectivity.

Taken together, the above results demonstrate that transplanted embryonic neurons integrate into preexisting cortical circuits. They show virtually no aberrant connections and importantly, after only a couple of weeks, the transplanted neurons are fully integrated, showing functional properties which are indistinguishable from their original counterparts. Thus, neuronal replacement therapies become more realistic and feasible, at least at times when a sufficient part of the preexisting neuronal network is still available. This approach now provides a ‘gold standard’ model to test other cellular sources for repair, e.g. ESC-derived neurons, as well as other lesion paradigms, such as stroke or traumatic injury. Most importantly, we now know that new neurons are able to acquire appropriate network connectivity and functional properties, and that hence repair of intricate neuronal circuits is in principle possible.

**Methods**

Mice and anesthetics

All animal experiments were carried out in compliance with the institutional guidelines of the Max Planck Society, the Ludwig-Maximilians-Universität and the local government (Regierung von Oberbayern).

Data for this study is derived from a total of 36 adult mice. For the characterization of the lesion model and the rabies virus-based circuit tracing we used C57BL/6J mice. For structural *in vivo* two-photon imaging we used Emx1-Cre16 crossed to CAG-CAT-GFP48 donor cells and Ai9 (Rosa-CAG-LSL-tdTomato reporter mice)17 host mice. For functional *in vivo* two-photon imaging we used Emx1-Cre16 crossed to Ai9 donor cells and C57BL/6J host mice.

Mice were housed in a 12:12 hour light-dark cycle. All mice were 2-4 months old at the time of the first surgery. Surgeries were performed aseptically under anaesthesia with a mixture of fentanyl (0.05 mgkg-1, Hexal or Janssen), midazolam (5 mgkg-1, Ratiopharm or Roche) and medetomidine (0.5 mgkg-1, Orion Corp. or Fort Dodge). After surgery, anaesthesia was terminated with atipamezol (2.5 mgkg-1, Orion Corp.), flumazenil (0.5 mgkg-1, Hexal) and naloxone (1.2 mgkg-1, Ratiopharm) or buprenorphin (0.01 mgml-1, Essex). Carprofen (5 mgkg-1, Pfizer) was administered as a postoperative analgesia.

Neocortical layer-specific lesion

Rhodamine-labeled latex beads (Lumafluor) were conjugated with chlorine e6 (Ce6, Frontier Scientific) as described previously49, with minor modifications. Briefly, 1.5 ml of Ce6 solution (0.597 mg/ml in 0.01 M phosphate buffer [PB], pH 7.4) were added to 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (MP Biomedicals) and kept at 4 °C for 30 min. 12.5 μl of rhodamine latex beads were then diluted in 100 μl PB, mixed with 750 μl of activated Ce6 solution, and incubated on an orbital shaker for 1 h at room temperature. The reaction was stopped by adding 335 μl of 0.1 M glycine buffer (pH 8.0). The conjugated latex beads were washed at least three times (30 min, 100 000 g) with 0.01 M PB and finally re-suspended in 50 μl PB. Beads were stored at 4 °C and used within one month. For beads injection mice were anaesthetized, and an unilateral craniotomy (2.5 mm diameter) was performed at the posterior end of the parietal bone, centered at 2.5 mm lateral from the lambda, in order to expose the visual cortex. Ce6-conjugated beads (9 µg/µl) were injected at 5 to 10 locations to reach a total volume of 0.5 to 1 µl, at a depth of 0.5 mm into the primary visual cortex (V1). Injections were restricted to the binocular zone of V1 with a total volume of 0.5 µl in structural and functional *in vivo* imaging experiments (Fig. 2-3; Fig. 5-6). The location of V1 and the binocular zone was identified using intrinsic optical imaging19 (Extended Data Fig. 2a) and/or the characteristic blood vessel pattern. One to two weeks after the beads injection, a second craniotomy was performed in order to expose the contralateral V1that was non-invasively subjected to laser-photoactivation of Ce6 for 4 min (670 nm, 27 mW; laser and beam shaping optics for a collimated parallel beam of 1 mm diameter, Schäfter+Kirchhoff) to induce apoptotic cell death of callosal projection neurons (CPNs). This lesion model thus targets only a fraction of neurons in an area and layer-specific manner, while the overall tissue structure is preserved.

*In utero* electroporation

For monosynaptic rabies virus tracing experiments, mouse cortical cells were labeled via *in utero* electroporation. Briefly, at E14.5/E15.5, pregnant mice were anaesthetized and the uterine horns were exposed by laparotomy. The plasmid pDsRedExpress2-2A-Glyco-IRES2-TVA or pDsRedExpress2-IRES2-TVA (1 µg/µl) was mixed with 2.5 µg/µl Fast Green dye (Sigma-Aldrich) and 1 µl of the solution was injected into one of the lateral ventricles of each embryo using a glass capillary. Tweezer-type circular electrodes oriented with a ~45° angle for cortical targeting were used to hold the head of the embryo and deliver electrical pulses (5 pulses, 35 V, 100 ms) produced by a square-wave electropora­tion generator (ECM 830, BTX, Harvard Apparatus). Uterine horns were returned into the abdominal cavity and embryos were allowed to continue their normal development. For transplantation, E18.5 embryos previously electroporated at E14.5 were sacrificed and cerebral cortices were collected for cell dissociation. Some cells were plated directly after dissociation at a density of 500 000 cells/well in 24-well plates in B27-containing DMEM high glucose (4.5 g/L) with glutamax, plus penicillin-streptomycin, and fixed after 2h for subsequent immunocytochemistry (see below).

For tracing of the afferents to endogenous upper layer neurons, electroporation was performed at E15.5 and animals allowed to develop until adulthood, when the RABV was then injected in the electroporated hemisphere.

Embryonic cell culture and viral labeling

In a subset of experiments, *in vitro* transduction with Moloney Murine Leukemia Virus (MMuLV)-derived retroviral vectors (n = 10 host mice) or adeno-associated virus (AAV; n = 2 host mice) was performed in order to label donor cells.

Briefly, neocortical tissue from E14.5/E15.5 embryos was mechanically dissociated in Hanks' balanced salt solution (HBSS, Invitrogen) buffered with 10 mM HEPES, or in Earl´s balanced salt solution (EBSS) with papain (20 U/ml Papain, 0.005% DNase, 1 mM L-cysteine, 0.5 mM EDTA; Papain dissociation system, Worthington) after 45 min of enzymatic treatment at 37 °C. Enzymatic activity was stopped by protease inhibitors (10 mg/ml ovomucoid). Cells were plated in 20 µg/ml poly-D-lysine (Sigma-Aldrich) coated 24-well plates, at a density of 300 000 cells/well. Cells were initially kept in 10% FBS-containing DMEM high glucose (4.5 g/L) with glutamax, plus penicillin-streptomycin (all from Invitrogen), and transduced after 2-4 h with viral vectors (1-2 µl/well; titers typically ranged from 107 to 1011 transducing units per mL). For monosynaptic rabies tracing experiments MMuLV-derived retroviral vectors (CAG-DsRedExpress2-2A-Glyco-IRES2-TVA or CAG-DsRedExpress2-IRES2-TVA) and for functional *in vivo* imaging experiments an AAV vector (AAV2/1-hSyn1-flex-mRuby2-P2A-CGaMP6s-WPRE-SV40) were used. Serum was gradually removed by replacing half of the medium with B27-containing DMEM high glucose (4.5 g/L) with glutamax plus penicillin-streptomycin on each of the following two days. Cells were harvested for transplantation after 2-5 days *in vitro*.

Transplantation of embryonic cells into V1

Embryonic cells for transplantation were fluorescently labeled either in the above mentioned mouse lines (Fig. 2-3; Fig. 5-6), by *in utero* electroporation of DNA plasmids (Fig. 1; Fig. 4) or via *in vitro* viral transduction (Fig. 4; Fig. 6).

Seven to ten days after laser-photoactivation, embryonic cells were transplanted into the previously illuminated area. In short, E18.5 embryos were sacrificed, and fluorescently labeled cortical hemispheres were collected for dissociation. Cortical tissue was mechanically dissociated either in HBSS buffered with 10 mM HEPES (Fig. 1; Fig. 4), or in EBSS with papain (20 U/ml Papain, 0.005% DNase, 1 mM L-cysteine, 0.5 mM EDTA; Papain dissociation system, Worthington) after 45 min of enzymatic treatment at 37 °C (Fig. 2-3; Fig. 5-6). In case of papain treatment, enzyme activity was stopped by protease inhibitors (10 mg/ml ovomucoid). A cell suspension (50 million cells/ml) was prepared in neurobasal medium (NB) or DMEM high glucose (4.5 g/L), both supplemented with B27, glutamax and penicillin-streptomycin.

Donor cells labeled via *in vitro* viral transduction were washed at least 5 times with pre-warmed phosphate-buffered saline (PBS), in order to remove any remaining viral particles. Gentle trypsinization (0.025%, 10 min at 37 °C) was performed and a cell suspension (25-50 million cells/ml) was prepared in B27-containing DMEM high glucose (4.5 g/L) with glutamax, plus penicillin-streptomycin.

Between 25 000 and 80 000 donor cells were transplanted into the V1 of adult mice. A total volume of 1 µl of cell suspension was injected with a syringe (ga 31-33, Hamilton) within the area located at 2.5 ±0.2 mm medio-lateral, 0.0 ±0.2 mm antero-posterior relative to lambda and distributed dorso-ventrally over 200-300 µm at a depth between 0.5 to 0.2 mm. The exact injection coordinates and pattern of pial vasculature were noted for later injection of the RABV. For two-photon imaging a cranial glass window was implanted, otherwise the bone lid was repositioned and the skin was sutured.

Cranial Window Implant

For chronic structural and functional *in vivo* imaging experiments (Fig. 2-3, Fig. 5-6) a cranial glass window was implanted on top of V118 after cell transplantation. Briefly, a coverslip (5 mm diameter, #1 thickness, EMS) was loosely placed on the dura, resting on the edge of the craniotomy, and sealed to the bone with cyanoacrylate. A small metal bar with screw holes (5 x 8 mm) for head fixation during image acquisition was attached to the skull medial to the window implant. Skin margins, cover-glass and metal bar were embedded in dental acrylic (Heraeus Kulzer) mixed with black pigment (Kremer).

Intrinsic Optical Imaging

In a subset of mice (n=16) the location of cell transplantation was verified with optical imaging of intrinsic signals (Extended Data Fig. 2a). In short, anaesthetised mice were presented with square wave drifting gratings (4 orientations, 600 ms stimulus duration; 0.03 cycles deg-1, 2 cycles sec-1) in a 2 x 2 array covering approximately -10° to 70° azimuth, -20° to 40° elevation of the ipsi- or contralateral visual field, respectively. For identification of the binocular zone, visual stimuli were presented to the ipsilateral eye while the contralateral eye was covered. The cortical surface was evenly illuminated through the cranial window with monochromatic light at 707 nm. A cooled, slow scan CCD camera (12 bit, Optical Imaging Inc) was focused 200-300 µm below the cortical surface and frames were recorded with 600 ms exposure time. Images of average responses (3 repetitions of 12 stimulus frames per location) were blank-corrected, range-fitted and low-pass-filtered50. The false color-coded maximum projection of visual responses is mapped on top of the blood vessel image acquired prior to visual stimulation.

Monosynaptic Rabies Virus Tracing

We examined the brain-wide synaptic input to transplanted cells by retrograde monosynaptic tracing with a modified rabies virus (EnvA-ΔG-RABV)20. RABV particles are pseudotyped to specifically infect cells expressing TVA-class receptors and express eGFP instead of their own glycoprotein (G). Trans-synaptic spread to pre-synaptic partners is only possible after complementing the RABV with its G-protein coat by the G/TVA expressing cells and is therefore limited to one monosynaptic jump. G/TVA expressing cells also encode for DsRed, while their pre-synaptic partners will be eGFP+ only. DsRed+/GFP+ neurons are termed starter cells.

In short, EnvA-ΔG-RABV was injected 4 or 12 weeks after transplantation of embryonic cells expressing DsRedExpress2-2A-Glyco-IRES2-TVA or DsRedExpress2-IRES2-TVA, in three locations surrounding the transplantation site (200 nl/location). In order to map the pre-synaptic connectivity of upper layer neurons in adult V1, E15.5 embryos were *in utero* electroporated with pDsRedExpress2-2A-Glyco-IRES2-TVA or pDsRedExpress2-IRES2-TVA. Embryos were allowed to develop and adult mice received 3 injections of RABV within V1. In both experimental groups, animals were sacrificed 7 to 9 days later for immunostainings and circuit analysis.

Structural *in vivo* two-photon imaging

*In vivo* two-photon imaging was carried out on an Olympus FV1000BX61 system equipped with a mode-locked Ti:sapphire laser (Mai Tai DeepSee, Spectra-Physics) through a 25x water immersion objective (1.05NA, Olympus). Laser settings and image acquisition were controlled by Fluoview software (Olympus).

For structural *in vivo* imaging mice were anaesthetized with fentanyl based anaesthesia (see above) and placed on a feedback controlled heating pad. Data were acquired at 910 nm with an average laser power of <30 mW, and the emission signal was directed through a dichroic mirror (DM570) and red/green bandpass emission filters (BA495-540HQ and BA570-625HQ, all Olympus); a typical imaging session lasted 2 h. Host mice were imaged as early as 3 days post transplantation. Individual transplanted cells were identified and followed in short, increasing intervals (2 to 5 days) within the first 4 weeks and weekly thereafter (up to 12 wpt). In 2 mice we acquired late time points at 9 to 11 months post transplantation (Figure 3d-i; Extended Data Fig. 4d).

In each imaging session high-resolution tiled volume stacks (510 x 510 µm field of view; 0,33 µm/px; 1-3 µm z-steps) were acquired up to a depth of 350 µm from the pial surface for overview and reconstruction of whole cell morphology of transplanted cells (Fig. 2, Extended Data Fig. 2). In addition, high-resolution close-up stacks (73 x 73 µm; 0.14 µm/px; 0.5-1 µm z-steps) of typical 3 individual dendritic and axonal processes were acquired in various depths between 50 and 300 µm. For the analysis presented in Fig. 3 we included both basal and apical dendritic processes.

*In vivo* two-photon calcium imaging

For functional *in vivo* imaging experiments donor cells were labeled with the genetically encoded calcium indicators (GECIs) CGaMP6s29 or Twitch2B30 (a FRET based sensor). Emx1-Cre X Ai9 donor cells were mixed with AAV encoding a double-floxed inverted open reading frame version of either GECI prior to transplantation (AAV2/1-hSyn1-flex-CGaMP6s-WPRE-SV40; AAV2/1-CAG-flex-Twitch2B-WPRE-SV40). In a subset of experiments (n=2 host mice) donor cells were labeled *in vitro* (AAV2/1-hSyn1-flex-mRuby2-P2A-CGaMP6s-WPRE-SV40, see above).

*In vivo* imaging was performed under light anaesthesia; mice received 0.4x dose for initial anaesthesia and a subsequent 0.2x dose every 90 min. Mice were kept on a feedback controlled heating pad. For ipsi- and contralateral visual stimulation either the left or the right eye was occluded, respectively, and full field moving gratings (square wave, high contrast; 0.04 cyc deg-1, 1.5 cyc s-1; 4 orientations, 8 directions) were presented to the open eye (30 cm distance monitor to eye). The 8 directions were presented in random order, each displayed for 3 seconds, followed by 3 seconds of an isoluminant grey screen. Presentation of 3 x 8 directions was flanked by 10 seconds of grey screen (constituting one stimulus sequence of 3 repeats). Typically, 2-3 stimulus sequences were presented per imaging plane (altogether 6-9 repeats).

Data was acquired either at 940 nm (GCaMP6) or at 860 nm (Twitch2B) with an average laser power of <30 mW; a typical imaging session lasted 2-3 h. Emitted light was directed through a longpass dichroic mirror (DM570, GCaMP6; 505DCXR, Twitch2B) and recorded through emission filters (BA495-540HQ and BA570-625HQ, GCaMP6; ET480/40M and ET535/30M, Twitch2B).

In each imaging session a tiled volume stack (760 x 760 µm field of view; 0.49 µm/px; 3-5 µm z-steps) was acquired up to a depth of 350 µm from the pial surface for an overview of transplanted (RFP+) neurons. Five to 10 candidate areas with RFP+/GECI+ neurons were recorded at a frame rate of 2.4 Hz during visual stimulation (73 x 73 µm field of view; 0.28 µm/px).

Host mice were imaged starting at 4 wpt, and individual responsive neurons were repeatedly recorded up to 15 wpt. In a subset of experiments (n=2 mice, see above) we specifically recorded late time points at 11 to 15 wpt.

Analysis of *in vivo* structural data

Image stacks were processed using the Fiji package of ImageJ (US National Institutes of Health) as follows: Fluorescence signals of rhodamine-coupled chlorine e6 beads were removed by channel subtraction (Extended Data Fig. 2d). Images were converted to 8 bit and subjected to a small 2D Gaussian filter (σ < 0.6 px). For display purposes only, maximum intensity z-projections are shown with adjusted brightness/contrast.

Whole cell morphology was reconstructed using Simple Neurite Tracer. Briefly, apical and basal dendrites were semi-automatically traced through the high-resolution tiled volume stack. Based on the traced skeleton, a single cell 3D volume model was rendered (Extended Data Fig. 2c).

In order to determine spine and bouton densities, dynamics and survival, putative synaptic structures were identified18 in image stacks at each recorded time point. We included all clearly visible structures in x, y and z. An ID was assigned to each individual identified structure at the time point of its first appearance and registered across time points. Density is reported as structures per µm, and turnover is calculated as fraction of structures (gained + lost) / (total t1 + t2). We calculated the average survival fraction of gained structures dependent on the time point of their first appearance according to the non-parametric Kaplan-Meier estimator (using GraphPad PRISM). This method takes into account that there is some uncertainty of the actual survival of spines present at (and presumably longer than) the last experimental time point. Hazard ratios compare the rate of structure loss between structures that were gained at different time points; median survival ratios compare the relative median survival of gained structures at different time points (see Extended Data Fig. 5).

16 dendritic stretches out of 5 mice and 33 axonal stretches out of 6 mice were analyzed (average dendritic length: 50.5 ± 12.4 µm; average axonal length: 77.7 ± 25.3 µm). A total of 13251 dendritic spines and 6266 axonal boutons across all time points were identified and registered to 2493 individual dendritic spines and 1600 individual axonal boutons on 0.8 mm total dendritic and 2.6 mm total axonal length.

Analysis of *in vivo* functional data

Functional imaging data was analyzed using Fiji and Matlab (Mathworks). Individual frames were background subtracted using a rolling ball algorithm (>100 px radius), converted to 8 bit and subjected to a small 2D Gaussian filter (σ < 0.8 px). Stacks were full-frame aligned using linear transformations (StackReg, Thévenaz P., EPFL) and ROIs were selected manually based on the aligned maximum intensity projection across a stimulation sequence. The fluorescence signal (F) was calculated as the average fluorescence of all pixels within a given ROI (Twitch2B: R = av.FYFP/ av.FCFP). Neuronal activity was measured as the normalized change in fluorescence signal over time: (Ft-F0)/F0 (Twitch: Rt-R0/R0). The baseline (F0, R0) was calculated as the average signal over typically 10 seconds before and after each stimulation sequence (see above).

We classified neurons as visually responsive if the average ΔF/F0 > 3σ (Twitch2B: ΔR/R0 > 0.0530) for at least one stimulus direction. Tuning properties of each neuron were depicted in complex space using the normalized average peak response for each direction expressed in polar coordinates.

Orientation and direction tuning preference was expressed as orientation and direction selectivity index (OSI/ DSI), respectively and calculated as follows: OSI = (Rpref- Rortho)/ (Rpref+ Rortho), DSI = (Rpref- Ropposite)/ (Rpref+ Ropposite). R is the average peak response to the preferred direction (Rpref), to the mean of the orthogonal directions (Rortho) and the opposing direction (Ropposite). As ratio based tuning properties do not take into account the distribution of responses across all tested directions, we also calculated single and double Gaussian fits51. Following the assumption that an ideal orientation (or direction) tuned neuron would be perfectly described by a double (or single) Gaussian fit, the goodness of fit (R2) serves as a measure of tuning quality. In short, curves were fit with non-linear regression using PRISM (GraphPad). Single Gaussian fits were calculated according to y = a + amp \* exp (-0.5 \* ((x-xmean)/σ)2), with a = offset from x-axis, amp = peak amplitude, x = stimulus directions in degree, xmean = x value at peak amplitude, and the following constraints: a > 0, amp = 0-1, xmean = 180°, σ ≥ 22.5°. Double Gaussian fits were calculated according to y = a + amp1 \* exp (-0.5 \* ((x-xmean1)/σ1)2) + amp2 \* exp (-0.5 \* ((x-xmean2)/σ2)2), with a = offset from x-axis, amp1 = peak amplitude, amp2 = amplitude at opposing direction, x = stimulus directions in degree, xmean1 = x value at peak amplitude, xmean2 = x value at amplitude of opposing direction, and the following constraints: a > 0, amp1 = 0-1, amp2 < amp1, Δxmean1, 2 = 180°, σ1, 2 ≥ 22.5°. R2 is computed as the normalized sum of least squares.

To further describe the changes in tuning of individual neurons over time, we calculated the difference in preferred orientation in successive imaging time points for all neurons recorded at least at two subsequent time points. With 4 orientations, the individual difference was limited to discrete values of Δ45° between 0° and 135°.

Immunocyto/histochemistry

Plated cells were fixed in 4% paraformaldehyde (PFA) for 30 min, washed and incubated in blocking and permeabilizing solution for 30 min (3% bovine serum albumin; 0.5% triton X-100) before applying the primary antibodies goat mCherry (1:500; Sicgen), rabbit anti-Cux1 (1:500; Santa Cruz), for overnight incubation at 4 °C. After washing, cells were incubated with species- and subclass-specific secondary antibodies conjugated to Cy3 and Cy5 (Dianova) used at 1:500 for 2h at room temperature. Nuclei were stained with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich; 5 min at room temperature) and coverslips were mounted on glass slides with Aqua-Poly/Mount (Polysciences).

For immunohistochemistry, brains were collected after transcardial perfusion of deeply anaesthetized mice with phosphate-buffered saline (PBS, 5 min) followed by 4% PFA, 30-40 min. Brains were then post-fixed in 4% PFA overnight, at 4 °C, serially cut on a vibratome into 60-70 µm sagittal or coronal sections, which were kept free-floating for further processing. TUNEL staining was performed according to the manufacturer’s instructions (Roche). Immunohistochemistry was carried out using the following primary antibodies: chicken anti-Green Fluorescent Protein (GFP, 1:1000; Aves Labs), rabbit anti-Red Fluorescence Protein (RFP, 1:1000; Rockland), goat mCherry (1:200; Sicgen), rabbit anti-Cux1 (1:200; Santa Cruz). After washing, sections were incubated with species- and subclass-specific secondary antibodies conjugated to Cy3 or Cy5 (Dianova) or Alexa Fluor 488 or 647 (Invitrogen), used at 1:500 or 1:1000 depending on high (>1:500) or low (<1:500) concentration of the primary antibody. Sections were incubated for 10 min with 1 µg/ml DAPI for nuclear labeling and mounted on glass slides with Aqua-Poly/Mount (Polysciences).

Images were acquired using a laser-scanning confocal microscope (Zeiss, LSM 710), and analyzed with ZEN 2012 (Zeiss) and ImageJ 1.48p software. Quantitative analysis of Cux1 expressing cells was done by counting positive cells among all RFP+ donor cells in the mouse. Cell countings were performed with the Cell Counter plug-in for Image J 1.48p, by careful inspection across serial optical sections (spaced at 1 µm) of confocal Z-stacks acquired with a 40x objective (NA 1.1). Results are represented as mean ± S.E.M. calculated between different mice. Image processing was performed with ImageJ and Adobe Photoshop/Illustrator (Adobe Systems) for preparation of multipanel figures.

Circuit analysis

For circuit analysis complete brains were carefully removed from the skull following perfusion (see above). After cutting, brain sections were kept in serial order and stained for GFP and RFP. Subsequently, sections with transplanted cells were selected and further stained for cortical layer markers. Of all sections, those with one or more GFP-labeled cell somas were scanned using an epifluorescence microscope with a motorized stage (Zeiss, Axio ImagerM2) equipped with a 10x objective (NA 0.3). We used automatic scanning and alignment of individual tiles, followed by image stitching to create a high resolution image of the whole section. These images were used to identify brain regions where GFP labeled cells were found, by comparison to the corresponding sections of the Allen Reference Atlas of the adult mouse brain (version 2; 2011; Allen Institute for Brain Science). Some sections of interest are not available in this reference atlas, namely in the sagittal atlas, which displays 21 sections spaced at 200 μm intervals, and only up to 4.0 mm lateral from bregma. In these cases, the Brain Explorer 2 software (Allen Institute for Brain Science) was used to retrieve the corresponding annotated section to overlap it with the experimental section and identify the anatomical location of the labeled cells. In sections with unclear cell numbers due to close apposition of two GFP cell bodies or with high densities of GFP cells, scanning of confocal Z-stacks with a 40x objective (NA 1.1) was carried out, and quantification was performed by careful inspection through serial optical sections spaced at 1 µm interval. In sections containing transplanted cells, four categories were considered for counting: GFP-only cells with neuronal morphology, GFP-only cells with glial morphology, GFP/RFP (or mCherry) cells with neuronal morphology, GFP/RFP (or mCherry) cells with glial morphology. Connectivity ratio for a given anatomical region was calculated by computing the ratio of the total number of GFP-only cells with neuronal morphology counted in that region and the total number of GFP/RFP (or mCherry) cells with neuronal morphology (starter neurons for RABV spread) in V1. Results are represented as mean ± S.E.M. calculated between different mice.

Statistics

Statistics was performed using PRISM (Graphpad). Spine and bouton data were analyzed with one way ANOVA and Tukey posttests for multiple comparisons. Survival curves were analyzed pairwise using the Gehan-Breslow-Wilcoxon test, and p-value thresholds were adjusted for multiple comparisons applying a Bonferroni correction. Functional data were subjected to non-parametric tests using Kruskal-Wallis with Dunn’s post-tests. The minimum level of significance was defined as p<0.05 and all values are reported ± S.E.M., if not stated otherwise.

**References**

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**Author contributions**

The original idea for the study came from MG. MG, MH, and TB then initiated the study and planned the experimental approach. SF, SG, MG, MH, and LD designed the experiments which were conducted and analyzed by SF and SG. MG and LD provided the lesion model. KC provided the Rabiesvirus and expertise for its use for monosynaptic tracing. Finally, MG, MH, SF, and SG wrote the paper with input from LD, KC, and TB.

**Author information**

**Tables**

**Figure Legends** (incl. Extended Data Tables and Figures)

Figure 1 – **Lesion model and identity of transplanted neurons.** **a**, Experimental procedure and timeline. Schematic drawing on the left shows injection of chlorine e6 (Ce6) coupled beads into the left hemisphere followed by laser photoactivation in the contralateral hemisphere 1-2 weeks later, inducing local apoptotic cell death in Ce6-containing callosal projection neurons (CPN). Schematic drawing on the right shows transplantation of fluorescently labeled embryonic cortical cells into CPN-depleted V1; subsequent analysis is depicted in the time lines on the right. **b**, Left, accumulation of Ce6 rhodamine beads (red) around neuronal cell nuclei (NeuN, white) in V1 contralateral to the beads injection. Right panels show high magnification panels with DAPI+ (blue) nuclei, not all of which contain red fluorescent beads, but those that do are NeuN+ neurons (arrowheads). **c**, TUNEL staining (red) performed 3 days after laser illumination overlaps with loss of NeuN immunoreactivity (white), indicating degenerating neurons, and accumulation of condensed apoptotic-like nuclei (DAPI, blue; inset shows high magnification). **d**, Micrograph depicting transplanted neurons (RFP-labeled; red) in host V1 at 5 wpt. **e**, Z-stack projection (left) and examples of cells in single optical sections (right) shows that the majority of transplanted neurons expresses Cux1 (white; arrowheads, Cux1-positive cells; arrow, Cux-1 negative cell). Scale bars: **b**, left, 50 µm; right, 20 µm; **c**, 50 µm; **d**, 100 µm; **e**, left, 25 µm; right, 25 µm.

Figure 2 – **Transplanted embryonic neurons develop pyramidal neuron morphology.** **a**, *In vivo* two-photon z-stack projection (inverted) of transplanted neurons in host V1 at 45 dpt. **b**, Reconstruction of neuron indicated in **a**, (blue arrowhead), side view. The cell body is located at a depth of 200 µm, apical dendrites extend up to 50 µm below the pial surface, and basal dendrites reach a depth of 300 µm (see also Extended Data Fig. 2). **c**, Development of an individual transplanted neuron (GFP+) 3 to 92 dpt. Note the overall stable morphology between 28 and 92 dpt. BV, bloodvessel. **d**, Left, inverted binary maximum projection of neurites at 3 dpt of neuron depicted in **c**. Right, transplanted neurons extending early neurites (blue arrowheads). Star, axonal growth cone; diamond, dendritic growth cone. **e**, Development of basal dendrites. Left, binary image and right, high magnification of neuron in **c**. Pruned process, red arrowheads; newly formed processes, green arrowheads. **f**, Development of apical dendrite. Left, binary of neuron in **c**. Right, high magnification of another neuron imaged repeatedly from 6 to 29 dpt. Early branch points remain stable (green arrowheads). **a-f**, Blue arrowhead indicates location of the cell body. **c-f**, *In vivo* two-photon z-stack projections. Scale bars: **a-d** 50 µm; **d** (star, diamond) 5 µm; **e-f** 10 µm.

Figure 3 – **Transplanted neurons form synaptic structures.** Repeated *in vivo* two-photon imaging of individual **a**, dendrites and **c**, axons at high magnification (z-stack projections). Examples of lost (red arrowheads), gained (green arrowheads) and stable (blue arrowheads) dendritic spines and axonal boutons. **b**, Early formed spines are dynamic, later formed spines are stable, as demonstrated by overlaying binary, color-coded images of early (left; blue, 12 dpt; red 17 dpt; green 22 dpt) and late (right; blue, 34 dpt; red, 41 dpt; green, 48 dpt) time points. **d**, Density of dendritic spines (16 cells, n=5 mice) and **e**, axonal boutons (33 processes, n=6 mice), expressed as mean ±s.e.m (dark blue, average; light blue, individual cells). **f**, Turnover (dark blue; sum of fractional gain, green, and fractional loss, red) of dendritic spines (16 cells, n=5 mice) and **g**, axonal boutons (33 processes, n=6 mice), expressed as mean ±s.e.m; elevated turnover until 8 wpt (one-way repeated measure ANOVA 4-10wpt, p<0.0001; Tukey posttests). **h**, Survival fraction of newly formed dendritic spines and **i**, axonal boutons 4 dpt to 11 month post transplantation. Spines and boutons formed at early time points (red spectrum) are more prone to be eliminated. Structures formed at 9 months post transplantation (blue) have a higher chance of survival (2493 Spines, n=5 mice; 1600 boutons, n=6 mice; Gehan-Breslow-Wilcoxon comparison with Bonferroni correction for multiple comparison: spines p<0.00064, boutons p<0.00018). Scale bars: **a, c** 10 µm.

Figure 4 – **Input connections of transplanted neurons**. **a**, Molecular tools and **b**, experimental timeline for the transsynaptic tracing strategy: donor cells express a red fluorescent protein (RFP), RABV G and the TVA receptor. TVA expression renders cells susceptible for later infection by EnvA-coated modified RABV (ΔG-GFP) in the host brain, and transcomplementation with G allows the RABV to retrogradely cross one synapse revealing their monosynaptic input. RFP-G-TVA expressing embryonic cells are transplanted into CPN depleted V1. Four or twelve wpt ΔG-GFP (EnvA) RABV is injected into the transplanted region for circuit tracing. **c,** Z-stack projection from a sagittal section of a transplanted hemisphere shows transplanted cells (red) in V1, surrounded by GFP-only neurons (green). These are direct pre-synaptic partners of transplanted neurons infected by the RABV (eGFP/RFP, ‘starter neuron’, example in **d**). **e**, RABV-traced input connectivity of transplanted neurons at 4 wpt (n=6). Abbreviations in yellow correspond to regions where GFP-only neurons were observed, and white ones denote nearby regions for anatomical reference. Insets show examples at high magnification. For abbreviations see Extended Data Table 1. **f**, Color-coded connectivity ratio for all brain regions providing synaptic input to transplanted neurons at 4 wpt and endogenous neurons (n=2, see Extended Data Fig. 8). **g**, Schematic drawings of sagittal sections highlighting brain regions that establish afferent connections with new neurons in V1, at 4 wpt (red dots and contours, n=6) and at 12 wpt (blue dot and contour, n=6). Connectivity ratio range is indicated by the number of dots per region. Note that innervation from the claustrum, pons and dorsal striatum was only detected at 12wpt, and all of these regions are known to project to V1, for dorsal striatum see also (online Allen Mouse Brain Connectivity Atlas, Experiment 112458831, section 90; Experiment 100142580, section 90; Experiment 112307754, section 82). Scale bars: **c**, 100 µm; **d**, 50 µm; **e**, 200 µm; insets, 50 µm.

Figure 5 – **Transplanted neurons show tuned responses to visual stimuli.** **a**, Embryonic donor cells were co-labeled with Emx1-Cre driven tdTomato and GECIs (GCaMP6s or Twitch2B, respectively; delivered via AAV, see Methods). **b**, Experimental timeline. Following transplantation, chronic two-photon calcium imaging was performed at 4 to 15 wpt. Mice were presented with full field gratings moving in 8 directions. **c-g**, Changes in GECI fluorescence was recorded in cell bodies (green traces), axons (blue traces) and dendritic spines (magenta traces) of transplanted neurons. **c**, Examples of transplanted neurons (tdT+) also expressing GECIs. Single optical planes, maximum projection of all frames of one stimulation sequence (see methods); scale bar, 5 µm. **d**, Stimulus evoked changes in fluorescence relative to baseline (dR/R, Twitch2B; dF/F, GCaMP6), example traces. **e**, Single (thin line) and average responses (thick line) to repeated stimulus presentations, sorted to the respective grating orientation and direction. Gray: stimulus on. **f**, Normalized average maximum peak response at each direction plotted in polar coordinates for cells depicted in **c**. **g**, Polar plots of further example cells. Transplanted neurons display tuning properties typical for upper layer pyramidal neurons in V1.

Figure 6 – **Tuning of transplanted neurons sharpens over time.** **a**, Changes in tuning at 5 to 9 wpt, measured from the axon of a transplanted cell (white arrowheads indicate two boutons; scale bar 5 µm). **b**, Stable tuning of a transplanted cell at 11 to 15 wpt (dashed line indicates neuronal cell body; scale bar 5 µm). **a, b**, Top row, individual and average stimulus evoked responses; Middle row, polar plots and orientation and direction selectivity indices (OSI/ DSI); Bottom row, single plane maximum projections of 150 frames during visual stimulation. **c**, OSI and DSI between 6 and 15 wpt (mean ±s.e.m; 27 cells, n=5 mice). **d**, Difference in preferred orientation of neurons recorded at least at two subsequent time points (one or two weeks interval) from 6 to 15 wpt (mean ±s.e.m; 12 cells, n=4 mice; Kruskal-Wallis test p<0.0183, Dunn's posttest for multiple comparison). **e**, Overlay of polar plots at 6, 10 and 15 wpt (preferred direction aligned to 90°) shows overall sharpening of tuning. **f-g**, Assessing orientation and/or direction selectivity with Gaussian fits (also see Extended Data Fig. 12 and Methods). Goodness of fit of **f**, double (DG) and **g**, single gauss (SG) functions (mean ±s.e.m; 17 cells, n=4 mice; Kruskal-Wallis test, DG p<0,01 and SG p<0,038; Dunn's posttest). Noteworthy, it is unlikely that the changes in tuning preference and specificity of transplanted neurons reported here are due to non-linearity effects of the calcium indicators, as we see both, marked changes in tuning preference at consistent average peak amplitudes across time points (d, see also Extended Data Fig. 11a), as well as stable tuning at late time points (b).

Extended Data Figure 1 – **Identity of embryonic neurons prior to transplantation**. **a**, *In vitro* Cux1 staining (white) on acutely dissociated cells from E18.5 cortex previously labeled by *in utero* electroporation at E14.5 with a RFP expressing plasmid (red). **b,c**, insets shown in **a**. Green arrowheads, examples of Cux1-negative cells validate the specificity of the immunolabeling; yellow arrows, examples of Cux1-positive cells which are the majority amongst RFP+ neurons. Scale bars: a, 50 µm; b,c, 20 µm.

Extended Data Figure 2 – **Intrinsic signal imaging, location of grafting site and neuron reconstruction.** **a**, Top, overlay of visual stimulus evoked intrinsic signal (color coded in green) and the blood vessel pattern through a cranial glass window above V1. Red dotted line, area of laser-photoactivation. Bottom, widefield fluorescence image through the same cranial window (V1, green dotted line). Grafting site (GFP+, white arrowhead) in the binocular region of V1. **b**, *In vivo* two-photon z-stack projection (inverted) of a grafting site 52 dpt, top view and side view. **c**, Left, *in vivo* two-photon z-stack projection of a grafting site 45 dpt (inverted; same as in Fig. 2a). Right, reconstruction (skeleton) of an example neuron present in the grafting site depicted left reveals typical layer 2/3 like morphology. Apical dendrites (magenta) branch from one prominent main dendritic trunk and extend to the surface. Basal dendrites (green) extend from the cell body and reach 300 µm below the pial surface. Upper row, top view; lower row, side view. Scale bars a, b, 100 µm; Scale bars c, 50 µm.

Extended Data Figure 3 – **Control for fusion events.** **a**, Genetic strategy to control for fusion events. Emx1-Cre driven GFP+ donor cells were always transplanted into tdTomato reporter mice (n=10). **b**, Absence of tdTomato fluorescence in GFP+ grafted neurons *in vivo*. Line plot (along the white dotted line) across a GFP+ example neuron (green arrowhead) shows that the neuron is tdT negative. Rhodamine beads (red arrowheads) are equally detected in both channels. Units, 8 bit greyscale.

Extended Data Figure 4 – **Modes of formation of spines and boutons, and long-term survival of grafted neurons.** **a**, Dendrites extend and arborize considerably before the first spines form on bare dendrites. Example of a naked dendrite bare from 4 dpt (not shown) to 8 dpt that forms the first spines 12 dpt (empty green arrowheads indicate the location on the dendrite and filled green arrowheads indicate the newly formed spines). Two spines remain stable until 35 dpt (blue arrowheads). **b**, Bouton formation precedes spine formation. Example axon at 4 dpt; arrowheads (green) indicate new boutons that remain stable over subsequent time points (blue arrowheads). **c**, Boutons are able to form within a few µm of the axonal growth cone (green arrowheads). Individual boutons that have formed in the vicinity of a growth cone are able to survive for days and weeks (blue arrowheads). **d**, Grafted neurons that survived the early phase of integration (>12 dpt) remained stable until the end of the experiment (here 10 months) and likely for the rest of the animals life. Scale bars a, b, 10 µm; Scale bar c, 20 µm

Extended Data Figure 5 – **Survival of newly formed dendritic spines and axonal boutons.** Comparison of early-formed spines and boutons with structures formed 4 to 9 wpt. Median survival ratios indicate the relative survival. Hazard ratios indicate the relative chance for structures to be lost. **a**, Early formed dendritic spines (<12 dpt) have a 1.5 to 2.4 times higher chance of being eliminated compared to spines newly formed 4 to 9 wpt. Hazard ratios remain elevated (>1.2) up to 24 dpt. Spines formed at 9 months post transplantation, however, have a very high chance of survival. While the median survival 4 to 9 wpt is 28 days, more than half of the spines formed at 9 months post transplantation survive at least for 51 days (blue triangle, arbitrary value as survival is >50%). **b**, Early formed axonal boutons (<7 dpt) are 2 to 8 times more likely to be eliminated compared to boutons newly formed 4 to 9 wpt. Boutons formed at 9 months post transplantation, have a 4 times higher chance of survival.

Extended Data Figure 6 – **Local and brain-wide monosynaptic input to transplanted neurons.** **a**, Examples of the transplantation site in three different animals selected to demonstrate the range of RFP+ transplanted neurons (left to right: many to few) but consistently surrounded by a large number of GFP-only labeled connecting neurons from the host. **b**, 3D diagram of the brain-wide monosynaptic input connectome for each one of the examples in a, shows in yellow the location of starter neurons in V1 and in green the innervating neurons, either local (green circle) or distant (green lines, thickness of the line represents the connectivity ratio for a given area and respects the ranges displayed in Fig. 4f). Note the strong input from thalamic nuclei, in particular the dLGN in all individual cases. Importantly, the number of areas and of input neurons in a given area correlates with the number of starter neurons in V1 (see Extended Data Fig. 6). **c**, Distribution of neurons providing synaptic input to transplanted neurons, example traced at 4 wpt. Number of GFP-only (green) and GFP/RFP double-labeled cells (yellow) throughout the transplanted hemisphere. Each horizontal bar corresponds to one coronal section, from anterior (top) to posterior (bottom) coordinates of the mouse brain. Sections including the visual cortex are highlighted in gray. Note the overrepresentation of local connections compared to long-range projections.

Extended Data Figure 7 – **Quantitative analysis of the monosynaptic synaptic tracing of regenerated and endogenous neuronal circuits**. **a**, Connectivity ratio for each anatomical area, obtained from RABV tracing at 4 and 12 wpt, or in naïve mice, and calculated as described in Methods. The number of mice with synaptic input from a given area is specified (from a total of 6 mice per group, or 2 mice in the endogenous circuit tracing). The data indicate that some areas project few axons to V1, and thus a higher number of starters results in increased probability of synapse formation, necessary to unveil these connections; while other areas project massively to V1 and are therefore traced even from only one starter neuron in V1 (Vis, dLGN). The number of starter neurons varied across mice due to variability of the number of transplanted cells and efficacy of RABV injection (starter neurons in each of the 6 mice at 4 wpt: 1, 3, 16, 24, 32, 80, and in each of the 6 mice at 12 wpt: 1, 1, 3, 10, 13, 29). s.e.m., standard error of the mean. **b**, Top, 3D diagram of the brain-wide input connectome at 4 wpt (green, n=6) and additional connections revealed at 12 wpt (blue, n=6; regions highlighted at the bottom diagram); in yellow the location of starter neurons in V1 and in green/blue the innervating neurons, either local (green circle) or distant (green/blue lines; thickness of the line represents the connectivity ratio for a given area and respects the ranges displayed in Fig. 3f). Note the pronounced input from visual cortex and dLGN. **c**, RABV-traced inputs to transplanted neurons observed exclusively at 12 wpt. Sagittal brain sections with nuclear staining (DAPI) indicate the field magnified below. Abbreviations in yellow correspond to regions where GFP-only neurons were observed, and white ones denote nearby regions for anatomical reference. Individual cells are shown at high magnification below. For abbreviations see Extended Data Table 1. Scale bars, 200 µm; insets, 50 µm.

Extended Data Figure 8 – **Normal circuitry of upper layer neurons in V1**. **a**, Schematic depicting experimental procedure. At E15.5, L2/3 neuronal progenitors were *in utero* electroporated to express RFP-G-TVA or RFP-TVA. Electroporation was targeted to the somatosensory cortex in order to achieve a sparse number of transduced cells in the nearby visual cortex. In adult mice, ΔG-GFP (EnvA) RABV was injected into V1 of the *in utero* electroporated hemisphere. **b**, Sagittal section stained with DAPI (blue) shows RFP-G-TVA electroporated neurons (red) restricted to the upper layers. Axon collaterals of these neurons cause dimmer red labeling in layer 5. In green, pre-synaptic neurons in and around the area of RABV injection and transduction of few RFP-G-TVA (red) cells. **c**, Higher power micrographs showing (left) RABV-traced cells (green) in V1 with RFP-G-TVA (top) or RFP-TVA (bottom)-expressing upper layer neurons (red). Right, high magnification shows that a modest number of starter cells (arrows; eGFP/RFP) connects robustly with local neurons, while, if primarily infected cells lack G, only occasional and local GFP-only are observed (arrowheads). Scale bars: **b**, 1 mm; **c**, left, 100 µm; right, 50 µm.

Extended Data Figure 9 – **Responses to visual stimulation of boutons on the same axon.** Left, Axon of a transplanted neuron (tdTomato+) expressing GCaMP6, single optical plane, maximum projection of all frames of one stimulation sequence (see Methods). Scale bar, 5 µm. Right, individual and average responses (dF/F) of 4 boutons (indicated left) to visual stimulation with gratings moving in 8 directions (grey bars, direction indicated on top). Note highly similar responses of all boutons.

Extended Data Figure 10 – **Transplanted** **neurons show binocular responses.** **a**, Individual transplanted neurons respond to ipsi- and contralateral eye stimulation (IL and CL, respectively). Top row, polar plots; bottom row, single plane, maximum projection of all frames of one stimulation sequence. Scale bar 5 µm. **b**, Transplanted neurons are tuned to all directions, with a slight overrepresentation of cardinal directions. Cumulative absolute number of grafted neurons across imaging time points, sorted according to their respective preferred direction.

Extended Data Figure 11 – **Peak amplitudes are constant and do not correlate with specificity.** **a**, Consistent average peak amplitudes across imaging time points. Kruskal-Wallis test, p=0.42, ns. **b**, Orientation and direction selectivity indices do not correlate with average peak amplitudes (OSI, R2=0.087; DSI, R2=0.15; dashed lines, 95% CI). Reported52 non-linearity effects of calcium indicators would predict higher selectivity indices at lower average peak amplitudes.

Extended Data Figure 12 – **Orientation and direction selectivity assessed with Gaussian fits.** **a**, Example cell displaying strong orientation selectivity (right, polar plot), fitted with a double Gaussian function (DG). **b**, Example cell displaying strong direction selectivity (right, polar plot), fitted with a single Gaussian function (SG). **c**, Individual DG fits of 17 cells in total (n=4 mice) at 6, 10 and 15 wpt. **d**, Individual SG fits of 17 cells in total (n=4 mice) at 6, 10 and 15 wpt.

Extended Data Table 1 – **Abbreviation list of anatomical areas in the adult mouse brain**.

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