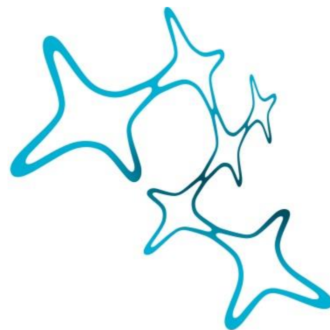
SYNAPTIC INTEGRATION OF TRANSPLANTED FETAL NEURONS INTO DIFFERENT NEOCORTICAL ENVIRONMENTS

Judith Thomas



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Abstract

Brain repair strategies are becoming more promising as the approach of neuron transplantation has been tested in clinical settings, e.g., as therapy for Parkinson disease (PD). One important feature that transplanted neurons need to fulfill is their precise synaptic integration into the existing host brain network to truly reconstruct neuronal circuits. Brain-wide connectivity as well as functionality of grafted neurons was shown to be highly adequate. Transplanted neurons were proven to become functional and integrate with high specificity into the host cortical circuitry in a condition of upper layer neuron ablation. However, there is still little knowledge about brain-wide input connectivity of grafted neurons particularly concerning conditions of severe brain injury that goes along with reactive gliosis (brain trauma) or neurodegenerative diseases and aging with slow progression of synapse loss.

Therefore, in the course of this PhD project I examined host-graft connectivity using monosynaptic rabies virus (RABV) tracing in cortical stab wound (SW) injury, intact, and inflamed cortical conditions in adult mice to evaluate if and to which extent these conditions integrate transplanted fetal neurons. In addition, I investigated graft integration in brain environments of progressive amyloidosis going along with synapse loss as observed in Alzheimer's disease (AD) and of healthy aging to explore any influence of the aging brain environment *per se.*

Indeed, in all these different host environments the grafted fetal neurons survived, differentiated, and integrated by forming connections with the correct host input regions. Surprisingly, brain-wide connectivity analysis showed that the grafts received excessive inputs from local neurons in the SW-injured, amyloid-plaque loaded, and aged environment. On the other hand, there was quantitatively fewer neuron integration in intact young control brains and in brains exposed to Lipopolysaccharide (LPS) induced inflammation as opposed to the massive input connections observed in the other conditions. Thus, new neurons integrate independent of prior neuron loss or mild reactive gliosis as grafted cells formed connections even in conditions where neuron loss did not occur. State-of-the-art proteome analysis using mass spectrometry (MS) revealed the protein compositions of these host cortical environments promoting excessive synaptic integration.

This data provides important and highly relevant insights for the design of cell-based therapies for brain trauma and neurodegenerative diseases that go along with synapse loss.

Understanding the mechanism that promote synaptic integration will open new avenues to modulate certain parameters in order to achieve adequate functional repair of lost neurons and synaptic connections.

INTRODUCTION

1. Introduction

The vertebrate brain is an overly complex interconnected network of millions of nerve and glial cells, which makes it the most complicated organ of the body. Trauma or central nervous system (CNS) disease disturb this delicate network, which can cause a variety of complications. Therefore, there is a compelling medical need for replacement of neurons that degenerated after brain injury or in any neurodegenerative disease (Grade and Götz, 2017; Barker, Götz and Parmar, 2018). Transplantation of exogenous neuronal cells is broadly studied since years and by now the most promising approach in the field of brain repair. Moreover, it has successfully reached clinical trials with some extent of improvement (Tabar and Studer, 2014; Barker, Götz and Parmar, 2018). New sources of cells for transplantation deriving from pluripotent stem cells (PSC) have now further promoted the attempts for clinical trials using transplantation approaches, e.g. to treat PD patients (Stoddard-Bennett and Reijo Pera, 2019). However, cell transplantation in clinical trials has led to very variable outcome, with two PD patients experiencing improved motor behavior and some extent of recovery (Madrazo *et al.*, 1987) whereas there was no improvement at all in other cases (Backlund *et al.*, 1985; Barker, Drouin-Ouellet and Parmar, 2015). Therefore, the field of cell-based brain repair, especially regarding transplantations, needs more basic research to understand why grafted neurons led to a beneficial outcome in only a few cases. To make neuronal replacement more reliable, efficient, and safe for patients, many aspects need to be studied more closely to know how new neurons survive, integrate, connect, and function in an existing brain network that is in need of repair. The following introduction will explain changes of the brain parenchyma due to injury or disease to understand the complexity of cellular and molecular changes that can occur. Moreover, the status quo in the field of brain repair, important conceptual questions, and the methodology applied in this PhD project will be described.

1.1 Brain injury and diseases

Before exploring repair mechanisms that could be used to treat CNS impairments, it is crucial to understand how injury and disease affect the brain dynamics including different cell types and their connections. Trauma, slowly progressing neurodegeneration, or physiological aging itself

can differently affect the brain and have been explored more intensively in recent years to understand how plastic the brain reacts to these conditions. Brain injury and age-related neurodegenerative diseases can both lead to dysfunction of sensory and motor networks and eventually to cognitive decline, depending on the affected region. The better we understand the processes and consequential reaction of the brain upon injury or disease, the better repair strategies can be applied and improve certain conditions.

1.1.1 Acute brain injury

The complex architecture of the brain with its precise network can be easily disturbed by traumatic brain injury (TBI). This affects not only neuronal cells and their connections but also other cell types like glial cells and blood-derived immune cells. TBI goes along with irreversible loss of neurons and thus disruption of normal brain functions, which is problematic since the mammalian brain is not able to replace lost neurons. Focal acute injuries, occurring at a specific location of the brain and diffuse (multifocal) or chronic injuries, being more widespread, cause glial cells to react with different mechanisms and responses triggered depending on the type of insult. Acute injury, often modeled through cortical SW in animals (for example described in Buffo *et al.,* 2005), leads to disruption of blood vessels and thus blood brain barrier (BBB). The consequent decrease in blood supply, oxygen, and glucose leads to neuron depolarization, increased neurotransmitter levels, excitotoxicity, and subsequent neuron death. Other death-promoting mechanisms after injury can involve formation of free radicals, inflammatory molecules, and apoptosis (Leker and Shohami, 2002). Besides neuronal loss, different non-neuronal cell types, mainly astrocytes, microglia, and NG2 glia rapidly react to the insult and contribute to inflammation processes.

Astrocytes were shown to be very heterogeneous after injury as they become reactive or proliferate, and even acquire stem cell properties (Bardehle *et al.*, 2013; Sirko *et al.*, 2015). Upon tissue damage, astrocytes are disturbed in their function, especially in their normal interactions with neurons to supply energy (Pellerin *et al.*, 2007), controlling the BBB and blood flow (Iadecola and Nedergaard, 2007), and modulating synapses (Allen and Eroglu, 2017; Eroglu and Barres, 2010; Pekny and Pekna, 2016). During the first days after injury, they are becoming reactive (represented by upregulation of glial fibrillary acidic protein, GFAP), are involved in tissue

remodeling, and develop a hypertrophic (swelling) morphology, which is triggered through cytokines such as transforming growth factor (TGF) or Interleukin 6 (IL-6) (Burda and Sofroniew, 2014; Dimou and Götz, 2014; Pekny and Pekna, 2016). Moreover, they are involved in scar formation to form a protective border between the lesion core and the surrounding healthy parenchyma to avoid entrance of inflammatory cells to the intact tissue.

Microglia, the brain resident immune cells, which have a ramified morphology and surveil the brain parenchyma (quiescent state) under healthy conditions, become hypertrophic and amoeboid in their morphology upon activation (reactive state). They start to proliferate and migrate to the lesion site as opposed to astrocytes that stay in place when becoming reactive (Bardehle *et al.,* 2013; Burda, Bernstein, and Sofroniew, 2016; Sofroniew and Vinters, 2010). Moreover, they remove debris through phagocytosis and are also involved in glial scar formation (Nimmerjahn, Kirchhoff and Helmchen, 2005; Burda and Sofroniew, 2014; Karve, Taylor and Crack, 2016; Pekny and Pekna, 2016). NG2 glia also quickly react to injury by becoming hypertrophic, proliferative, and accumulating around the injury site through migration, and thus being part of the glial scar and wound healing process (Hughes *et al.*, 2013; Dimou and Götz, 2014).

Lastly, days after the insult, the injured tissue will be remodeled and convert into a glial scar formed by astrocytes, microglia, and NG2 glia, which was long time believed to be a barrier for CNS regeneration. By now it is known that the glial scar has also beneficial effects that support the repair of the brain and protect neuronal tissue from the inflamed lesion core (Burda and Sofroniew, 2014). The complex interplay of different cell types leading to remodeling of brain tissue needs to be considered for brain repair strategies as it can affect the performance of new neurons. The diverse processes involved in acute injury differ from age-related neurodegenerative processes. Therefore, they need to be well distinguished for neuronal replacement approaches so that repair mechanisms can be adjusted depending on the condition.

1.1.2 Aging and Neurodegeneration

Compared to the above-described changes that occur after acute injury, the aging or neurodegenerating brain reacts differently as the involved processes slowly progress. The aging brain is characterized by plenty of changes on cellular and molecular levels and is the main risk factor to develop neurodegenerative disorders (Hou *et al.*, 2019). The functional capabilities of cell organelles decline with age, which includes e.g. mitochondria that support synaptic transmission, cell maintenance, and repair under healthy conditions (Mattson, Gleichmann and Cheng, 2008). They become dysfunctional as observed for example by mitochondrial enlargement, fragmentation, or DNA damage (Mattson and Arumugam, 2018). Moreover, neurons accumulate molecules that were damaged through oxidative imbalance or/and were not properly degraded by the cellular machinery due to impaired lysosome and proteasome functions. Additionally, neurons lose their ability to maintain calcium homeostasis, which is very important for fine-tuning of signals. The integrity of synaptic activity becomes perturbed due to excitatory imbalance as a consequence of impaired GABA signaling. These process are involved in cognitive decline or depression, which is often observed in elderly patients, depending on the affected brain region (Mattson and Arumugam, 2018). Another characteristic of brain aging is the progressively increasing inflammation. Especially microglia and astrocytes become more and more reactive, which is to some extent similar to the described glial reactivity in acute injury conditions except that it rises more slowly over a long period of time during aging (Norden and Godbout, 2013; Rodríguez-Arellano *et al.*, 2016).

Microglia are known to develop a primed phenotype during aging or neurodegeneration, which is depicted in less mobility and exaggerated response to inflammatory challenges (Niraula, Sheridan and Godbout, 2017). Moreover, it was shown that astrocytes can lose their capacity to proliferate properly in the aged injured cortex (Heimann *et al.*, 2017), which indicates that although astrocytes become more reactive with age they also become dysfunctional. It was believed for a long time that a hallmark of aging is neuron loss and thus decreasing brain mass (Ball, 1977; Coleman and Flood, 1987), which was later shown not to be the case. It is now accepted that age-related changes and disease rather base on region-specific network dysfunction and general impaired synaptic plasticity but also on the aforementioned cellular changes (Burke and Barnes, 2006; Yankner, Lu and Loerch, 2008).

One main characteristic of brain degenerative conditions is misfolding, abnormal accumulation and aggregation of proteins like amyloid beta (Aß) (Stroo *et al.*, 2017). The term "neurodegeneration" defines a state in which neurons progressively lose structure and function, which is caused by abnormal protein accumulation and related diseases are until today still incurable. Under physiological conditions proteins undergo constant quality control for example through chaperones stabilizing them or proteasomes degrading misfolded proteins. With age or in neurodegenerative conditions this quality control declines, which results in abnormal aggregation of proteins (Stroo *et al.*, 2017). Neurodegenerative diseases can vary in their pathology because different anatomical regions of the CNS and different neuronal subtypes can be affected. For example, in PD, one prominent characteristic is the loss of dopaminergic neurons in the substantia nigra, which can lead to severe motor symptoms, whereas in AD broad amyloid-plaque deposition in mostly hippocampus and cortex leads to dementia (Przedborski, 2016; Stroo *et al.*, 2017).

In AD, extracellular Aß plaques and intracellular neurofibrillary tangles progressively accumulate. Aß plaques are a consequence of miscleavage of the amyloid precursor protein (APP). During the amyloidogenic enzymatic pathway, APP is cleaved by ß-secretase into soluble APPβ and β-c-terminal fragment, and subsequent cleavage through γ-secretase leads to Aß release into the extracellular space (Haass *et al.*, 2012). These neurotoxic Aß peptides are insoluble and accumulate to fibrils and eventually form dense plaques. There are many different transgenic animals available to study AD (listed and described in detail on www.alzforum.org/research-models). Amongst the most popular ones is for example the double transgenic mouse line APP/PS1 that overexpresses a mutated human APP (KM670/671NL) and Presenilin 1 (PS1, L166P) under the neuron-specific Thy1 promotor (Radde *et al.*, 2006). This leads to robust overexpression of APP and hence more extracellular Aß release, as well as overexpression of PS1, which is a catalytic subunit of the γ-secretase (Wolfe *et al.*, 1999; Haass *et al.*, 2012). One advantage of this AD mouse model is the early and robust onset of pathology as Aß plaque deposition appears from six weeks of age on and progressively increases. This goes along with hypertrophic reactive microglia accumulating around plaques and increasing astrogliosis whereas no global neuron loss is observed (Radde *et al.*, 2006; Rupp *et al.*, 2011; Figure 1). With respect to reactive gliosis, it was found that amyloid-plaque affected astrocytes differ from injury affected astrocytes. GFAP+ astrocytes barely showed proliferative potential in APP/PS1 mice and

most proliferative cells were found to be NG2 glia or microglia (Sirko *et al.*, 2013). Generally, astrocytes are known to undergo molecular changes in AD compromising neuronal communication for instance (Pekny, Wilhelmsson and Pekna, 2014; Osborn *et al.*, 2016). In addition to the increasing Aß plaque load, APP/PS1 mice develop early dendritic spine loss, especially around plaques (Bittner *et al.*, 2012). These characteristics make this mouse model very suitable to study AD because the described features represent many of the neuropathological hallmarks of AD. For these reasons, I used the APP/PS1 mice to examine how such a host neurodegenerative brain environment synaptically integrates new neurons and thus affects repair strategies.

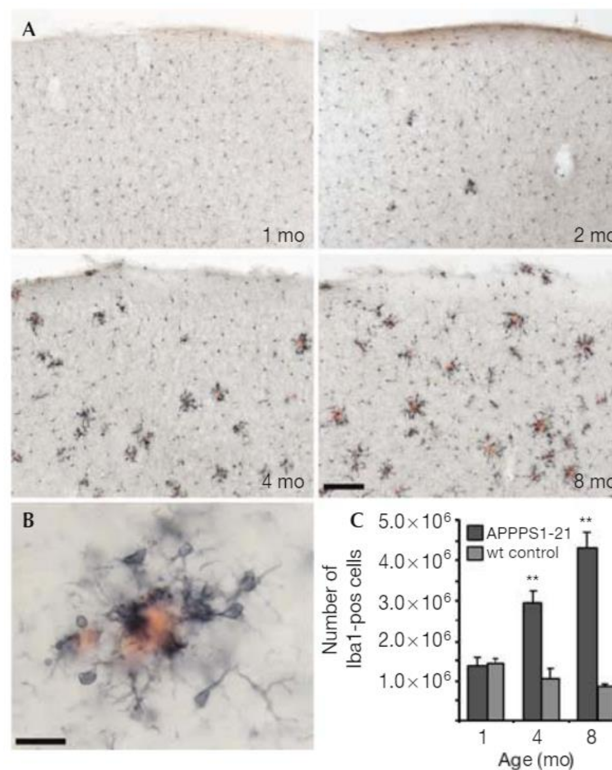

Figure 1: Activated microglia and amyloid plaque deposition in APP/PS1 mouse model (A) Amyloid plaque deposition is accompanied by gliosis in APP/PS1 transgenic mouse cortex at different ages (months = mo) showing congo-red stained amyloid and activated microglia (Iba1) surrounding the plaques. Scale bar 100 µm. **(B)** Clustered microglia around plaques in high magnification. Scale bar 20 µm. **(C)** Quantified number of Iba1+ microglia of transgenic and non-transgenic (wildtype = wt) control mice showing significant increase of microglia with age. (from Radde *et al.* 2006; License number 4957140905020, 27.11.2020).

The above-described changes and impairments in the aging or neurodegenerative brain make these environments especially interesting to investigate neuronal replacement strategies.

Neuron transplantation aims to be applied in mostly elderly patients, which often suffer from neurodegeneration-related pathologies that involve gliosis and synaptic changes or loss rather than actual neuron loss. So far, different regenerative approaches were studied in mostly younger brains or in injury environments in which neurons were lost, but the field of brain repair is beginning to examine aging and neurodegeneration as well more closely. The status quo and examples of brain repair strategies applied to brain trauma and neurodegenerative conditions will be explained in the next chapter.

1.2 Brain repair strategies

Three different approaches for neuronal replacement have been described so far: recruitment of endogenous cells, reprogramming of endogenous glial cells, and transplantation of exogenous cells. However, not all are equally promising and applicable in patients, which will be explained in this chapter. The adult mammalian brain does not naturally generate new neurons, which makes neuronal replacement very interesting but also an extremely challenging field of research. The CNS shows some extent of plasticity upon injury or disease to compensate for lost neurons or synaptic connections but in cases of more severe injury or disease, the brain is not capable of self-healing.

1.2.1 Endogenous self-repair

The adult brain contains neural stem cell (NSC) niches that can continuously generate new neurons even during adulthood. The most well-known neurogenic niches are the subependymal zone (SEZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. The SEZ is at the lateral wall of the lateral ventricles and continuously produces new neuronal precursors, which migrate to the olfactory bulb, but it also generates oligodendrocyte precursors that migrate to the cortex or corpus callosum (Kazanis, 2009; Lim and Alvarez-Buylla, 2016). The NSCs of the SGZ produce only granule neurons that are thought to contribute to cognitive functions such as learning and memory (Jawad *et al.*, 2018). Upon brain insult, neuroblasts from neurogenic niches can spontaneously migrate towards injured or diseased tissue (Figure 2a, 3a). For example, studies in rodents showed that SEZ-derived neuroblasts migrated into the stroke-injured cortex

and differentiated into mature neurons (Kreuzberg *et al.*, 2010). Moreover, new neurons migrated towards the injured striatum and even developed a striatal neuronal phenotype (Arvidsson *et al.*, 2002). There is also evidence that the aged human brain generates increased numbers of immature neurons in the SEZ upon ischemia (Macas *et al.*, 2006) suggesting that there is a certain capacity of the mammalian brain for self-repair after neuron loss. However, such recruited endogenous neurons have a poor survival rate and their ability to integrate into the existing network needs to be elucidated (Lindvall and Kokaia, 2015). Therefore, the potential of these mechanisms as therapeutic approach for patients is still under debate (Grade and Götz, 2017). It is not well understood if stimulation of new endogenous neurons is efficient to replace lost neurons and thus can be applied in patients, and therefore brain repair strategies focus on two more promising strategies that are discussed below.

1.2.2 Reprogramming of endogenous cells

Another strategy to replace lost neurons is the use of non-neuronal endogenous cells within a certain brain area that can be differentiated into new neurons and thus serve for brain regeneration (Figure 2b, 3b). Several different approaches regarding the conversion of somatic cells into neurons have been investigated so far. It was shown that postnatal cortical astroglia can be reprogrammed into neurons by applying a single transcription factor and that these neurons even become functional and form synapses when cocultured with embryonic neurons (Berninger *et al.*, 2007). Reactive astrocytes from the adult injured cortex can be converted into subtype specific neurons with a similar strategy (Heinrich *et al.,* 2010). But also other somatic, non-neuronal cell types can be converted into mature neurons such as human brain-derived pericytes (Karow *et al.*, 2012), mouse fibroblasts (Vierbuchen *et al.*, 2010), peripheral blood T-cells (Tanabe *et al.*, 2018), or even human glioblastoma cells (Yuan *et al.*, 2018). After years of investigating different factors and adjusting cell culture protocols, neuronal reprogramming was shown to be successful *in vivo*. Early studies were able to prove that proliferating glia cells can be reprogrammed into immature neurons in the injured cortex (Buffo *et al.*, 2005) including NG2 glia that can convert into young neurons after injury (Heinrich *et al.*, 2014). Unfortunately, the conversion rate was rather low, and the *in vivo* generated neurons were immature and survived only a short amount of time. However, more promising results for neuronal reprogramming

appeared more recently. By administering not only neurogenic factors but also Bcl2 and vitamin D in an injury condition, reprogramming could be successfully boosted to a remarkably high conversion rate and longer survival (Gascon *et al.*, 2016). Moreover, it was recently shown that local reactive astrocytes reprogram with high efficiency into functional neurons that acquire correct layer identity and even form long-range output connections (Mattugini *et al.*, 2019). One main critique in the field of reprogramming is that it remains to be investigated if the reprogrammed cells properly integrate into the preexisting network (Bocchi and Götz, 2020). A recent publication gives hope that the restoration of function can be achieved by glia to neuron conversion. It was successfully shown, that the conversion of midbrain astrocytes into dopamine-releasing neurons was followed by reinnervation of nigrostriatal circuit and restoration of motor function in a PD mouse model (Qian *et al.*, 2020).

There are obvious advantages of using endogenous cells for brain repair. Converting local reactive glia to replace lost neurons can also inhibit scar formation (Zhang *et al.,* 2020). At the same time, there is no need to find a source of cells along with clinical manufacturing them as reprogramming targets endogenous cells anyway. In addition, reprogramming overcomes the problem of unwanted immune reactions and potential cell rejection, an adverse effect that can occur when transplanting exogenous cells. However, there are still hurdles that refrain this approach from becoming a therapeutic option for brain repair. For instance, finding the perfect route of applying reprogramming factors to the CNS or guaranteeing a good efficiency of these factors. Moreover, it still remains to be more intensively studied if induced neurons form correct output and input connections and if those connections lead to actual behavioral improvements to consider this strategy for regenerative medicine (Grade and Götz, 2017; Bocchi and Götz, 2020). Overall, transplanting exogenous cells to replace lost neurons is by now the most advanced approach showing great potential in the field of brain repair.

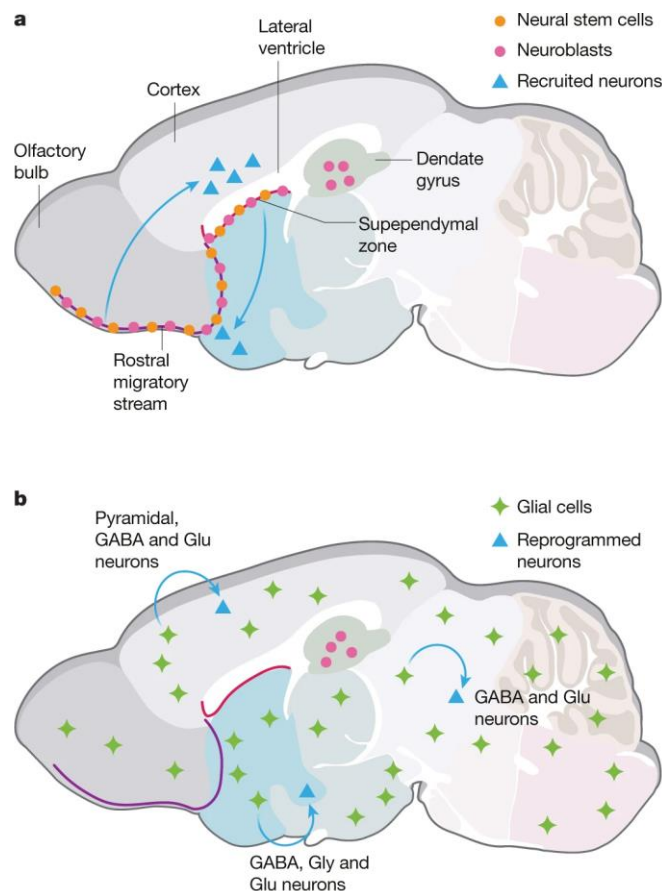

Figure 2: In situ brain repair (a) New neurons can be recruited from neurogenic niches to the olfactory bulb (via the rostral migratory stream) or to the cortex. **(b)** Direct reprogramming of local glial cells of different brain regions into neurons of even specific subtypes. (from Barker, Götz, and Parmar 2018; License number 4957141342130, 27.11.2020).

1.2.3 Transplantation of exogenous cells

When a critical number of neurons is lost, reaching functional recovery through transplantation is a very promising option and has been studied broadly. To yield great results there are important aspects that need to be considered. For instance the perfect neuronal cell type, an efficient number of cells, the suitable anatomical location for engraftment, and whether additional treatments are needed to guarantee a successful repair (Barker, Götz and Parmar, 2018). These issues are being investigated since a while and examples of the achieved successes will be pointed out in this chapter. Transplanting immature neurons into injured or diseased brains is a very favorable approach and the first clinical trials were already conducted in the late eighties when human fetal dopamine neurons were transplanted into PD patients (Lindvall *et al.,* 1988; Lindvall *et al.,* 1990). Unfortunately, the results from several clinical trials showed high

variability regarding motor improvements (Barker *et al.*, 2013) suggesting that neuron transplantation still needs improvement to be the best therapeutic approach.

Different types of donor neurons can be used for transplantation and most promising results were achieved with fetal neurons of adequate regional identity as donor cells (Grade and Götz 2017, Figure 3c). For example, after transplanting fetal mesencephalic dopaminergic neurons into PD rodent models the grafted cells were shown to survive, mature, and reinnervate the striatum and alleviate motor symptoms (Barker *et al.,* 2013; Grealish *et al.*, 2010). In other studies, the potential of fetal projection neurons transplanted into the neocortex was investigated. It was shown that donor neurons formed long-distance connections to host brain areas after induced neuronal loss in the somatosensory cortex (Hernit-Grant and Macklis, 1996) or donor cells reaching even far-distant anatomical regions like the spinal cord after transplantation into the lesioned motor cortex (Gaillard *et al.*, 2007). More recently, it was revealed that fetal cortical neurons transplanted into the lesioned visual cortex form adequate afferent and efferent connections with the host circuitry that even very closely match the endogenous visual cortex circuitry. In addition, the grafted cells became functional, which was demonstrated by *in vivo* calcium imaging of donor cells after visual stimulation (Falkner *et al.*, 2016). This proof of principle study showed that grafted cells not only integrate into the host network, but the level of synaptic integration is very precise as it almost perfectly reconstructs the endogenous synaptic connections of the specific visual cortex connectome. Primary fetal neurons have also been probed in AD-related animal models. For example, immature interneurons have been transplanted into the hippocampus of an AD mouse model and they matured, integrated into the host brain, and also restored learning and memory function (Tong *et al.*, 2014). Furthermore, genetically modified embryonic medial ganglionic eminence neurons were engrafted into AD mice and led to improved cognition as well (Martinez-Losa *et al.*, 2018).

Besides fetal neurons, neurons from other cell sources have been tested in transplantation studies such as embryonic stem cells (ESC), PSCs, or NSCs (Figure 3c). The latter are expandable *in vitro* into neurospheres and can be differentiated into any desired neuronal cell type. Moreover, an important feature of NSCs is that they are multipotent and generate neurons and glia cells, which makes them an interesting source of cells for different CNS diseases affecting specific neuronal subtypes (Bonnamain, Neveu and Naveilhan, 2012). Early transplantation studies have shown that NSCs differentiate into neurons when grafted into the adult lesioned

mouse cortex (Snyder *et al.*, 1997). Shortly after, neural precursors were differentiated into dopaminergic neurons *in vitro* and grafted into the striatum, which lead to recovery in a PD rat model (Studer, Tabar and McKay, 1998). Moreover, even human tissue was tested for its capacity to generate subtype specific neurons. Indeed, human fetal midbrain precursors transplanted into the striatum of Parkinsonian rats survived and differentiated into a dopaminergic subtype (Sánchez-Pernaute *et al.*, 2001). Human NSCs have also been tested in mouse models of AD and were shown to differentiate into neurons and glia, promoting synaptic growth, and improving cognition (Ager *et al.*, 2015). On the other side, opposing evidence occurred as transplanted human NSCs failed to improve cognition or enhance synapse growth in an AD model (Marsh *et al.*, 2017) indicating that more research is needed to investigate the mechanisms that lead to actual improvement and assure efficacy.

Using ESCs also offers a cell state of pluripotency and thus, employing these cells for transplantation is yet another interesting option. Therefore, it is not surprising that these cells were broadly investigated for brain repair, e.g., by transplanting mouse ESC-derived neurons into the lesioned mouse brain in which the grafted cells migrated towards the injured tissue and proliferated (Srivastava *et al.*, 2006). Moreover, several studies examined the potential of ESC-derived neurons in the developing or postnatal rodent brain. These revealed migration and synapse formation of grafted neurons (Wernig *et al.*, 2004) and differentiation and maturation of human ESC-derived neurons transplanted into newborn mice (Koch *et al.*, 2009). Moreover, it was shown that human neurons placed into the rat striatum form long-distant axonal projections (Denham *et al.*, 2012), or they mature, develop correct target projections, and functionally integrate into the cortex of newborn mice (Espuny-Camacho *et al.*, 2013). ESC-derived neurons were also studied for their therapeutic potential towards PD and AD. For example, dopamine neurons derived from human ESCs and grafted into a PD rat model showed long-term survival and functionality, formation of specific afferent and efferent connectivity, and motor function restoration (Grealish *et al.*, 2014). Optogenetic silencing experiments even proved that human ESC-derived neurons grafted into a mouse model of PD were responsible for the recovery of motor deficits (Steinbeck *et al.*, 2015). ESC-derived neurons being amyloid precursor protein deleted (APP-/-) were grafted into an AD mouse model and lead to improved cognition and reduced AD-pathology (Zhao *et al.*, 2020) suggesting that ESC-derived neurons can become a therapeutic tool to mitigate AD. However, the use of human ESCs still raises ethical

issues as it is debated whether a blastocyst is considered as human life or not and can hence be used for brain repair strategies (Baldwin, 2009).

Induced PSCs (iPSCs) overcome these ethical issues because easily accessible adult human tissue can be converted back into a pluripotent state followed by conversion into the desired CNS cell type. Another advantage of iPSCs is that patient-specific donor cells can be created, and thus immune rejection can be bypassed. Therefore, many studies examined iPSCs-derived neurons for their therapeutic potential. In one of the earlier studies, neurons derived from converted fibroblasts were transplanted into fetal mouse brains and migrated, differentiated into neurons and glia, and became functionally integrated as shown by electrophysiological recordings (Wernig *et al.*, 2008). Human iPSCs transplanted into stroke-injured rodent striatum and cortex matured into different neuronal subtypes and integrated into the host brain by forming afferent and efferent connections with the host neurons (Oki *et al.*, 2012). Moreover, transplanted human iPSCs GABAergic neurons synaptically integrate into the neonatal mouse brain by showing mature electrophysiological properties (Sun *et al.*, 2016) and a small-molecule approach accelerating the induction of cortical neurons also showed that grafted neurons become functional, fire action potentials, and form axonal projections in the mouse cortex (Qi *et al.*, 2017). A recent promising study demonstrated that human iPSCs-derived cortical neurons transplanted *ex vivo* onto human organoids mature and form functional synapses, providing first experimental evidence for a successful human-to-human cortical engraftment (Grønning Hansen *et al.*, 2020).

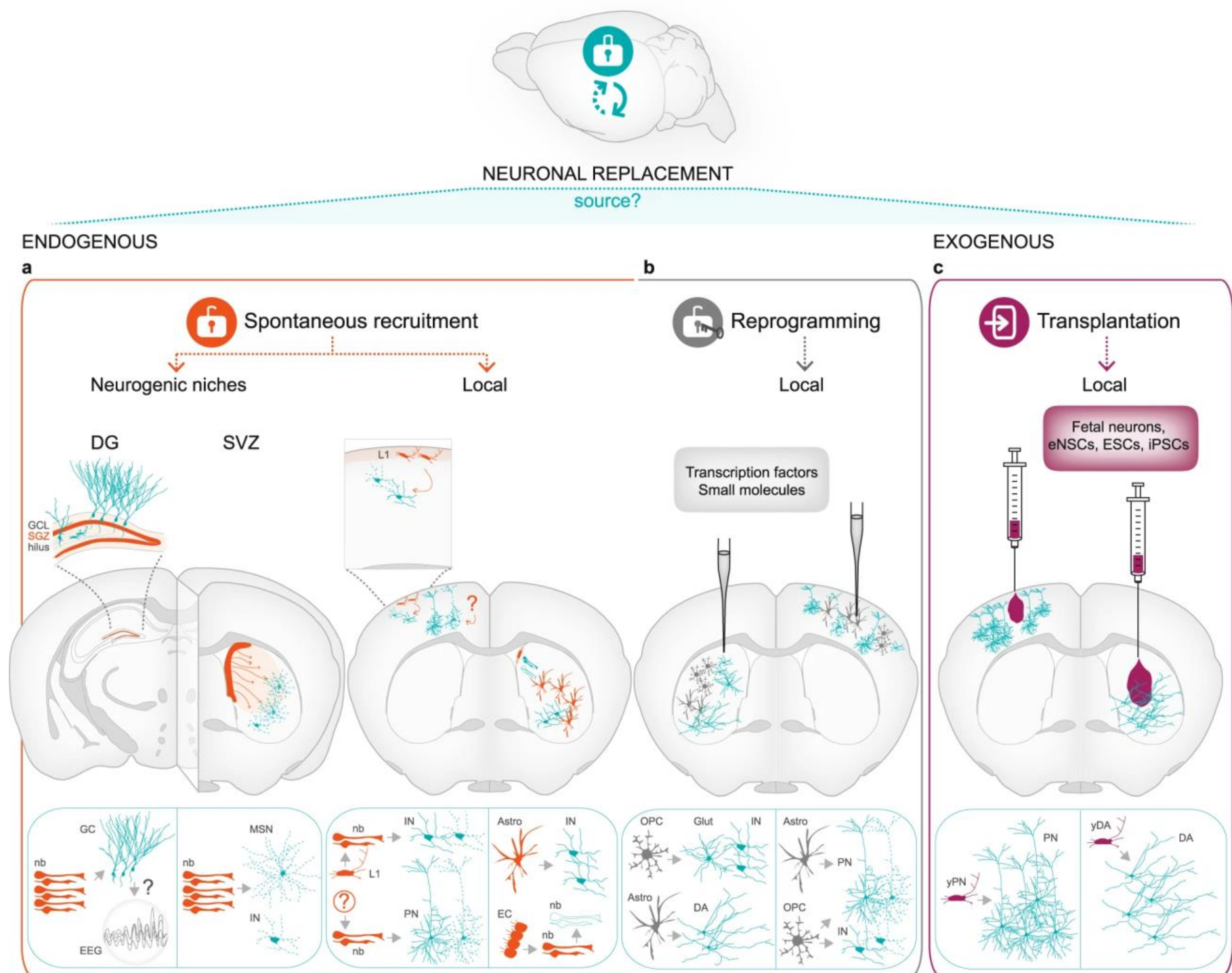

Figure 3: Neuronal replacement strategies (a) endogenous cell sources from neurogenic niches like the subgranular zone (SGZ) of the dentate gyrus (DG) or the subventricular zone (SVZ) at the lateral ventricle walls that can produce neural progenitors. **(b)** Local endogenous sources of new neurons through reprogramming of glia cells. **(c)** Exogenous cell sources for transplantation of immature neurons into different brain regions. (adapted from Grade and Götz 2017, this is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

Besides the aforementioned achievements of the different cell sources that can be used for transplantation studies, other aspects need to be investigated in more detail to successfully bring the field towards clinical application. For instance, it still needs to be examined if donor axons become properly myelinated, if their speed of signal transmission is similar to the lost neurons, and if grafted cells develop into the specific neuronal subtype that is needed. Furthermore, the precise connectome of donor cells within a pre-existing host network is another aspect to address in the field of brain repair (Grade and Götz, 2017; Barker, Götz and Parmar, 2018). Output connectivity of grafted cells has been broadly studied (Hernit-Grant and

Macklis, 1996; Gaillard *et al.*, 2007) but input connectivity was investigated only more recently (Falkner *et al.*, 2016; Espuny-Camacho *et al.*, 2018; Linaro *et al.*, 2019; Grønning Hansen *et al.*, 2020; Palma-Tortosa *et al.*, 2020) and still needs to be studied further. Appropriate integration is key to fully repair lost neurons and their synaptic connectivity instead of causing over- or under-connectivity, which could harm the existing host network. To learn about how transplanted neurons integrate into the host circuitry *in vivo*, transneuronal tracers can be used to visualize synaptic connections and will be explained in the next chapter.

1.3 Connectivity analysis of transplanted cells

Connectomics is a subfield of neuroscience that aims at comprehensively mapping the organization of neural connections. There is only one organism to date of which a full microscale map of all neuronal connections was established, which is *Caenorhabditis elegans* (White *et al.,* 1986; Cook *et al.,* 2019). The brain connectome can be analyzed on a microscale (network of neurons), mesoscale (network of cortical columns and circuits), and macroscale (network of brain areas) level (Oh *et al.*, 2014; Sporns and Bullmore, 2014). In the context of my PhD project, I examined the input connectome formed between the host and the graft neurons on a mesoscale level.

As mentioned before, the generation of appropriate connections of grafted cells within the host brain network is necessary to fully repair for lost neurons and synaptic contacts. There are different ways of exploring newly generated connections between host and graft cells. To understand if correct and long-distance output connections of transplanted neurons are formed, axonal outgrowth to different host brain areas can be studied. It has been shown in many different settings employing various neuronal cell types that the graft forms correct and long-distance output connections (Gaillard *et al.*, 2007; Denham *et al.*, 2012; Jaber *et al.*, 2013; Cardoso *et al.*, 2018; Wuttke *et al.*, 2018; Besusso *et al.*, 2020; Dong *et al.*, 2020). Aside of that, input connections of new neurons can be investigated as well. For example by fluorogold injections that trace retrograde along the axon one can easily visualize input connections to a cell group of interest (Deschênes *et al.*, 2005; Schmued, 2016; Mattugini *et al.*, 2019). This technique allows intense long-term labeling but does not offer a synapse-specific labeling of

directly connected neurons. This can be achieved by viral tracers (Falkner *et al.*, 2016; Grade and Götz, 2017), which will be explained in more detail.

To map synapse-specific connections of a given neuronal circuit, viral tracers are applied. These are taken up by a neuron and transported to the connected partner making connections between individual cells visible. Many viral tracers have the advantage of being neurotropic and replicating within the transfected neuron and thus, guaranteeing intense labelling of the connected partner (Xu *et al.*, 2020). Anterograde tracers such as herpes simplex virus are helpful to investigate postsynaptic output connections and axonal projections (Zeng *et al.*, 2017; Beier, 2019; Xu *et al.*, 2020). On the other side, retrograde tracers visualize synaptic input connections to a certain neuron population.

The most specific and often probed option for short and long-range transsynaptic retrograde tracing is the rabies virus (RABV). It is an enveloped, bullet-shaped, negative single-stranded ribonucleic acid (RNA) virus belonging to the family of rhabdoviruses. It encodes for five structural proteins, a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a RNA polymerase (L), and a surface glycoprotein (G), which is involved in virus infectivity (Schnell *et al.*, 2010; Davis, Rall and Schnell, 2015). The virus binds to the host cell receptor at the axon terminal, enters through endocytosis, and is transported in vesicles along the axon to the cell body. The viral genome is released to the cell cytoplasm and new viral particles are produced and assembled using the host cell machinery to be released again (Schnell *et al.*, 2010; Davis, Rall and Schnell, 2015). This virus is known to propagate retrogradely along axons (Klingen, Conzelmann and Finke, 2008), which makes it therefore useful for investigating input connections. Unless other viral tracers, RABV does not cause cell lysis, which means that viral particles will not be randomly released (Ginger *et al*., 2013).

Since 2007, RABV started to become a famous tracing tool in neuroscience as it allows to specifically map monosynaptic connections. It was first developed into a deletion-mutant encoding enhanced green fluorescent protein (eGFP), which allows detailed visualization of neuronal morphology with bright fluorescence that is visible already two days after infection. By deleting the G-protein from the virus genome, the RABV became unable to transsynaptically spread but was still capable to replicate in the infected host cells and to produce high levels of eGFP (termed SADΔG-eGFP; Figure 4; Wickersham *et al.,* 2007). Moreover, the tropism of the RABV was modified to make it target only genetically manipulated defined cells. It was enveloped

(pseudotyped) with the avian sarcoma leucosis virus glycoprotein (EnvA) so that exclusively cells expressing the related avian tumor virus receptor A (TVA) can be infected by RABV. This receptor does not naturally occur in mammals but only genetically modified cells that express TVA will be infected. Additionally, RABV spread will be monosynaptic because only the initially infected cells (expressing TVA) were also genetically modified to express the G-protein. Through these modifications, the virus will transsynaptically spread to the directly connected presynaptic partners but will not spread further as the presynaptic partners lack the G-protein (Figure 4; Wickersham, Lyon, *et al.*, 2007; Xu *et al.,* 2020).

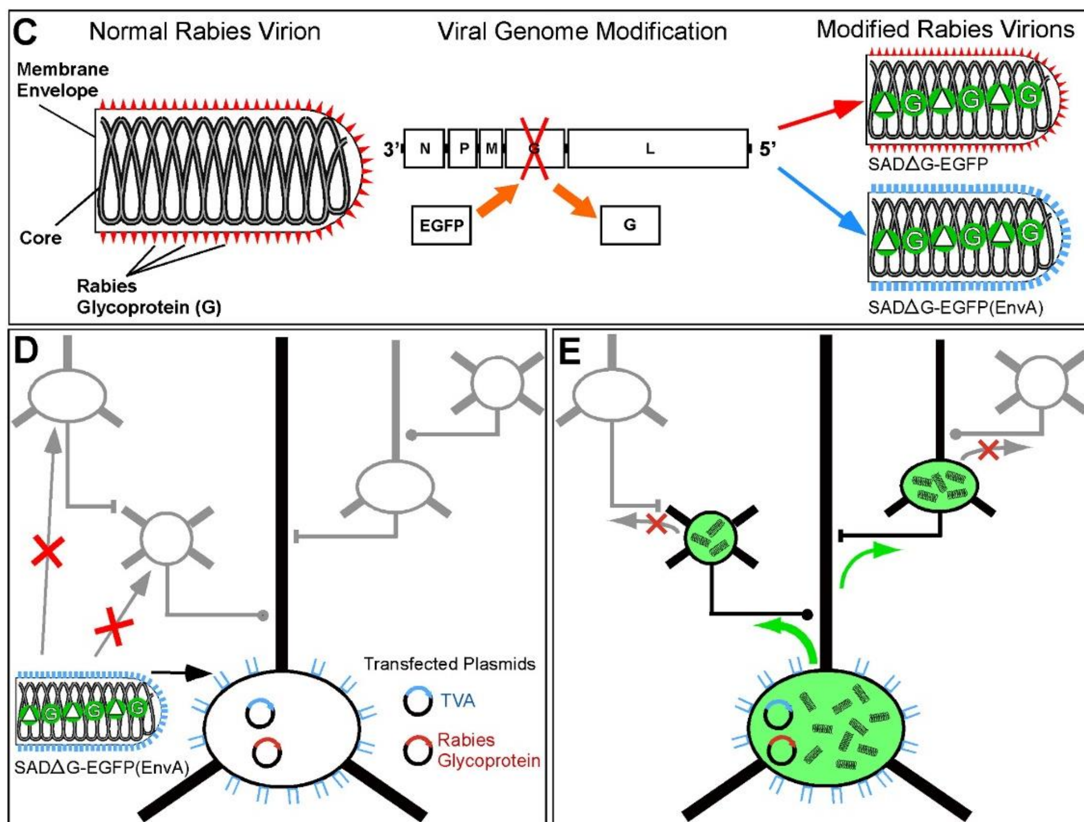

Figure 4: Deletion-mutant and pseudotyped rabies virus (RABV) in which the glycoprotein (G) is deleted from the viral genome to inhibit viral spread and eGFP is inserted to visualize infected cells (SADΔG-eGFP). This deletion mutant is unable to infect any mammalian cell. Additionally, the RABV is enveloped with EnvA to infect exclusively cells expressing the TVA receptor (SADΔG-eGFP(EnvA)). By providing rabies G-protein and TVA receptor to a certain neuron (for example via transfected plasmids), RABV infection and its transsynaptic spread to presynaptic partners is possible. (adapted from Wickersham, Lyon, *et al.,* 2007; License number 4957150181404, 27.11.2020).

There are several approaches to deliver the transgenes TVA and G-protein into a desired cell population like transfection through electroporation, via retrovirus, lentivirus, or adeno-associated virus-mediated transduction, or Cre-recombination in transgenic mice (Ginger *et al.*,

2013). This sophisticated technique enables us to examine particularly monosynaptic short and long-range connections of defined neurons (for example host-graft connected neurons). Moreover, the RABV tracer tool can be combined with other transgenes to manipulate circuit function. This tracer provides information about type, localization, and number of synaptic contacts and is thus preferably used these days in transplantation studies to examine graft input connectivity (Grealish *et al.*, 2015; Tornero *et al.*, 2017; Cardoso *et al.*, 2018).

This tracing system does not only enable analysis of presynaptically connected neurons to a single starter-neuron, but it also makes exact quantification of brain-wide first-order input connections feasible. In this study, I used the RABV monosynaptic tracing system to quantify brain-wide synaptic inputs to new neurons transplanted into the primary visual cortex (V1). By counting monosynaptic input connections in a given anatomical brain area and the RABV-infected donor cells (starter-neurons), a ratio of input connectivity was calculated (as described before in the study of Falkner *et al.,* 2016). By normalizing the number of directly connected presynaptic neurons to the number of starter-neurons, we can measure the extent of graft cell integration. This tracing tool proved that the endogenous input connectivity of a certain network can be properly restored by cell transplantation in a mild injury condition (Falkner *et al.*, 2016). During my PhD studies, I examined if this would be feasible as well in brain conditions of more severe injury and neurodegenerative disease.

1.4 Aim of the study

The aim of my PhD project was to investigate how different brain environments, such as acute cortical injury, aging, and neurodegeneration, would influence the synaptic integration of transplanted neurons. Achieving a better understanding of how different brain microenvironments shape the connections of grafted neurons with the host is clinically very relevant. Transplantation of exogenous cells for brain repair can only be safely applied to patients if it is known how different states of the brain will react to such repair approaches. Grafted cells need to adequately connect into the existing host network as too little or too many synaptic contacts could cause network dysfunction instead of actual repair.

The first aim was to examine the influence of a traumatic brain injury condition and of an uninjured brain condition that was triggered by systemic inflammation on the integration of new neurons. The rationale behind this is to understand how neuron death and an inflammatory stimulus affect integration of transplanted neurons. For this purpose, we transplanted fetal neurons into the core of the cortical SW-lesion, or into the intact cortex of LPS-injected animals followed by monosynaptic viral tracing of the newly connected cells and whole-brain input connectome analysis. To thoroughly investigate the composition of these two differently manipulated brain environments, we performed comprehensive proteome analysis of visual cortex tissue punches.

Secondly, I investigated brain environments that are affected by broad amyloidosis or physiological aging to understand if these conditions influence the rate of synaptic integration. Therefore, I transplanted fetal neurons into the cortex of either transgenic APP/PS1 mice that develop early brain-wide amyloid plaques or into healthy aged wildtype mice followed by monosynaptic viral tracing and brain-wide input connectivity analysis. Additionally, I also performed a comprehensive proteome analysis of these conditions to unravel mechanisms that shape the integration of new neurons in these neurodegenerative disease related environments.

My overall PhD project aim was to gain new important knowledge for brain repair approaches by investigating different brain environments with regard to their ability to integrate new cells and form new connections.

RESULTS

2. Results

2.1 Aim of the study I – Injury and inflammation

The aim of this project was to investigate how the host cortical environment impaired by an acute injury or inflammatory stimulus influences the integration of transplanted fetal neurons.

"The injury environment critically influences the brain-wide input connectome of transplanted neurons"

Sofia Grade, Judith Thomas, Karl-Klaus Conzelmann, Stefanie M. Hauck, Magdalena Götz

For this publication as co-author, I was involved in animal surgeries, and in preparation, and collection of tissue samples for mass spectrometry. I analyzed and visualized the proteome data and was involved in manuscript writing and editing.

Submitted manuscript.

Note that due to elevated number of pages Table S2 and Movie S1 are not included in the PDF version of this thesis but are available as separated files.

The injury environment critically influences the brain-wide input connectome of transplanted neurons

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Abstract

Cell transplantation is a promising approach for reconstruction of neuronal circuits after brain damage. Transplanted neurons integrate with remarkable specificity into circuitries of the mouse cerebral cortex affected by neuronal ablation. However, it remains unclear how neurons perform in a local environment undergoing reactive gliosis, inflammation, macrophage infiltration and scar formation, as in brain trauma. To elucidate this, we transplanted cells from the embryonic murine cerebral cortex into stab-injured, inflamed-only, or intact cortex of adult mice. Brain-wide quantitative connectomics unraveled graft inputs from correct regions across the brain in all conditions, with pronounced quantitative differences: scarce in intact or inflamed brain, versus exuberant after trauma. In the latter, excessive synapse pruning follows the initial overshoot of connectivity resulting in only a few input connections left. Proteomic profiling identifies candidate molecules involved in the synaptic yield, a pivotal parameter to tailor for functional restoration of neuronal circuits.

Keywords

Transplantation, Neuronal replacement, Circuit mapping, Synaptic yield, Inflammation, Injury proteome, Cell therapy

Introduction

The adult mammalian brain poorly regenerates in the aftermath of an injury and thus strategies of neuronal transplantation have been pursued, to rebuild circuits and restore behavioral function (Grade and Götz, 2017; Espuny-Camacho *et al.*, 2018; Barker *et al.*, 2019; Palma-Tortosa *et al.*, 2020). Quantitative connectomics has enabled a fine comparison of the connections developed by transplants to their endogenous counterparts (Grealish *et al.*, 2015; Falkner *et al.*, 2016; Tornero *et al.*, 2017). Previous analysis in the mouse cortex has shown that new neurons mature and connect with the host brain in a remarkably correct manner, with high specificity both in their synaptic integration and in the response to external stimuli (Falkner *et al.*, 2016). The injury, in this case, consisted of the selective ablation of a cohort of neurons in the cortex by inducing apoptosis in the targeted neurons, and implied little inflammatory reaction, no glial scar or brain-blood barrier breakdown (Sohur, Arlotta and Macklis, 2012).

While these findings are encouraging, it remains open whether accurate connectivity is achieved in more clinically relevant injuries like a traumatic brain injury (TBI), which provide a distinctive cellular and molecular milieu. While several transplantation studies described a bystander effect via neuroprotection and immunomodulation in TBI (Mahmood, Lu and Chopp, 2004; Walker *et al.*, 2012; Zhang *et al.*, 2015; Xiong *et al.*, 2018) still little is known about neuronal replacement and brain-wide connectivity in this injury. This has been probed only using grafts with low amounts of mature neurons and lacking full and quantitative connectivity analysis (Xing *et al.*, 2019). Herein, we analyzed graft connectivity in a mouse model of penetrating TBI. TBI results in severe reactive gliosis and scar formation, with infiltration of phagocytic macrophages and accumulation of high content of inflammatory molecules (Fitch and Silver, 2008; Frik *et al.*, 2018; Kjell and Götz, 2020). Molecular and cellular components of an injured brain parenchyma have been implicated in synapse pruning (Stephan, Barres and Stevens, 2012; Hong *et al.*, 2016). One may hence predict deficits in the initial synaptic integration, as synapse formation may concur with uncontrollable pruning. Microglial cells, in particular, are critical for brain wiring and synapse pruning (Schafer *et al.*, 2012) and are the first responders to brain injury, swiftly adopting an activated phenotype and initiating neuroinflammation (Kreutzberg, 1996; Prinz and Priller, 2014). To examine their contribution, we utilized a systemic injection of lipopolysaccharide (LPS), a well-documented inflammatory stimulus as additional condition complementing the TBI. LPS activates TLR4 receptor which is specifically expressed by microglial

cells in the rodent brain (Lehnard *et al.*, 2002). To achieve the least damaged environment we also transplanted into the intact cerebral cortex, only inflicted by the thin transplantation needle, as a 3rd paradigm.

Importantly, we also set out to study the maintenance of initial connectivity. A phase of active synapse remodeling with a net gain was observed by live imaging in the neurites of transplanted neurons during the first month (Falkner *et al.*, 2016). This synapse turnover then gradually reached the basal turnover rate observed in the cortex of adult mice (Hofer *et al.*, 2009) at about 2-3 months after transplantation. However, the balance may be tilted towards an excessive elimination of new synapses in an environment characterized by a reactive and inflammatory state with large amount of pruning cells. The stability of the initial connectivity in TBI conditions remains unknown. Long-term connectivity is crucial for the successful outcome of a neuronal replacement therapy and needs to be ensured prior to clinical trials. Here, we investigated the role of the host microenvironment in initial and long-term connectivity of neuronal transplants by using rabies virus (RABV) mediated tracing and brain-wide quantitative connectomics, and leverage proteomics to identify candidate molecules involved in the connectivity differences.

Results

Transplanted neurons develop complex morphologies and synaptic protrusions in cortical stab injury

To produce a brain injury in the cerebral cortex of the adult mouse brain we inflicted a stab injury or so-called stab wound (SW) within the primary visual cortex. The choice of the injury model rests on its high reproducibility and extensive knowledge about the temporal dynamics of wound healing, reactive gliosis, scar formation, as well as transcriptome and proteome (Sirko *et al.*, 2013, 2015; Frik *et al.*, 2018; Mattugini *et al.*, 2018; Kjell and Götz, 2020). Donor cells from mouse embryonic cortex expressing GFP or RFP were transplanted into the center of the incision a week later and analyzed 5 weeks post-transplantation (wpt; Figure 1A, B). By this time, the graft was confined to the site of transplantation and injury, with no or little cell dispersion, and cells had developed complex arborizations of their neurites (Figure 1C-E). Cux1 immunolabeling demonstrated that a majority of transplanted neurons displayed cortical upper layer identity (Figure 1D). These observations are in line with those in the neuronal ablation model with low

inflammation (Falkner *et al.*, 2016). Inspection at high magnification shows that a complex network of axons and dendrites has outgrown from single transplanted neurons with appreciable density of axonal boutons and dendritic spines throughout their length (Figure 1E, insets; Movie S1). Spontaneous cell-to-cell fusion events can occur between transplanted cells and central nervous system neurons although these are extremely rare (Alvarez-Dolado *et al.*, 2003; Brilli *et al.*, 2013). We tested for the occurrence of cell fusion by using Emx1-Cre/GFP donor cells (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006) and tdTomato reporter mice as hosts (Madisen *et al.*, 2010) (Figure S1A). Cell fusion upon transplantation would result in Cre-mediated recombination and expression of tdTomato. We observed no tdTomato neurons and no GFP+/TdTomato+ neurons (Figure S1B). Thus, cell fusion can be excluded in our experimental paradigm.

Together, these observations demonstrate that cells from the embryonic cortex transplanted into adult mouse cortex subjected to an invasive injury survive and develop morphological traits of mature cortical neurons, such as complex dendritic arbors and synaptic specializations.

Host environment dictates neuronal integration: injury promotes initial integration of neuronal transplants

To uncover the synaptic network established between transplanted neurons and the host brain we used a modified RABV that allows monosynaptic tracing of inputs to targeted cells (Wickersham *et al.*, 2007). In this deletion mutant rabies virus, ΔG RABV, the glycoprotein gene (G), necessary for the transsynaptic spread, is deleted, and replaced by a fluorescent reporter. Additionally, the virus envelope is EnvA-pseudotyped to infect only cells expressing TVA. As a result, the primary infection is targeted to cells co-expressing TVA receptors and the rabies G allowing trans-complementation and assembly of new infectious particles. We therefore engineered donor cells derived from embryonic day (E) 14 cerebral cortex cultured *in vitro* by retroviral infection to express RFP/G/TVA prior to transplantation, or alternatively, transplanted acutely dissociated cells from E18 Emx1-Cre/G-TVA/GFP embryos (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006; Takatoh *et al.*, 2013). Complementary GFP or mCherry-expressing ΔG RABV, injected 1 month after transplantation thus propagates retrogradely and across one synapse to the direct pre-synaptic partners. As they lack G protein, infection is halted

at these cells allowing unambiguous identification and mapping at fine scale the pre-synaptic partners of transplanted neurons brain-wide (Figure 2A). Most importantly, by combining the comprehensive anatomical registration of single cells across the entire brain with computation of the connectivity index (number of neurons in a given anatomical region per primarily infected neuron also called “starter” neuron e.g., Figure 2B) we can run comparative analysis between connecting areas or experimental groups (Falkner *et al.*, 2016).

To investigate whether neurons can connect properly within a gliotic microenvironment and the influence of a preceding injury, we transplanted reporter/G/TVA-expressing cells into either the SW-inflicted or intact primary visual cortex of adult mice, injected ΔG RABV (expressing a different reporter protein) 1 month later in the transplantation site and examined the brains 1 week afterwards (Figure 2A, C). Mapping and quantification of pre-synaptic input neurons showed the highest connectivity index for the visual cortex (primary and high-order areas) among all innervating areas in both experimental conditions (Figure 2D). However, these short-distance connections within the visual cortex were abundant for transplants in the SW, while they were extremely scarce for neurons in the intact cortex with ˜8x lower connectivity index (Figure 2D-F). Also, the global input landscape across the brain was strikingly different between these conditions, with more synaptic connections per region for neurons in the SW-injured cortex and with overall more connecting regions, compared to neurons in the intact cortex (23 versus 15 afferent regions for transplants in SW and intact cortex, respectively) (Figure 2D, E, Figure S2; see Table S1 for abbreviations). In both groups, neurons receive input from various cortical and subcortical regions of the ipsilateral hemisphere including sensory cortices, associative areas, and thalamic nuclei, but less regions are represented in the connectome of transplants in the intact cortex. A major source of inputs to the mouse primary visual cortex is the dorsal lateral geniculate nucleus (dLGN), in the thalamus, which relays visual information from the retina to the primary visual cortex (López-Bendito and Molnár, 2003). While transplants in the SW visual cortex receive a considerable input from the dLGN, those in the intact group hardly do so (Figure 2F, G). Likewise, in the latter, input from the contralateral hemisphere is reduced to a minor innervation from the visual cortex, whereas a few other cortical and subcortical regions of the contralateral hemisphere contribute to graft connectivity in the SW injury condition (Figure 2E). Thus, the injury condition has a profound impact on the input connectome of neuronal transplants.

To determine how close these connectomes were to the connectome of endogenous neurons, we used our previously published data obtained by electroporation of visual cortex neurons with the RFP/G/TVA plasmid during mouse cortical development and subsequent RABV-mediated tracing during adulthood (Falkner *et al.*, 2016). Notably, the RABV strain and helper constructs, experimentator and data analysis pipeline were the same in the present study and previous study. The overall number of afferent regions was found to be very similar with 25 regions for endogenous neurons and 23 regions for regenerated circuits after SW. However, the quantitative comparison revealed significantly higher connectivity ratios for neurons transplanted in the SW cortex (Figure 2E). Here, transplants receive input from more neurons in their surrounding visual areas. We could also appreciate a seemingly stronger innervation from the neighboring retrosplenial and ectorhinal cortices, and from the distant thalamic dLGN, a key component of the visual pathway as aforementioned. In stark contrast, the connectivity of transplanted neurons in the intact brain lags significantly behind the endogenous rates, with a local connectivity ~6x lower, and lower connectivity index for most of the afferents throughout the brain (Figure 2E). Importantly, for both groups all the identified regions are known to project to the visual cortex (Oh *et al.*, 2014; Zingg *et al.*, 2014, Allen Connectivity dataset).

Thus, neuronal integration into pre-existing circuits of the mouse cerebral cortex requires an altered local environment, but a highly inflammatory and gliotic parenchyma is overly permissive to synaptogenesis resulting in supernumerary graft-host connections.

Neuronal survival and differentiation of transplants in the intact cortex resemble those in injured cortex

One possibility explaining the poor input connectivity to neurons derived from transplants into the intact cortex, could be poor differentiation and/or survival. To explore this, we analyzed grafts at early time points after transplantation (Figure S3A). Five days after transplantation into the intact cortex neurons were immunopositive for the immature neuronal marker doublecortin (Dcx) and already projected many neurites through the host parenchyma (Figure S3B, C). These neurites display enlarged terminal structures at their tips which were reminiscent of growth cones, suggesting ongoing pathfinding. A few had reached the corpus callosum at about 300-400 μm from the transplant, indicating no major obstacle to their navigation through the cortical

tissue. By 2 wpt, spine-bearing dendrites were identifiable (data not shown) and neurons had acquired expression of the mature neuronal marker NeuN (Figure S3D). Many co-expressed the upper layer neuron marker Cux1 as observed for transplants in a SW injury (Figure 1D) or in a neuronal ablation injury (Falkner *et al.*, 2016). Finally, graft size was considered as a proxy for graft survival and no significant difference on graft size was detectable between intact or SW cortex despite a trend of the latter towards a larger size (Figure S4). We have also confirmed the absence of graft-host cell fusion in an intact environment using the same Cre-loxP combination of mouse lines as before (data not shown).

Altogether, these data indicate that also the environment of the intact cerebral cortex can nurture early neuronal development but fails to allow synaptic integration of new neurons.

LPS-elicited inflammation is insufficient to promote graft-host connectivity

One obvious difference between intact or SW-injured cortex is the activation of glial cells and the inflammatory stimuli elicited by this invasive injury (Sirko *et al.*, 2013; Götz *et al.*, 2015; Frik *et al.*, 2018; Mattugini *et al.*, 2018; Kjell and Götz, 2020). To determine if such an inflammatory component of an injury may be sufficient to elicit the circuit plasticity required for the adequate integration of new neurons, we injected bacterial lipopolysaccharide (LPS) intraperitoneal (i.p.), transplanted cells 1 week later and then analyzed their connectivity. First, we monitored the reactive gliosis elicited by different LPS concentrations at 1 week post-injection in comparison to the reaction elicited by the SW injury (Figure 3A). Chosen concentrations were previously used as an inflammatory challenge (Dénes, Ferenczi and Kovács, 2011; Erickson and Banks, 2011; Chen *et al.*, 2012) without neuronal cell death (Qin *et al.*, 2007; Chapman *et al.*, 2015). Reactive astrocytes and microglia were stained by GFAP and Iba1 respectively and showed comparable reactivity, with hypertrophic astrocytes and de-ramified microglia, after SW and with the higher LPS dose. In contrast, immunoreactivity, and glial cell morphology with the lower LPS dose was comparable to the contralateral visual cortex from SW brains (Figure 3A) or naïve mice (data not shown).

We therefore used the higher LPS dose eliciting stronger reactive gliosis to test the influence of inflammation in graft connectivity (Figure 3B). Connectivity of neuronal transplants in the cortex

with LPS-induced reactive gliosis was rather similar to those in the intact cortex, despite a small trend for a higher local connectivity (15 versus 13 afferent regions: 6,64 versus 3,91 connectivity ratio for visual-visual connections, for LPS and intact, respectively). These experiments revealed a minor or neglectable role for inflammation and the ensuing reactive gliosis in graft-host synaptic connectivity.

Proteome analysis highlights increased levels of complement proteins and reduced levels of synapse-related proteins in the SW cortex

As LPS-induced inflammation showed little effect boosting the integration of the neurons differentiating in the transplants, we pursued an unbiased proteomics approach to identify mechanisms fostering integration of new neurons in the host environment. Using a biopsy punch, we collected the host region at the time when cells would normally be transplanted, i.e., 1 week after inflicting the SW injury or of LPS injection and collected the same region from age- and sex-matched control visual cortex ("intact"; Figure 4A). By shotgun proteomics using liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) we reproducibly detected a total of 5225 proteins and quantitatively compared the samples testing for significant differences (t-test). First, we compared each of the conditions (SW/LPS) with the control samples (intact brain).

After SW injury, 221 proteins differed significantly in their abundance compared to the control (Figure 4B, Table S2a). A total of 168 proteins were significantly enriched in the injury condition including some expected proteins, such as GFAP (7.65-fold) and Vimentin (3.21-fold; Vim) which are upregulated in reactive astrocytes (Brenner, 2014; Götz *et al.*, 2015; Frik *et al.*, 2018). Likewise, the typical injury associated extracellular matrix (ECM) proteins Transglutaminase 1 and 2 and Inter trypsin alpha inhibitors (see e.g., Kjell and Götz, 2020) were significantly enriched. Consistent with an ongoing inflammatory response, GO term enrichment analysis for biological processes showed significantly enriched categories such as "acute inflammatory response" and "activation of immune response" including e.g. C3, Ighg1, Igkc, C1qa, b, c, Alpha-1-acid glycoprotein 1 (Orm1), Immunoglobulin heavy constant (Ighm), Signal transducer and activator of transcription 3 (Stat3) and Ig gamma-2B chain C region (Ighg2b) (highlighted GO terms in Figure 4D, Table S2b). We noted several complement system-related proteins, namely

complement factor C3 (C3), complement C4 B (C4b), complement C1q subcomponent subunits A, B and C (C1qa, C1qb, C1qc), complement factor b (Cfb), complement factor h (Cfh). These are involved in the classical and alternative pathways of the complement system that propagate neuroinflammation (Elvington *et al.*, 2012; Alawieh *et al.*, 2018; Roselli *et al.*, 2018) and have been implicated in synapse and dendrite remodeling in different conditions (Hong *et al.*, 2016; Presumey, Bialas and Carroll, 2017; Alawieh *et al.*, 2018). Accordingly, GO term enrichment analysis revealed the significantly enriched categories "complement activation" and "synapse pruning" that include proteins like C4b, C3, C1qa, C1qb, C1qc, Cfh, Cfb, Immunoglobulin kappa constant (Igkc), Immunoglobulin heavy constant gamma 1 (Ighg1), Integrin alpha-M (Itgam) amongst others. Consistent with the damage to the blood brain barrier (BBB) in this injury model (Petersen, Ryu and Akassoglou, 2018a), each of the three polypeptide chains forming the blood protein fibrinogen (fibrinogen alpha, beta and gamma chains, i.e., Fga, Fgb and Fgg) was significantly more abundant. Interestingly, fibrinogen induces spine elimination via microglia activation in a model of Alzheimer's disease with vascular dysfunction (Merlini *et al.*, 2019). Fibrinogen binds the same receptor as complement protein (CR3 or CD11b) (Stephan, Barres and Stevens, 2012; Petersen, Ryu and Akassoglou, 2018b) and presumably both ligands thus contribute to microglia-mediated synapse pruning in conditions involving a breach of the cerebral vasculature. We also detected 53 proteins with significantly decreased abundance after SW. These showed the significant enrichment of the GO category "regulation of neuronal synaptic plasticity" including disk large homolog 4 (Dlg4), SH3 and multiple ankyrin repeat domains protein 3 (Shank3), Glutamate receptor ionotropic NMDA 1 and NMDA 2b (Grin1 and Grin2b), Ras/Rap GTPase activating protein (Syngap1) suggesting a decrease of synaptic structures after brain injury (Figure 4B, Table S2a,b). These data are consistent with reduced neuronal excitability and firing in the acute phase after TBI (Chandrasekar *et al.*, 2019).

Comparing the LPS-induced cortex biopsies to controls, we found 123 proteins of significantly different abundance (Figure 4C, Table S2c). Amongst the 60 proteins more abundant upon LPS injection, we observed Ighg2b, Igkc and Orm1, similar to the increased proteins in the brain injury. We also found the increased GO term "acute inflammatory response" to be enriched in LPS condition (Table S2d). Amongst the 63 proteins with lower abundance were synapse related proteins like Shank3, Syngap1 and Regulating synaptic membrane exocytosis protein 4 (Rims4) suggesting some, albeit small (Figure 4E), overlap and similarity between SW and LPS conditions.

We next focused on the proteins uniquely regulated upon SW and not upon LPS as they may contribute to fostering the integration of new neurons (Figure 4E-G, Table S2e). Indeed, the activation of the complement system, synapse pruning, and immune system proteins like C4b, C3, C1qa, b, c, Itgam, Ighg1 were specific in this environment, as was the enrichment of GFAP, Vim, Fga, Fgb and Fgg. Also, the downregulated proteins Dlg4, Grin1, Grin2b, and others that are involved in synaptic plasticity were injury specific.

Taken together, proteome comparison of microenvironments eliciting low and high graft connectivity reveals fibrinogen and complement activation as possible mechanisms that may promote integration of new neurons into synaptic networks of the host brain. At the same time, these findings led us to ask if their integration is stable or synapse pruning lingers for a longer period and eventually affects also newly formed host-graft connections.

Excessive graft-host connectivity in cortical stab injury is transient

Given the above observation that fibrinogen and complement factors are particularly abundant in the SW condition, and their documented role in synaptic pruning, we set out to determine the persistence of new synapses after the initial synaptic integration in this condition. In the neuronal ablation model, we had observed a net increase in spine number in the first month, followed by 1 more month of active spine turnover with no overall change in input connections and a stable connectivity ratio between 1 and 3 mpt (Falkner *et al.*, 2016). We therefore traced input connectivity at 3 mpt in SW cortex and compared to the data obtained at 1 mpt (Figure 5A). Surprisingly, by 3 months, connectivity with the host brain was severely diminished, with about 3-fold fewer local connections (Figure 5B, C) and a total of 13 afferent areas as opposed to the earlier 23 (Figure 4C, D). Importantly, connectivity had dropped to metrics below those of normal circuits (endogenous connectivity) with only 5 as compared to 12 afferents with connectivity ratio ≥ 0.05 respectively (Figure 4E). Most of the weaker connections (connectivity ratio ≤ 0.03) observed at 1 mpt had been lost, with e.g., only one contralateral innervation persisting, from the contralateral visual cortex. These results imply that early formed synapses with transplants in SW are eliminated in the course of the following months and thus call for caution and the need to explore specific injury conditions prior to use in neuronal replacement therapy.

Discussion

Despite the broad experimental use of neuronal transplantation for cell replacement in cortical brain injury, an outstanding open question is still whether the injured environment, variable across pathologies, plays a role in the integration of transplanted neurons. Our data show that the integration of neurons into pre-existing circuits is highly influenced by the local environment where they develop. While the transplant survival and gross morphological maturation share features across different hosting environments, its degree of input connectivity with the host circuits and stability of these new connections differs substantially.

By transplanting the same donor cell type as previously in a neuronal ablation model (Falkner *et al.*, 2016) now in a penetrating traumatic brain injury we demonstrated pronounced differences in initial and long-term input connectivity, with initial excessive inputs from the host networks that were eventually pruned to sub-normal levels. In contrast, neurons grafted in intact or LPS-induced networks were scarcely innervated by the host brain.

Neurons derived from mouse embryonic stem cells fail to project long-distance axons after transplantation if no injury is previously inflicted, as opposed to an injured brain, despite good graft survival in both experimental groups (Michelsen *et al.*, 2015). Transplantation into the intact dentate gyrus or striatum of adult immunodeficient Rag2-/- mice yielded widespread inputs to large transplants, but their quantitative nature is unknown (Doerr *et al.*, 2017). Interestingly, cells from the spinal cord of rat embryos transplanted into the adult rat spinal cord fail to contact nearby corticospinal axons if no spinal cord injury precedes (Kadoya *et al.*, 2016). Also, transplantations of embryonic neurons in the postnatal mouse cortex have suggested that a preceding apoptotic injury promotes morphological maturation and synapse formation between the host and graft (Andreoli *et al.*, 2020). Clearly, there are obstacles to the development of graft outputs and inputs in the intact central nervous system. Our findings add knowledge by showing a priming effect of an injury opening brain networks to accommodate new neurons and form new synapses in the adult cerebral cortex. On the flipside, it became now obvious that not all injuries lead to the same level of host-graft connectivity in the long-term.

Several previous studies have shown that neuronal grafts in the injured postnatal or adult rodent cortex receive inputs from the host brain (Michelsen *et al.*, 2015; Falkner *et al.*, 2016; Tornero *et al.*, 2017; Xing *et al.*, 2019; Andreoli *et al.*, 2020). However, a comprehensive comparison of

the connectivity levels with native circuits has been a more challenging endeavor addressed by only a few studies (Falkner *et al.*, 2016; Tornero *et al.*, 2017). Based on our findings herein using the stab wound injury, we could hypothesize that an excessive input is also observed in transplants developing in another inflammatory condition like stroke. However, using a semi-quantitative analysis, Tornero *et al.* (2017) have reported input connectivity that resembles the normal circuitry when quantifying the contribution from each input region in 3 categories. This quantification may not resolve all the differences we found here. In addition, possible differences may be attributable to several reasons. First and foremost, transplantation of human induced pluripotent stem cells (iPSC)-derived neurons requires immune suppression and hence alters the environment. Second, the time and site of transplantation differs, since the authors grafted iPSC-derived cortically fated neurons nearby the stroke area and only 48h after the stroke (compared to 1 week post-injury in our study) when microglia numbers and reactivity at the site of transplantation is peaking and thus synapse pruning may be exaggerated leaving the initial connectome at lower metrics. Third, the degree of maturation differs profoundly with a much slower maturation speed of human neurons that are still rather immature at the time of RABV injection (2 mpt; only 20% NeuN; Tornero *et al.*, 2013) and thus the mapped connectivity may be a premature assessment of an expanding connectome. Importantly, data from brain imaging and network neuroscience has been converging into the view that TBI is characterized by an increased endogenous connectivity degree, while the transience of this hyperconnectivity is still under debate (Caeyenberghs *et al.*, 2017). Slicing the brain also correlates with a substantial, rapid increase in synapse number compared to tissue fixed *in vivo* (Kirov, Sorra and Harris, 1999). Most importantly, however, stroke differs in many parameters from TBI, and may hence indeed influence connectivity differently, highlighting the need to examine quantitative connectivity specifically in different injury conditions. This is also confirmed by our accompanying manuscript (Thomas et al.) showing the influence of the aging brain and amyloid-loaded host environment in promoting the input connectome locally.

Our data showed a sharp decline in the number of host-graft connections in the stab wound cortex at 3 mpt resulting in an extremely scarce connectome, likely insufficient for functional repair. These findings are difficult to compare with transplants of human PSC-derived neurons as these mature at a very different pace and develop in an immunosuppressed environment. Interestingly, however, previous work showed a surprisingly early innervation already at 1.5-2

mpt after transplantation that is comparable at more mature stages at 6 mpt, in stroke and PD models (Grealish *et al.*, 2015; Tornero *et al.*, 2017). It will be intriguing to analyze these transplants at even later stages, given the slow pace of human neuron maturation (Petanjek *et al.*, 2011; Linaro *et al.*, 2019).

Analysis of the proteome from environments that promote graft connectivity (SW) as compared with those where transplant connectivity remains under normal levels (intact/LPS) suggests that fibrinogen and complement activation pathways play a role in synapse yield. Both have been implicated in synapse pruning during brain development and/or neurological disease (Stevens *et al.*, 2007; Hong *et al.*, 2016; Merlini *et al.*, 2019). Interestingly, gene expression analysis in the neuronal apoptosis model where we have previously observed an adequate and stable level of connectivity, showed all the three components of C1q (C1qa, C1qb and C1qc) differentially overexpressed in injured regions (Sohur, Arlotta and Macklis, 2012). Collectively, our findings indicate that complement protein levels and its temporal dynamics at a brain injury may play an essential role dictating the outcome of transplantation and stability of new synaptic connections.

Successful brain repair by cell transplantation does not only involve axonal outgrowth and generation of new synapses with the correct regions, but also a tight quantitative match of the new input and output connections. In the mammalian cortex, excessive excitatory connections may result in hyperexcitability and formation of epileptic foci in the transplanted site, while scarce connections may be insufficient to restore the functional deficit caused by the injury. Herein, we provide the first evidence that injury type-dependent extrinsic cues influence the integration of transplanted neurons into the host synaptic circuitry. This work further advances our understanding of the prerequisites for neuronal replacement in damaged brain circuits and represents an important step in clinical translation of neuronal replacement strategies to restoring an authentic circuitry after brain damage and promote functional recovery in the injured brain.

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Author Contributions

M.G. initially conceived the idea. M.G. and S.G. conceived and coordinated the project. S.G. designed, performed, and analyzed all experiments except the proteomics. J.T. and S.H. conducted the proteome study: J.T. performed injuries, collected samples and post-analyzed the data. S.H. prepared the samples, performed measurements and quantitative analysis. K.-K.C. provided the expertise and viral vectors for monosynaptic tracing. S.G. and M.G. wrote the manuscript with input from all co-authors.

Declaration of Interests

The authors declare no competing interests.

STAR Methods

Key resource table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken anti-GFP	Aves Lab	Cat#GFP-1020; RRID: AB_10000240
Rabbit anti-RFP	Rockland	Cat#600-401-379; RRID: AB_2209751
Goat anti-mCherry	SICGEN	Cat#AB0081-200; RRID: AB_2333094
Rabbit anti-Cux1	Santa Cruz Biotechnology	Cat#sc-13024; RRID: AB_2261231
Mouse IgG1 anti-GFAP	Sigma-Aldrich	Cat#G3893; RRID: AB_477010
Rabbit anti-Iba1	Wako	Cat#019-19741; RRID: AB_839504
Mouse IgG1 anti-NeuN	Millipore	Cat#MAB377; RRID: AB_2298772
Rabbit anti-Dcx	Abcam	Cat#ab18723; RRID: AB_732011
Anti-chicken Alexa Fluor 488	Thermo Fisher Scientific	Cat#A11039; RRID: AB_2534096
Anti-rabbit Cy3	Dianova	Cat#711-165-152; RRID: AB_2307443
Anti-goat Cy3	Dianova	Cat#705-165-147; RRID:AB_2307351
Anti-rabbit Cy5	Dianova	Cat#111-175-144; RRID: AB_2338013
Anti-mouse IgG1 Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21121; RRID: AB_2535764
Anti-mouse IgG1 Alexa Fluor 647	Thermo Fisher Scientific	Cat#A21240; RRID: AB_2535809
Bacterial and Virus Strains		
Retrovirus CAG-DsRedExpress2-2A-Glyco-IRES2-TVA	In-house production	N/A
Retrovirus CAG-GFP	In-house production	N/A

Rabies virus (EnvA ΔG-GFP)	Wickersham et al., 2007; provided by K.-K.C	N/A
Rabies virus (EnvA ΔG-mCherry)	Provided by K.-K.C	N/A
Chemicals, Peptides, and Recombinant Proteins		
HEPES 1M	Thermo Fisher Scientific	Cat#15630-056
HBSS 1x	Thermo Fisher Scientific	Cat#24020-091
Poly-D lysine (PDL)	Sigma-Aldrich	Cat#P0899
Trypan Blue	Thermo Fisher Scientific	Cat#15250-061
DMEM (1x) Glutamax high glucose	Thermo Fisher Scientific	Cat#61965-026
Fetal bovine serum (FBS)	Pan system	Cat#P30-3302
B27 Supplement	Thermo Fisher Scientific	Cat#17504-044
Penicillin-streptomycin	Thermo Fisher Scientific	Cat#15140-122
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific	Cat#25300-054
Phosphate buffered saline (PBS) 10x	Thermo Fisher Scientific	Cat#14200083
Paraformaldehyde (PFA)	Roth	Cat#0335.4
Bovine Serum Albumine (BSA)	Sigma-Aldrich	Cat#A9418
Normal Goat Serum (NGS)	Thermo Fisher Scientific	Cat#16210-064
Triton X-100	Sigma-Aldrich	Cat#T9284
DAPI	Sigma-Aldrich	Cat#28718-90-3
Aqua Polymount	Polysciences	Cat#18606-5
Lipopolysaccharide (LPS) from E. coli	Sigma-Aldrich	Cat#L8274
Ketamine (Ketavet)	Pfizer	PZN 7506004
Xylazine (Rompun)	Bayer	PZN 1320422
Fentanyl	Janssen	PZN 2084366
Midazolam (Dormicum)	Roche	PZN 3096124
Medetomidine (Dorbene vet)	Fort Dodge	N/A
Atipamezol (Antisedan)	Janssen	N/A
Flumazenil	Hexal	PZN 4470990
Buprenorphin (Temgesic)	Essex	PZN 0345928
Ammonium bicarbonate	Sigma-Aldrich	A6141-500g

Tris	Roth	4855.5
Di-thiothreitol	Merck	1.11474.0025
Iodacetamide	Merck	8.04744.0025
Lys-C	Wako	125-05061
Trypsin	Promega	V5111
Acetonitrile	Roth	HN44.2
Trifluoroacetic acid	Thermo Fisher Scientific	400003
Vivacon 500® Centrifugal Filter (30,000 MWCO HY)	Sartorius	VN01H22ETO
Deposited Data		
Proteomic data	This paper	PRIDE #PXD023660
Experimental Models: Organisms/Strains		
C57BL/6J mice	in-house breeding	N/A
Emx1-Cre mice	Iwasato *et al.*, 2000	MGI:1928281
G-TVA mice	The Jackson Laboratory; Takatoh *et al.*, 2013	JAX: 024708; MGI:5550559
GFP reporter mice	Nakamura, Colbert and Robbins, 2006	JAX: 024636; MGI:3849685
TdTomato reporter mice, Ai9	Madisen *et al.*, 2010	JAX: 007909; MGI:3809523
Software and Algorithms		
ZEN imaging software	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
Brain Explorer 2	Allen Institute for Brain Science	https://mouse.brain-map.org/static/brainexplorer
Prism	Graphpad	Version 8.0
BioWheels	DiBS	https://dibsvis.com/biowheel
Gorilla	Eden *et al.*, 2009	http://cbl-gorilla.cs.technion.ac.il/

Proteome discoverer 2.4 software	Thermo Fisher Scientific	https://www.thermofisher.com/de/de/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html

Experimental model and subject details

Animals

Animal handling and experimental procedures were performed in accordance with German and European Union guidelines and were approved by the Government of Upper Bavaria. All efforts were made to minimize suffering and number of animals. Both male and female mice were used. Mice were kept in specific pathogen-free conditions and in 12:12 hour light/dark cycles with food and water *ad libitum*. All mice were 2-4 months old at the time of the first surgery. C57BL/6J wildtype mice were used as host mice for all studies except those to test for the occurrence of cell fusion wherein tdTomato reporter mice were used (Ai9; Madisen *et al.*, 2010). Surgeries were performed aseptically under anesthesia with a mixture of fentanyl (0.05 mg/kg, Janssen), midazolam (5 mg/kg, Roche) and medetomidine (0.5 mg/kg, Fort Dodge). After surgery, anesthesia was terminated with atipamezol (2.5 mg/kg, Janssen), flumazenil (0.5 mg/kg, Hexal) and buprenorphine (0.1 mg/kg, Essex). Meloxicam (1 mg/kg, Metacam) was administered for postoperative analgesia.

Cells for transplantation were obtained from C57BL/6J wildtype or Emx1-Cre/EGFP E14.5/E15.5 mouse embryos (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006) and cultured (see below), or alternatively, they were mechanically dissociated from E18.5 Emx1-Cre/G-TVA/GFP embryos (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006; Takatoh *et al.*, 2013) and readily transplanted. Triple transgenic embryos amid the litter were identified by GFP expression in the dorsal telencephalon and resulted from matings involving a G-TVA homozygous parent.

Primary culture of cortical neurons

Neocortex from E14.5/E15.5 mouse embryos was mechanically dissociated in Hanks' balanced salt solution (HBSS) buffered with 10 mM HEPES (both from Life Technologies). Cells were plated in 20 µg/ml poly-D-lysine (PDL, Sigma-Aldrich) coated 24-well plates, at a density of 200 000 cells/well. Cells were initially kept in 10% fetal bovine serum (FBS, Pan Biotech)-containing DMEM high glucose (4.5 g/L) with glutamax, plus penicillin-streptomycin (both from Life Technologies). Serum was gradually removed by replacing half of the medium with B27 (Life Technologies)-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 4-5 days *in vitro* upon validation of reporter expression.

All cells for primary culture were obtained from wildtype C57BL/6J mice except those used to test for the occurrence of cell fusion with the host cells. For this purpose, Emx1-Cre/EGFP (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006) embryos were used. Double transgenic embryos amid the litter were identified by GFP expression in the dorsal telencephalon.

Method details

<u>Virus treatment in cell cultures</u>

Neocortical neurons from C57BL/6J wildtype mouse embryos were cultured. Two to four hours after plating the cells were transduced with MMuLV-derived retroviral vectors CAG-EGFP or CAG-DsRedExpress2-2A-Glyco-IRES2-TVA (0.8-1 µl/well; titers ranged from 10^7 to 10^{11} transducing units per mL). Cells were then transplanted into C57BL/6J wildtype adult mice. For cell fusion test experiments, neuronal cultures were prepared from Emx1-Cre/EGFP E14.5/E15.5 embryos and transplanted into tdTomato reporter mice. Although these cells are endogenously labeled, we additionally treated them with CAG-EGFP retrovirus to account for a putative impact of retroviral transduction in the propensity of cells to fuse upon transplantation in the adult brain parenchyma.

<u>Treatments and Surgical procedures</u>

LPS inflammatory stimulus

C57BL/6J wildtype mice were subjected to an inflammatory stimulus by a single i.p. injection of 1 mg/kg or 3 mg/kg lipopolysaccharide (LPS) from E. coli (Sigma). A batch of mice was used to run a comparative analysis of cortical gliosis in SW, LPS and control conditions. For this purpose, a craniotomy was performed at the time of the LPS injection to account for glial reactivity due to the craniotomy *per se*. These mice were perfused 7 days after the LPS treatment, in order to monitor cortical gliosis at the time when transplants would normally be performed. Other mice were used for subsequent neuronal transplantation and connectivity analysis (see below).

Stab wound injury (SW)

C57BL/6J wildtype or tdTomato reporter mice were used for cortical stab wound injury as described in Mattugini *et al.* (2018). Briefly, mice were anesthetized, and a craniotomy of 2.5 mm diameter was open to expose the primary visual cortex (V1) of the left cortical hemisphere. Using an ophthalmological lancet, a 0.5 mm-deep / 1 mm-long incision was performed within V1 borders (coordinates from lambda: 0.0±0.2 anteroposterior, 2.0±0.2 to 3.0±0.2 mediolateral; coordinates were chosen to avoid large pial vasculature). The bone flap was placed back and the skin sutured. For analysis of cortical gliosis, mice were perfused 7 days after SW, while for analysis of graft integration the mice were subjected to further surgical procedures (see below).

Cell transplantation

Cells were transplanted into V1 of the left hemisphere of the mouse cerebral cortex. Host mice belonged to three groups: SW, intact (naïve) and mice subjected to an inflammatory stimulus by i.p. injection of 3 mg/kg LPS. Transplantation into SW or LPS mice was performed 7 days after the respective insult. In SW group, donor cells were placed right into the center of the incision, still lightly visible.

Donor cells were fluorescently labelled in Emx1-Cre/EGFP or Emx1Cre/G-TVA/EGFP mouse lines or via *in vitro* viral transduction with the aforementioned constructs. Cultured cells were washed 3 times with pre-warmed phosphate-buffered saline (PBS) to remove any remaining viral particles and cell debris. Gentle trypsinization (0.025%, 10 min at 37 °C) detached the cells and trypsin was then inactivated by FBS-containing medium (1:1). The cell suspension was then prepared in B27-containing DMEM high glucose (4.5 g/L) with glutamax, plus penicillin-streptomycin. 25 000-50 000 donor cells were transplanted in a total volume of 1 µl of cell suspension using a ga33 Hamilton syringe (coordinates from lambda: 2.5 ±0.2 mm mediolateral, 0.0 ±0.2 mm anteroposterior). Cell deposits were distributed dorsoventrally filling up a depth between 0.5 to 0.2 mm that results in a cellular graft encompassing cortical layers 1 to 4/5. Injection coordinates and pattern of pial vasculature were noted for later identification of the transplantation site and injection of the RABV. The bone lid was repositioned, and the skin was sutured. For analysis of transplanted neurons survival, some mice were perfused 5 days afterwards, while for analysis of neuronal integration mice were subjected to RABV injection as detailed next.

Rabies virus injection

We used retrograde monosynaptic tracing with a modified rabies virus (RABV: EnvA-pseudotyped ΔG-EGFP or ΔG-mCherry, complementary to the reporter in transplanted cells) (Wickersham *et al.*, 2007; Falkner *et al.*, 2016) to map brain-wide synaptic input to grafted neurons. In short, the RABV was injected 1 or 3 months after cell transplantation, in three locations surrounding the transplantation site (200 nl/location), using an automated nanoinjector at slow delivery speed. RABV titers typically ranged between 1.5-3.5 x 10^8 plaque forming units (pfu)/mL. Mice were sacrificed 7/8 days later for immunostainings and circuit analysis.

<u>Immunostaining</u>

Mice were deeply anaesthetized with Ketamin (100 mg/kg) and Xylazin (10 mg/kg) and perfused transcardially with PBS (5 min) followed by 4% PFA in PBS for 30-40 min. Brains were collected and post-fixed in 4% PFA overnight, at 4 °C, serially cut on a vibratome into 70 µm sagittal sections and slices were further processed as free-floating. Sections were washed and incubated in blocking and permeabilizing solution for 2h (3% bovine serum albumin or 10% normal goat serum; 0.5% triton X-100). The following primary antibodies were then used: 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), mouse anti-GFAP (1:1000; Sigma-Aldrich) rabbit anti-Iba1 (1:500; Wako), rabbit anti-Dcx (1:1000 Abcam) mouse anti-NeuN (1:200; Merck/Millipore) for overnight to 48h-incubation, at 4 °C). After washing, sections were incubated with appropriate 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 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for nuclear labeling and mounted on glass slides with Aqua-Poly/Mount (Polysciences).

For connectivity analysis, brain-wide, all sections were immunostained for GFP/RFP. For some brains, sections with the transplant were selected and subsequently stained for Cux1 colocalization analysis and all were mounted for microscopy and serial analysis.

<u>Slice processing and imaging</u>

For brain-wide connectivity analysis, brain sections were kept in serial order throughout their processing. Sections with one or more GFP (or mCherry)-labeled cell somas were scanned using an epifluorescence microscope with a motorized stage (Zeiss, Axio Imager M2) equipped with a 10x objective (NA 0.3). Automated scanning, tile alignment, and image stitching was performed to create a high-resolution image of the whole section. In sections with unclear cell numbers due to close apposition of two GFP (or mCherry) cell bodies or with high densities of GFP (or mCherry) cells, scanning of Z-stacks in a laser-scanning confocal microscope (Zeiss, LSM 710) with a 40x objective (NA 1.1) was carried out.

For all immunofluorescence studies, images were acquired using a epifluorescence microscope with a motorized stage (Zeiss, Axio ImagerM2) and a laser-scanning confocal microscope (Zeiss, LSM 710).

<u>Mass spectrometry</u>

3-4 months old male and female C57BL/6J mice (n=10 intact controls, n=10 SW-injured, n=5 LPS injected) were sacrificed through cervical dislocation, brains were removed and placed into cold PBS. Biopsy punches (2.5 mm diameter) of the visual cortex of both hemispheres were dissected whereas meninges and white matter were carefully taken off. The contralateral (uninjured) cortices of the SW-injured brains were not considered for proteome analysis (intact: n=20, SW: n=10, LPS: n=10). Samples were placed into low-protein binding Eppendorf tubes, frozen on dry ice, and stored at – 80 °C until further processing.

Tissue samples were lysed in NP40 buffer (1% NP40 in 10 mM Tris, pH 7.4, 150 mM NaCl) in a Precellys homogenizator (VWR) and 10 µg total protein *per* sample were proteolyzed with Lys-C and trypsin using a modified FASP procedure (Grosche *et al.*, 2016). LC-MS/MS analysis was performed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) online coupled to a nano-RSLC (Ultimate 3000 RSLC; Dionex). Tryptic peptides were accumulated on a nano trap

column (Acclaim PepMap 100 C18, 5 µm, 100 Å, 300 µm inner diameter (i.d.) × 5 mm; Thermo Fisher Scientific) at a flow rate of 30 µl/min followed by separation by reversed phase chromatography (µPAC™ column, 200 cm length, with pillar array backbone at interpillar distance of 2.5 µm, PharmaFluidics) using a non-linear gradient for 240 minutes from 3 to 42% buffer B (acetonitrile [v/v]/0.1% formic acid [v/v] in HPLC-grade water) in buffer A (2% acetonitrile [v/v]/0.1% formic acid [v/v] in HPLC-grade water) at a flow rate of 300 nl/min. MS spectra were recorded at a resolution of 60,000 with an AGC target of 3 x 106 and a maximum injection time of 50 ms, at a range of 300 to 1500 m/z. From the MS scan, the 10 most abundant ions were selected for HCD fragmentation with a normalized collision energy of 27, an isolation window of 1.6 m/z, and a dynamic exclusion of 30 s. MS/MS spectra were recorded at a resolution of 15,000 with an AGC target of 105 and a maximum injection time of 50 ms.

Quantification and statistical analysis

General image analysis and statistics

Images were analyzed with ZEN (Zeiss) and ImageJ software. Cell countings were performed with the Cell Counter plug-in for ImageJ by careful inspection across serial optical sections (spaced at 1 µm interval) of confocal Z-stacks acquired with a 40x objective (NA 1.1). Image processing was performed with ImageJ and multipanel figures assembled in Adobe Photoshop/Illustrator (Adobe Systems).

Statistical analysis was performed using GraphPad Prism Version 8.0 Software (Graphpad). All biological replicates (n, mice) are derived from at least 2 independent experiments. Values are reported as mean ± S.E.M. calculated between different mice. Statistical significance was defined at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. For comparison of connectivity ratio or graft size analysis between two conditions non-parametric Mann-Whitney tests was used. Data visualization leveraged Microsoft Excel, GraphPad Prism Version 8.0, Adobe Illustrator and Biowheel plots (DiBS).

Connectivity analysis

Whole slice tile scans were used to identify brain regions with GFP (or mCherry) labeled cells, by alignment with 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 and overlap it with the experimental section to identify the anatomical location of the labeled cells. In sections with unclear cell numbers analysis of the confocal Z-stacks was carried out, and quantification was performed by careful inspection through serial optical sections spaced at 1 µm interval. In sections including transplanted cells, four categories were considered for counting: GFP-only (or mCherry-only) with neuronal morphology, GFP-only (or mCherry-only) with glial morphology, RFP/GFP (or GFP/mCherry) cells with neuronal morphology so called

“starter” neurons, and RFP/GFP (or GFP/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 cells with neuronal morphology in V1, so called “starter cells” (or mCherry-only neurons in a region per number of starter GFP/mCherry neurons in V1). Results are represented as mean ± S.E.M. calculated between different mice.

Proteomic data processing – Label-free quantification

The individual raw-files were loaded to the Proteome discoverer 2.4 software (Thermo scientific) allowing for peptide identification and label-free quantification using the Minora node. Searches were performed using Sequest HT as search engine in the Swiss Prot database, taxonomy mouse (17038 sequences) with the following search settings: 10 ppm precursor tolerance, 0.02 Da fragment tolerance, full tryptic specificity, two missed cleavages allowed, carbamidomethyl on cysteine as fixed modification, deamidation of glutamine and asparagine allowed as variable modifications, as well as oxidation of methionine and Met-loss combined with acetylation at the N-terminus of the protein. The Percolator node was used for validating peptide spectrum matches and peptides, accepting only the top-scoring hit for each spectrum, and satisfying a false discovery rate (FDR) <1% (high confidence). Protein groups were additionally filtered for an identification FDR <5% (target/decoy concatenated search validation). Peak intensities (at RT apex) of all allocated unique peptides were used for pairwise ratio calculations. A background-based t-test was employed for calculation of statistical significance of the reported ratios.

Data was filtered to ensure direct identifications (not based on match-between run) in at least 30% of samples within at least one experimental group. For data visualization, volcano plots with log2 abundance ratio of sample replicates of SW and LPS condition versus intact control with corresponding log10 p-values were created using Microsoft Excel. Gene ontology enrichment analysis was performed by running significantly differentially expressed up- or downregulated proteins against a background list of all detected proteins using the webserver GO-rilla (http://cbl-gorilla.cs.technion.ac.il/; Eden *et al.*, 2009). A heatmap of all significantly regulated proteins of the SW condition was created with GraphPad Prism Version 8.0.

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Main figure legends

Figure 1: Transplanted neurons develop mature morphologies and synaptic structures within a cortical stab injury

(A-B) Schematic and timeline of the experimental procedure. **(C)** Confocal images of a representative transplantation site in the mouse cortex inflicted by a SW injury, 5 wpt (see all nuclei stained with DAPI; n=5). Boxed area highlights dendritic branches of RFP transplanted neurons. **(D)** Example image of an optical section in the transplant shows colocalization of RFP with Cux1, a marker of upper layer cortical identity (n=2). **(E)** Z-stack projection of an example of grafted neurons and respective high magnification insets shows the extension of apical dendrites as well as profuse basal dendrites (1) and axonal arborizations (2) from the cell bodies (n=5). Notice the appearance of spines and boutons in dendrites and axons, respectively (arrowheads in the highlighted neurite). Scale bars: (C) left, 100 µm, right, 50 µm (D) 50 µm, (E) left, 50 µm, right, 10 µm. Ctx, cortex; Hipp, hippocampus.

Figure 2: Input connectivity of neuronal transplants in SW and intact cortex

(A) Molecular tools and rationale of brain-wide monosynaptic tracing. **(B)** Example of a 'starter' neuron (RFP+/GFP+): a neuron within the RFP transplant (RFP+) that has been infected by the GFP rabies virus. **(C)** Experimental timeline. **(D-E)** Local and brain-wide inputs (GFP-only) to transplants, traced at 4 wpt (n=4/5). **(D)** Schematics depict brain regions that innervate the transplant. Red grading in sagittal sections and thickness of the lines in 3D connectograms reflect the connectivity ratio for a given connection. **(E)** Color-coded connectivity ratio for transplants in SW or intact cortex (n=4/5 respectively), as well as for endogenous neurons. The data shown for endogenous neurons (Endo) have been published before (Falkner *et al.*, 2016) and are used here solely for comparison. Note the excessive connectivity in SW and scarce in intact, as compared to the native. **(F)** Quantification of Vis-Vis and dLGN-Vis connectivity (n=4/5, *p<0.05 using Mann-Whitney test). **(G)** Pre-synaptic neurons (GFP+) in the dLGN of the thalamus. Scale bars: (B) 50 µm, (D,G) 100 µm. See Table S1 for abbreviations. Contra, contralateral; Ipsi, ipsilateral.

Figure 3: Gliosis and input connectivity of neuronal transplants in LPS-induced cortex

(A) Analysis of the reactive state of the visual cortex in the brain of mice treated with 1 or 3 mg/kg LPS as compared to that in a SW injury and control cortex (contralateral to SW) (n=2 for LPS groups and n=4 for SW and control groups. Timeline (top) and confocal images of immunostained sections for microglia and reactive astrocyte markers (Iba1 and GFAP respectively; bottom). Note the similar cellular response with the highest concentration of LPS and in the SW-inflicted cortex. **(B)** Analysis of the brain-wide monosynaptic input. Color-coded connectivity ratio for transplants in intact and 3 mg/kg LPS-induced cortex (n=5/6 respectively). Scale bar: (A) 50 µm. See Table S1 for abbreviations. Con/Contra, contralateral; Ipsi, ipsilateral.

Figure 4: Comprehensive proteome analysis of SW-injured and LPS-inflamed visual cortex

(A) Timeline for tissue punch collection. **(B-C)** Volcano plots showing log2 mean abundance ratio and corresponding log10 p-value comparing SW-injured (n=10, B) and LPS treated (n=10, C) with intact control (n=20) cortical tissue. Upregulated proteins in pink area and downregulated proteins in blue area of the plots. **(D)** Selection of enriched GO terms (biological process) of significantly enriched proteins in SW vs. intact cortex. **(E)** Heatmap shows the significantly regulated proteins in the SW cortex, along with their regulation in the LPS cortex. **(F)** Venn diagram depicts differentially regulated proteins that overlap or are exclusive for each condition. **(G)** Selection of proteins that are exclusively up- or downregulated in the SW-injured cortex. See Table S2 for protein and GO analysis. wpi, weeks post-injury (SW)/injection (LPS).

Figure 5: Comparison of early and late connectivity shows transience in cortical stab injuries

(A) Experimental timelines. **(B)** Local inputs (GFP-only) to transplants, traced at 4 and 12 wpt. **(C)** Color-coded brain-wide connectivity at 1 or 3 mpt in SW (n=4/6 respectively). Decreased connectivity at 3 mpt shows that many of the early synaptic connections have been pruned. **(D)** Distribution and strength of single host-graft connections evidenced by thickness of the lines between the graft (yellow) and each area (green). **(E)** Biowheel plot (using DiBS software for data visualization) highlights the increased amount of regions in the top-half of the color spectrum (white to red) at 1 mpt in SW (SW1) as compared to the normal connectivity (Endo, endogenous) and drop to the lower half at 3 mpt (SW3). Anatomical regions are sorted to the endogenous connectivity, in descending order of values. Scale bar: (B) 100 µm. See Table S1 for abbreviations.

Supplemental figure legends

Figure S1: Control for cell fusion between host and graft cells

(A) Genetic strategy: Emx1-Cre/GFP cells were transplanted into the SW-injured visual cortex of tdTomato reporter mice (n=4). **(B)** Confocal images and fluorescence intensity analysis along a line drawn across the transplant show absence of tdTomato fluorescence in GFP transplants. Scale bar: (B) 100 µm.

Figure S2: Brain-wide distribution of input neurons in SW and intact cortex, at 4 wpt

Example from both experimental groups shows (y-axis) the number of input neurons (GFP-only; in green) and "starter" neurons (double-labelled GFP/RFP; in yellow) throughout the brain. Each bar corresponds to one sagittal section, from the most lateral (left) to the most medial (right) in the mouse brain (x axis). The transplanted brain hemisphere (ipsilateral) and the contralateral are represented above and below the x-axis respectively. Note the overrepresentation of local connections compared to long-range in both conditions, and the scarce amount of input neurons for transplants in the intact brain, for a comparable number of "starter" neurons.

Figure S3: Early development of transplanted neurons in the intact brain

(A) Experimental procedure and time of analysis. **(B-C)** Confocal images of GFP-labeled neuronal transplants at 5 days after transplantation (dpt) immunostained for the immature neuronal marker Dcx and DAPI for nuclear labeling (the latter only in C). **(B)** Note the immature nature of the transplant, largely Dcx-positive, but robust outgrowth of neurites. **(C)** Neurites display growth cone like structures at their tips (see white and empty arrowheads for GFP+/Dcx+ and GFP-/Dcx+ growth cones respectively) and some have reached the corpus callosum (cc; evidenced by a distinct alignment and density of the DAPI nuclei characteristic of the white matter). Boxed areas are magnified in insets on the right. **(D)** High magnification confocal images within the transplant show that GFP grafted cells express the mature neuronal marker NeuN and a large fraction co-express the marker of upper layer cortical identity Cux1 (white arrows; blue arrows for GFP+/NeuN+/Cux1- cells) at 14 dpt.

Figure S4: Analysis of the graft size in SW and intact cortex

(A) Sagittal section from a mouse brain transplanted with RFP-labeled cells in the primary visual cortex (V1), either intact or previously inflicted with a SW. **(B)** Number of subsequent sections with RFP cells as a proxy for graft size (n=5/6 for intact and SW respectively). **(C)** Only sections with RFP cells within the cortical parenchyma were considered for the analysis, and those with cells above L1 or meningeal pia that result from leakage or reflux while retracting the transplantation needle were excluded. ns, not significant, using Mann-Whitney test.

Other Supplemental Items

Table S1: Abbreviations of anatomical regions.

Table S2: Protein and GO term enrichment analysis (additional excel file that is not included in this thesis).

Movie S1: Z-series stack shows the complex neuronal morphology 5 weeks after transplantation in a cortical stab injury (additional file that is not included in this thesis).

Figure 1

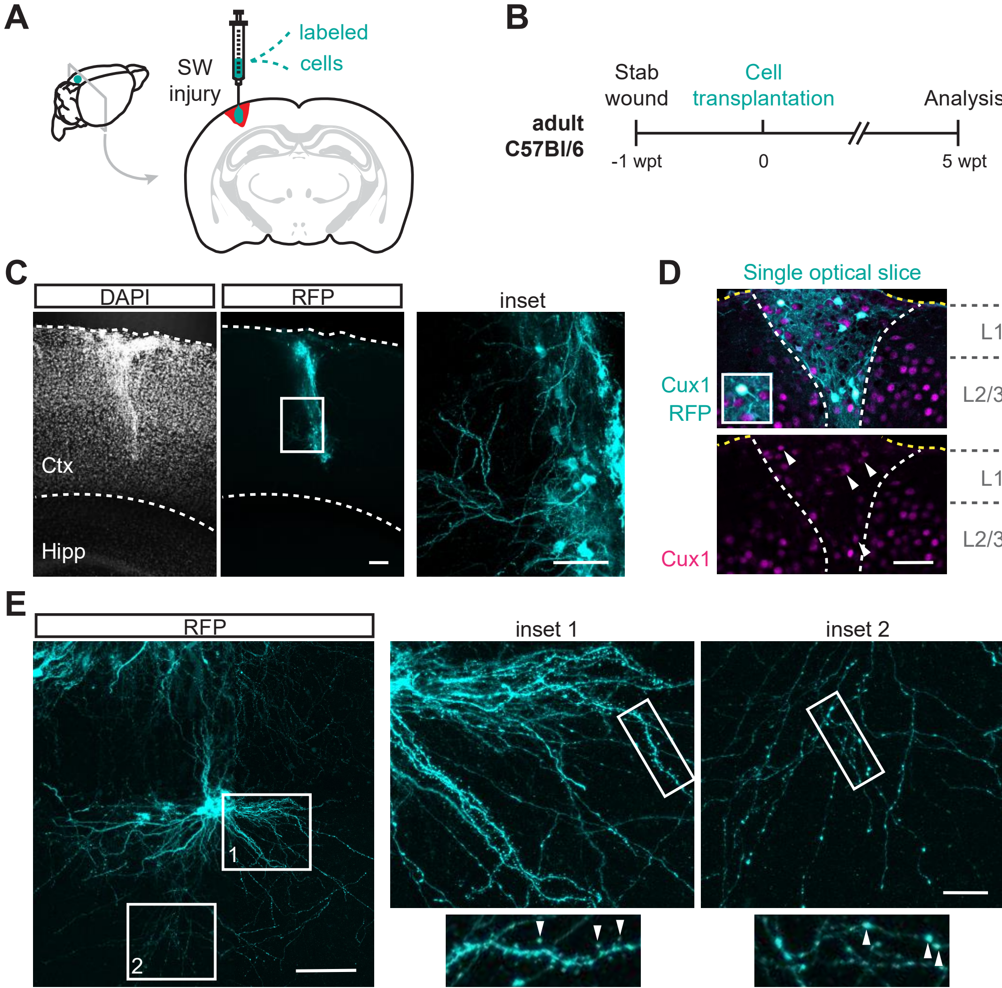
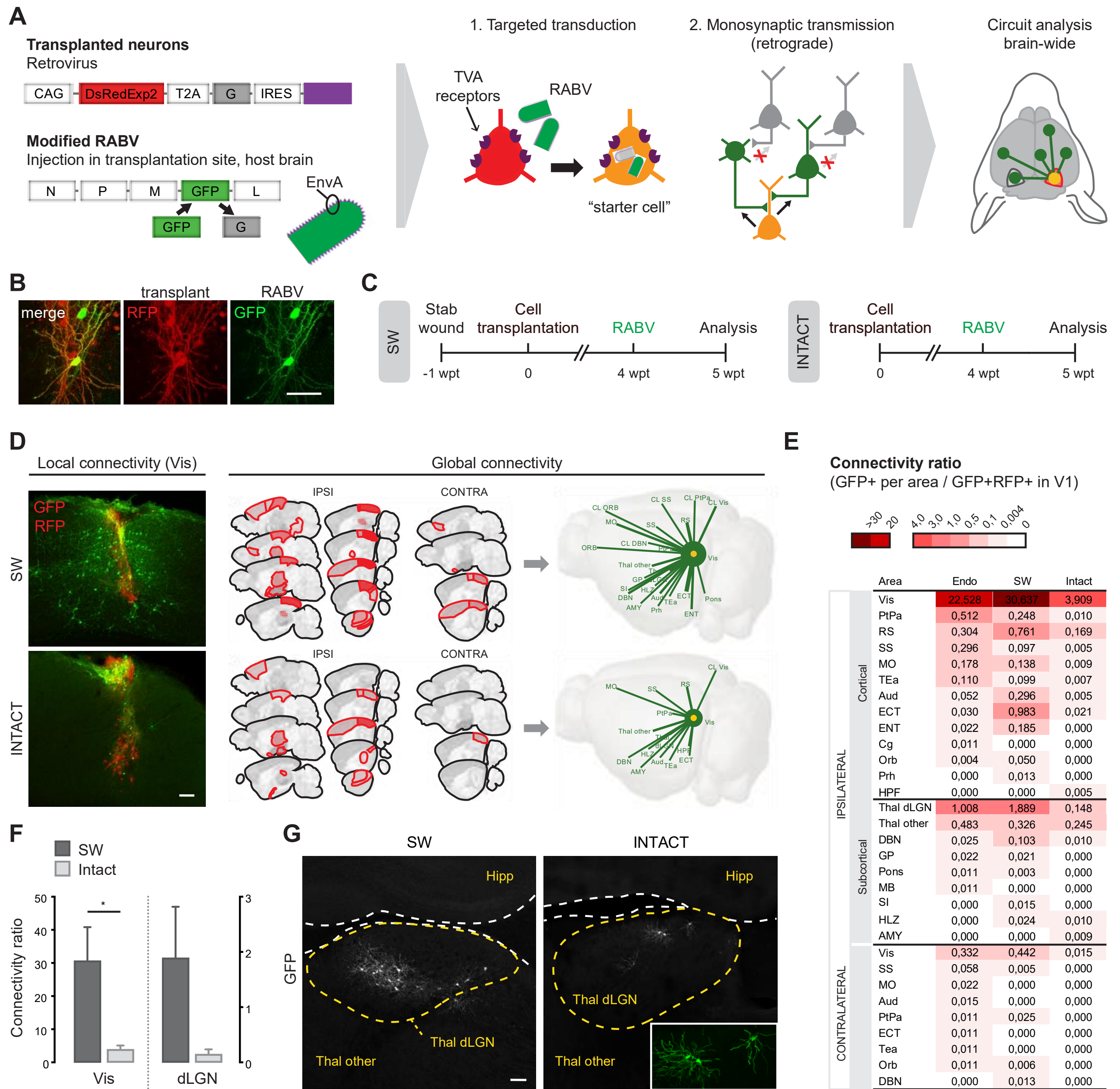
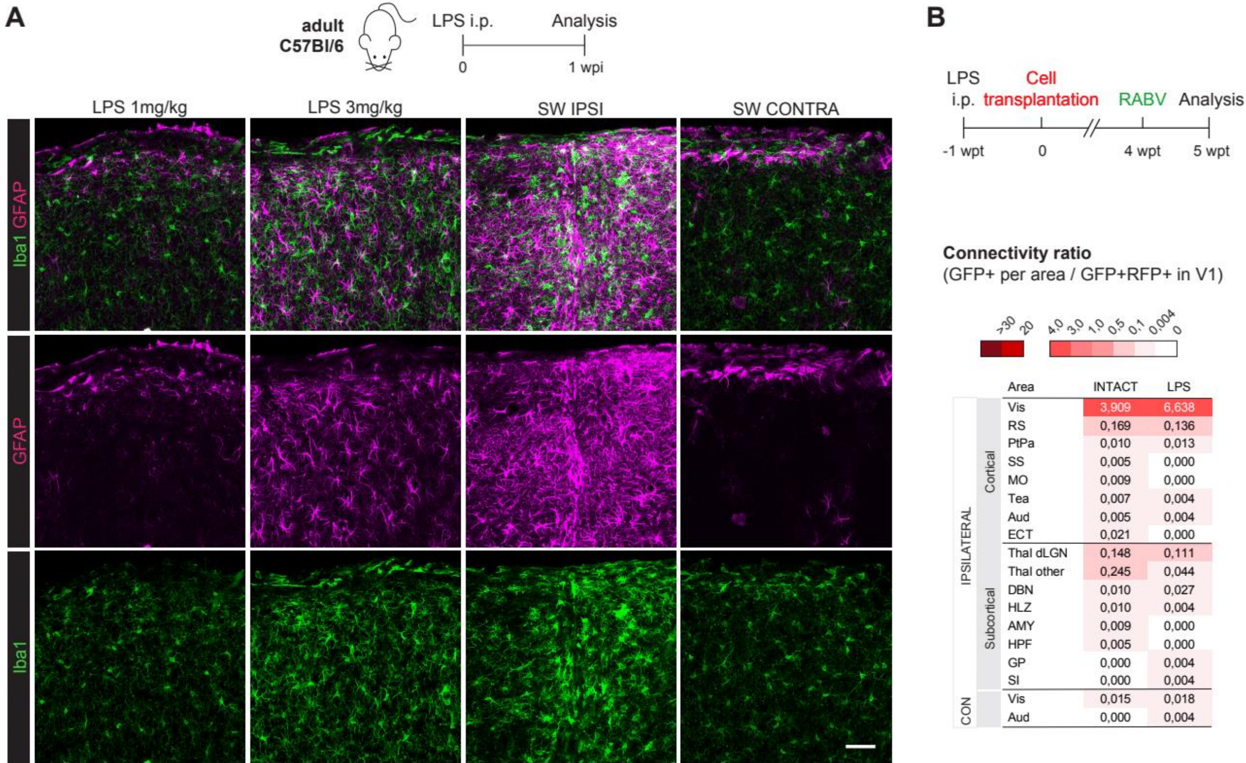


Figure 2



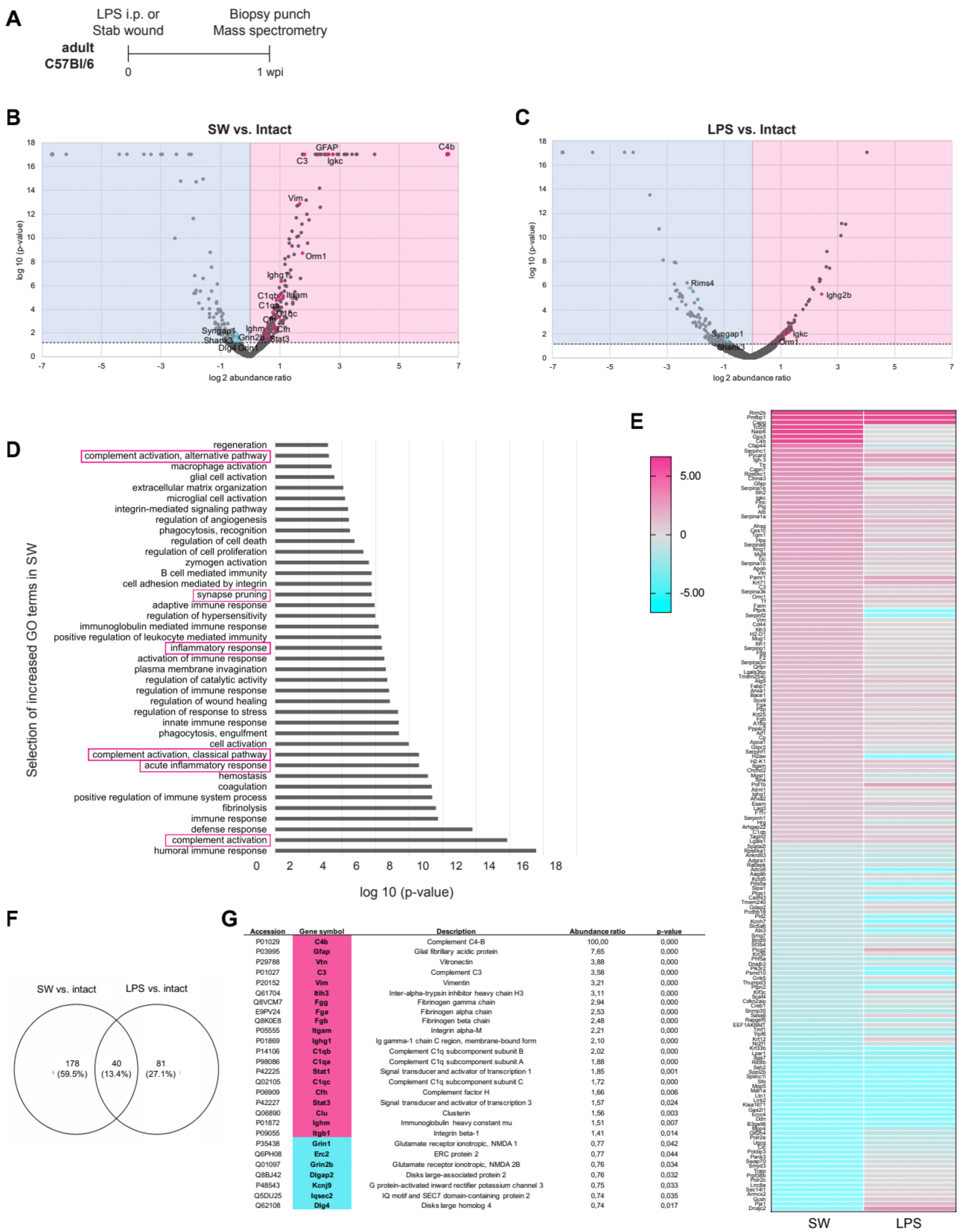
		Area	Endo	SW	Intact
IPSILATERAL	Cortical	Vis	22,528	30,637	3,909
		PtPa	0,512	0,248	0,010
		RS	0,304	0,761	0,169
		SS	0,296	0,097	0,005
		MO	0,178	0,138	0,009
		TEa	0,110	0,099	0,007
		Aud	0,052	0,296	0,005
		ECT	0,030	0,983	0,021
		ENT	0,022	0,185	0,000
		Cg	0,011	0,000	0,000
		Orb	0,004	0,050	0,000
		Prh	0,000	0,013	0,000
		HPF	0,000	0,000	0,005
	Subcortical	Thal dLGN	1,008	1,889	0,148
		Thal other	0,483	0,326	0,245
		DBN	0,025	0,103	0,010
		GP	0,022	0,021	0,000
		Pons	0,011	0,003	0,000
		MB	0,011	0,000	0,000
		SI	0,000	0,015	0,000
		HLZ	0,000	0,024	0,010
		AMY	0,000	0,000	0,009
CONTRALATERAL		Vis	0,332	0,442	0,015
		SS	0,058	0,005	0,000
		MO	0,022	0,000	0,000
		Aud	0,015	0,000	0,000
		PtPa	0,011	0,025	0,000
		ECT	0,011	0,000	0,000
		Tea	0,011	0,000	0,000
		Orb	0,011	0,006	0,000
		DBN	0,000	0,013	0,000

Figure 3



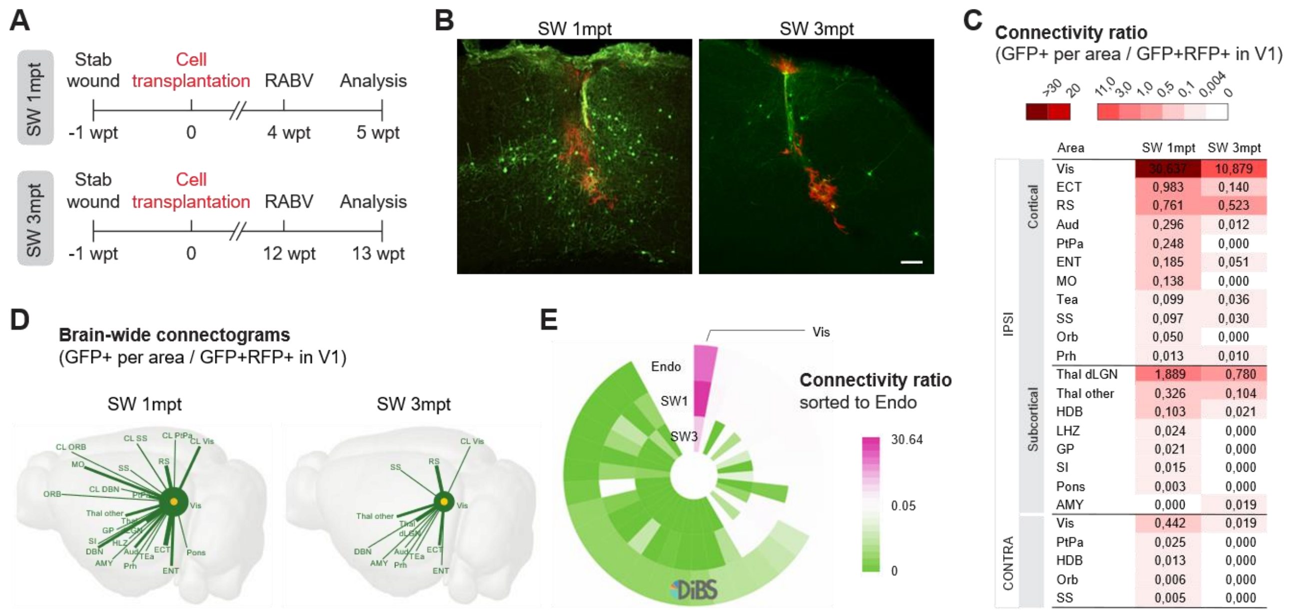
		Area	INTACT	LPS
IPSILATERAL	Cortical	Vis	3,909	6,638
		RS	0,169	0,136
		PtPa	0,010	0,013
		SS	0,005	0,000
		MO	0,009	0,000
		Tea	0,007	0,004
		Aud	0,005	0,004
		ECT	0,021	0,000
	Subcortical	Thal dLGN	0,148	0,111
		Thal other	0,245	0,044
		DBN	0,010	0,027
		HLZ	0,010	0,004
		AMY	0,009	0,000
		HPF	0,005	0,000
		GP	0,000	0,004
		SI	0,000	0,004
CON		Vis	0,015	0,018
		Aud	0,000	0,004

Figure 4



Accession	Gene symbol	Description	Abundance ratio	p-value
P01029	C4b	Complement C4-B	100,00	0,000
P03995	Gfap	Glial fibrillary acidic protein	7,65	0,000
P29788	Vtn	Vitronectin	3,88	0,000
P01027	C3	Complement C3	3,58	0,000
P20152	Vim	Vimentin	3,21	0,000
Q61704	Itih3	Inter-alpha-trypsin inhibitor heavy chain H3	3,11	0,000
Q8VCM7	Fgg	Fibrinogen gamma chain	2,94	0,000
E9PV24	Fga	Fibrinogen alpha chain	2,53	0,000
Q8K0E8	Fgb	Fibrinogen beta chain	2,48	0,000
P05555	Itgam	Integrin alpha-M	2,21	0,000
P01869	Ighg1	Ig gamma-1 chain C region, membrane-bound form	2,10	0,000
P14106	C1qb	Complement C1q subcomponent subunit B	2,02	0,000
P98086	C1qa	Complement C1q subcomponent subunit A	1,88	0,000
P42225	Stat1	Signal transducer and activator of transcription 1	1,85	0,001
Q02105	C1qc	Complement C1q subcomponent subunit C	1,72	0,000
P06909	Cfh	Complement factor H	1,66	0,006
P42227	Stat3	Signal transducer and activator of transcription 3	1,57	0,024
Q06890	Clu	Clusterin	1,56	0,003
P01872	Ighm	Immunoglobulin heavy constant mu	1,51	0,007
P09055	Itgb1	Integrin beta-1	1,41	0,014
P35438	Grin1	Glutamate receptor ionotropic, NMDA 1	0,77	0,042
Q6PH08	Erc2	ERC protein 2	0,77	0,044
Q01097	Grin2b	Glutamate receptor ionotropic, NMDA 2B	0,76	0,034
Q8BJ42	Dlgap2	Disks large-associated protein 2	0,76	0,032
P48543	Kcnj9	G protein-activated inward rectifier potassium channel 3	0,75	0,033
Q5DU25	Iqsec2	IQ motif and SEC7 domain-containing protein 2	0,74	0,035
Q62108	Dlg4	Disks large homolog 4	0,74	0,017

Figure 5



		Area	SW 1mpt	SW 3mpt
IPSI	Cortical	Vis	30,637	10,879
		ECT	0,983	0,140
		RS	0,761	0,523
		Aud	0,296	0,012
		PtPa	0,248	0,000
		ENT	0,185	0,051
		MO	0,138	0,000
		Tea	0,099	0,036
		SS	0,097	0,030
		Orb	0,050	0,000
		Prh	0,013	0,010
	Subcortical	Thal dLGN	1,889	0,780
		Thal other	0,326	0,104
		HDB	0,103	0,021
		LHZ	0,024	0,000
		GP	0,021	0,000
		SI	0,015	0,000
		Pons	0,003	0,000
		AMY	0,000	0,019
CONTRA		Vis	0,442	0,019
		PtPa	0,025	0,000
		HDB	0,013	0,000
		Orb	0,006	0,000
		SS	0,005	0,000

Figure S1

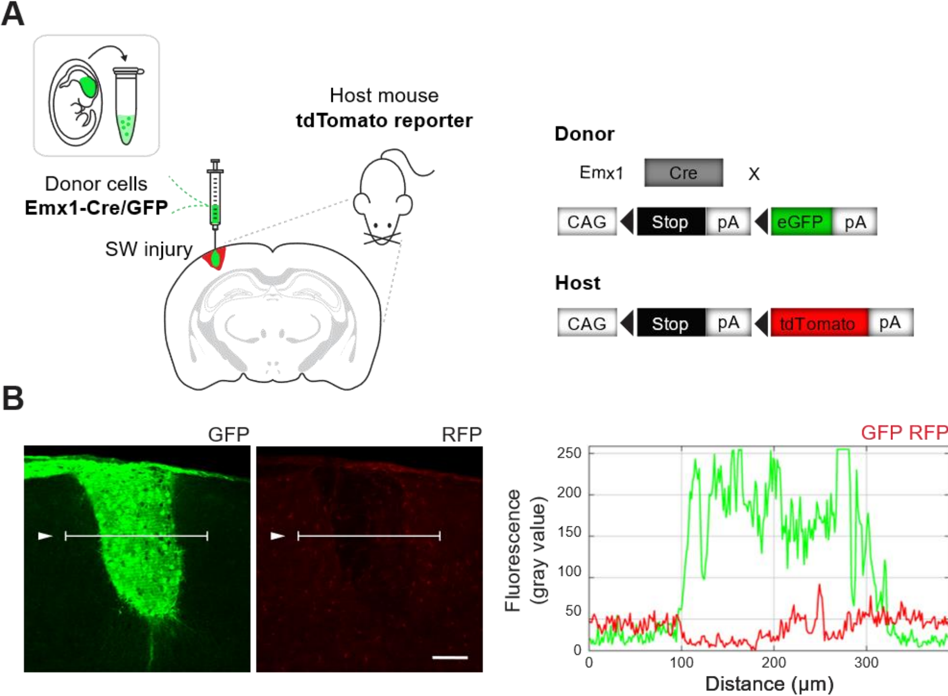


Figure S2

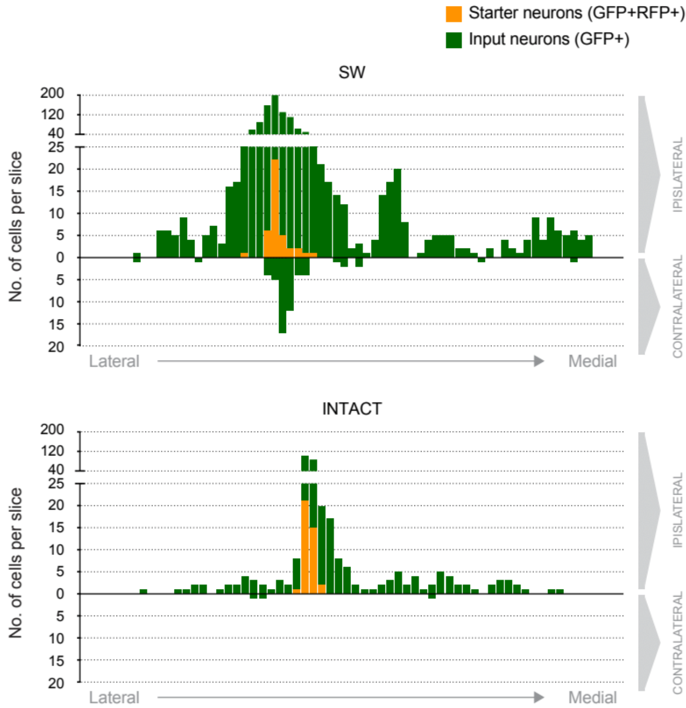


Figure S3

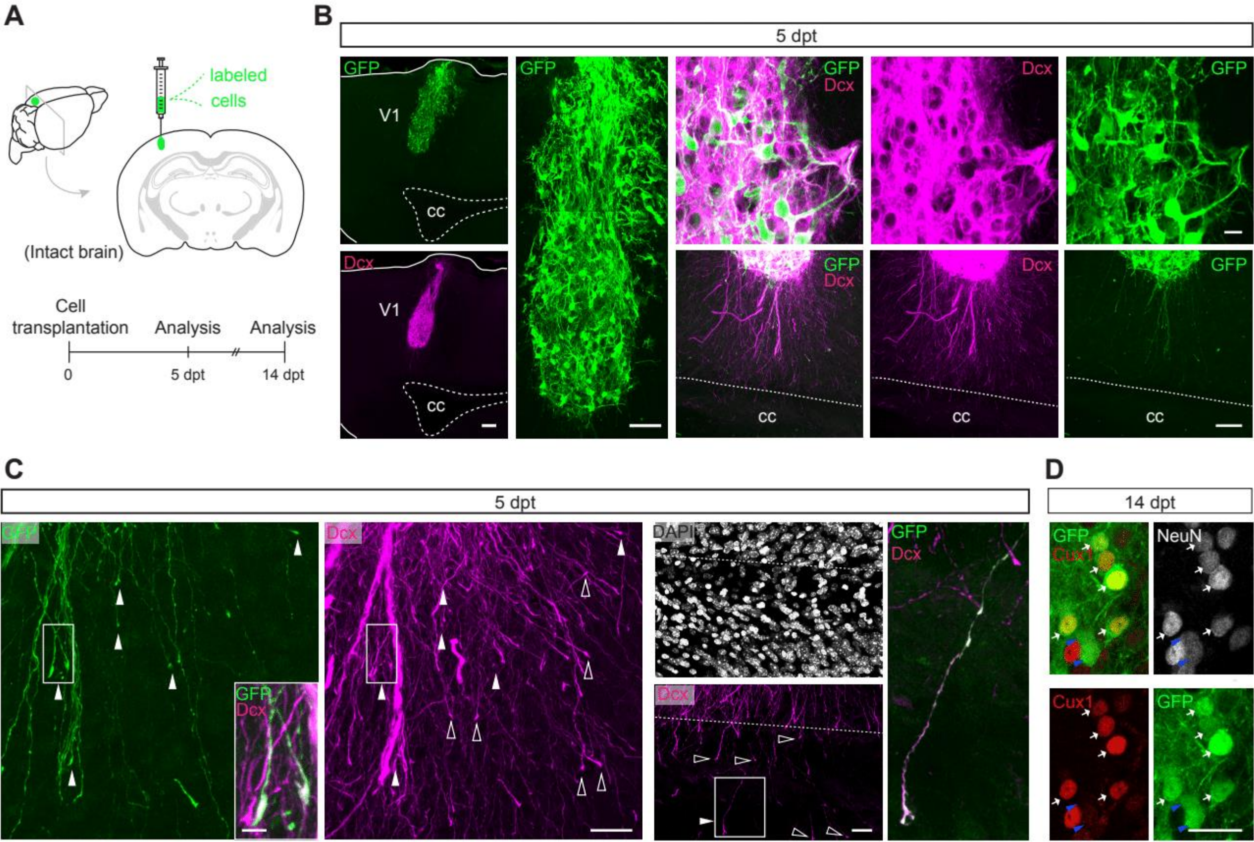


Figure S4

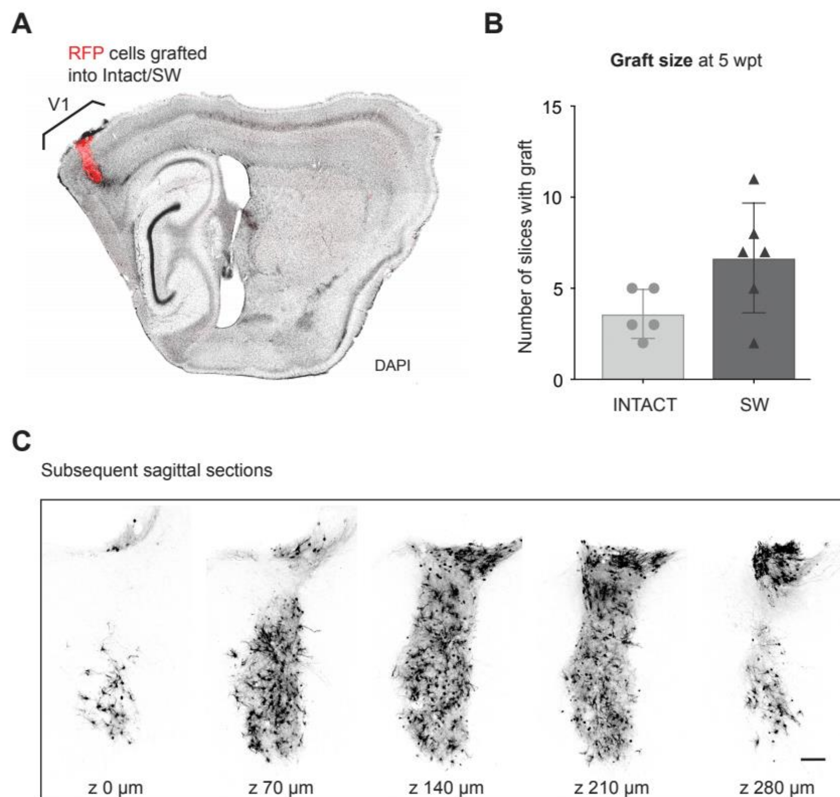


Table S1

Abbreviation	Anatomical region
AMY	Amygdala
Aud	Auditory cortex
Cg	Cingulate cortex
DBN	Diagonal band nucleus
ECT	Ectorhinal cortex
ENT	Entorhinal cortex
GP	Globus pallidus
HLZ	Hypothalamic lateral zone
Hipp	Hippocampus
HPF	Hippocampal formation
MB	Midbrain
MO	Motor cortex
Orb	Orbital cortex
Prh	Perirhinal cortex
Pons	Pons
PtPa	Posterior parietal association area
RS	Retrosplenial cortex
SI	Substantia innominata
SS	Somatosensory cortex
TEa	Temporal association areas
Thal dLGN	Thalamic dorsal lateral geniculate nucleus
Thal other	Other thalamic nucleus
Vis	Visual cortex

2.2 Aim of the study II – Aging and neurodegeneration

The aim of the second study was to examine how the healthy aged and neurodegenerative amyloid plaque-loaded cortical environments influence synaptic integration of transplanted fetal neurons.

"Excessive local host-graft connectivity in ageing and amyloid-loaded brain"

Judith Thomas, Karl-Klaus Conzelmann, Stefanie M. Hauck, Sofia Grade, Magdalena Götz

For this publication being the first author, I performed all animal surgeries along with all data analysis. I wrote and revised the manuscript together with Magdalena Götz and Sofia Grade. I was also involved in further editing and reviewing the manuscript together with the other authors.

Submitted manuscript.

Note that due to elevated number of pages Table S1 is not included in the PDF version of this thesis but is available as separated excel file.

Excessive local host-graft connectivity in ageing and amyloid-loaded brain

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Abstract

Transplantation is a clinically relevant approach for brain repair, but much remains to be understood about influences of the disease environment in the host on transplant connectivity. To explore the influence of ageing and amyloid pathology in Alzheimer's disease (AD) we examined graft connectivity using monosynaptic rabies virus tracing in APP/PS1 mice and in 16-18 month-old wildtype (WT) mice. Neurons differentiated within 4 weeks and integrated well into the host visual cortex, receiving input from the appropriate brain regions for this area. Surprisingly, we found a prominent several-fold increase in local visual cortex inputs, in both amyloid-loaded and aged environment. State-of-the-art deep proteome analysis using mass spectrometry highlights complement system activation as common denominator of environments promoting excessive local input connectivity. These data therefore reveal the key role of the host pathology in shaping the input connectome calling for caution in extrapolating results from one pathological condition to another.

Keywords

Transplantation, Neural repair, Alzheimer's disease, amyloidosis, synapse loss, connectomics, RABV tracing

Introduction

There is an urgent medical need for replacement of degenerated neurons after brain injury or neurodegenerative disease (Grade and Götz, 2017; Barker, Götz and Parmar, 2018). Transplantation of fetal neurons is at the forefront of this approach and has successfully achieved clinical improvements (Tabar and Studer, 2014; Barker *et al.*, 2017; Studer and Tabar, 2020). New sources of cells for transplantation like induced pluripotent stem cells have now further boosted the attempts for clinical translation in Parkinson's disease patients (Parmar, Grealish and Henchcliffe, 2020). However, rather little is known about neuronal graft integration in other neurodegenerative diseases or physiological ageing.

In conditions where neurons and synaptic connections are lost, transplanted neurons have to connect properly in order to repair neural circuit structure and function. Otherwise, they may cause dysfunction and perturb circuitry function rather than repair it. While output connectivity was found to be remarkably specific many years ago (Hernit-Grant and Macklis, 1996; Gaillard *et al.*, 2007; Gaillard and Jaber, 2011; Jaber *et al.*, 2013; Wuttke *et al.*, 2018; Poulopoulos *et al.*, 2019), the brain-wide analysis of input connectivity and in vivo observation of neuronal activity have been probed more recently (Falkner *et al.*, 2016; Espuny-Camacho *et al.*, 2018; Linaro *et al.*, 2019; Grønning Hansen *et al.*, 2020; Palma-Tortosa *et al.*, 2020). Fetal cells grafted into the cerebral cortex after neuronal ablation receive brain-wide inputs from the host, as revealed by rabies virus (RABV)-based transsynaptic tracing (Wickersham, Lyon, *et al.*, 2007). These inputs closely resemble the input connectome of the cortical neurons that had died (Falkner *et al.*, 2016). Co-registration of RABV-based tracing and 3D magnetic resonance imaging has further demonstrated synaptic integration of human neural transplants grafted into different brain regions in mice (Doerr *et al.*, 2017). Similar results have been obtained with cells grafted in a stroke model (Tornero *et al.*, 2017; Palma-Tortosa *et al.*, 2020) or in a traumatic brain injury (TBI) model (Xing *et al.*, 2019), but these studies did not examine the brain-wide input connectome and mainly used acute injury models.

Contrary to acute injuries, synapse and neuron loss progresses slowly during ageing and in many neurodegenerative diseases (Petralia, Mattson and Yao, 2014; Rajendran and Paolicelli, 2018; Henstridge, Tzioras and Paolicelli, 2019). Moreover, ageing or amyloid-loaded AD brains present vastly different host environments where transplants survive but their connectivity remains

unknown (Tong *et al.*, 2014; Shetty and Hattiangady, 2016; Espuny-Camacho *et al.*, 2017; Martinez-Losa *et al.*, 2018). Beyond the lack of connectivity analysis of transplants in these highly relevant conditions, basic principles for new neuron integration into a pre-existing circuitry are not known. Transplantation into the adult brain has mostly been performed in models with neuronal loss, but little is known if it is indeed a prerequisite to integrate new neurons. Synaptic loss is often the first step prior to neurodegeneration, and it is not known if and how this may influence the integration of new neurons into pre-existing but degenerating circuits.

The contribution of reactive gliosis and inflammation to host-graft connectivity have been hardly explored. Indeed, the influence of inflammation is difficult to deduce from previous studies of human cell transplants, as these xenografts require immunosuppression (Espuny-Camacho *et al.*, 2013, 2017; Tornero *et al.*, 2017; Palma-Tortosa *et al.*, 2020). The ageing brain is characterized by gradual cellular and molecular changes like oxidative damage and mitochondrial dysfunction, accumulation of aggregated proteins and mild inflammation accompanied by mild reactive gliosis (Norden and Godbout, 2013; Lupo *et al.*, 2019; Figure S1). These age-related changes go along with synapse loss, which ultimately lead to impaired function and cognitive decline (Mattson and Arumugam, 2018). Additionally, ageing is the main risk factor for neurodegenerative diseases like AD (Hou *et al.*, 2019). Hallmarks of AD include accumulation of extracellular beta amyloid proteins that clump together forming amyloid plaques, also eliciting astro- and microgliosis and loss of synapses, which ultimately lead to dysfunctional neuronal networks and cognitive impairments (Crews and Masliah, 2010). Importantly, reactive gliosis after acute and invasive brain damage, such as stroke and TBI (Burda and Sofroniew, 2014; Sims and Yew, 2017), differs profoundly from that in the ageing brain or in AD models (Radde *et al.*, 2006; Sirko *et al.*, 2013; Pekny, Wilhelmsson and Pekna, 2014; Heimann *et al.*, 2017). It is thus important to understand how these environments may affect synaptic integration of transplanted neurons.

To explore this, we carefully choose mouse models of ageing and AD, and use allografts, allowing us to probe these basic principles, while simultaneously exploring graft integration into highly relevant disease environments. The mouse model of AD overexpresses human amyloid precursor protein (APP) and Presenilin 1 (PSEN1) in neurons (APP/PS1; Radde *et al.*, 2006) lacking neuronal death in the cerebral cortex (Rupp *et al.*, 2011). These transgenic mice develop early cerebral amyloidosis accompanied by hypertrophic microglia and reactive astrocytes, and

dendritic spine loss around deposited amyloid plaques (Radde *et al.*, 2006; Bittner *et al.*, 2012; Sirko *et al.*, 2013). Thus, this mouse model allows determining the influence of progressive amyloidosis and synapse loss on new neuron integration in the absence of neuron loss, as opposed to acute neuronal loss investigated in previous studies. Furthermore, we compared with transplantations into the ageing brain that also exhibits altered synaptic dynamics (Grillo *et al.*, 2013; Mostany *et al.*, 2013), but much reduced gliosis compared to AD models with a high plaque load (Sirko *et al.*, 2013; Orre *et al.*, 2014; Osborn *et al.*, 2016; Heimann *et al.*, 2017; Figure S1).

Results

New neurons survive and integrate into cortical circuits in APP/PS1 and aged mice

To explore the influence of the distinctive cellular and molecular environments of APP/PS1 and aged brain on synaptic integration of transplanted neurons, we used our previously established paradigm of cell transplantation, circuit mapping and quantitative connectomics (Falkner *et al.*, 2016). Mouse cortical cells were isolated from embryonic day (E) 14 C57BL/6J embryos and transduced with a retrovirus encoding the rabies glycoprotein (G) required for its retrograde transport, the receptor TVA allowing for selective infection of these cells by the RABV (Wickersham, Lyon, *et al.*, 2007), and a red fluorescent protein (RFP; Figure 1A). After 3-5 days in culture, cells were collected and transplanted into the primary visual cortex (V1) of 8 months old APP/PS1 transgenic mice. At this age plaque deposition and reactive gliosis is wide-spread (Figure S1) as is the decrease in dendritic spines in the absence of neuron loss (Radde *et al.*, 2006; Rupp *et al.*, 2011; Bittner *et al.*, 2012). As controls, mice of the same age and background (C57BL/6J) including littermates were used. These were also compared to transplants into 16-18 months old ageing mice (Figure 1A). Alternatively, acutely dissociated cells from E18 Emx1Cre-G/TVA-GFP transgenic mice (Iwasato *et al.*, 2000; Nakamura, Colbert and Robbins, 2006; Takatoh *et al.*, 2013) were used for transplantation. Analysis at 5 weeks post transplantation (wpt) showed that donor cells survived well in all conditions and no difference in graft size was observed (Figures 1B-D; Figure S2A, B).

To trace the synaptic inputs to transplants developed in all the conditions, we injected the modified monosynaptic RABV (Wickersham, Lyon, *et al.*, 2007) expressing green fluorescent protein (GFP; or expressing mCherry, dependent on donor cell fluorescence) and pseudo-typed with EnvA. This allowed specifically targeting TVA-expressing donor cells at 4wpt followed by analysis one week thereafter (Figure 1A). Grafted cells that were RABV infected are referred to as starter cells that were present within the graft in all conditions (Figure 1B'-D'). Transplants were surrounded by GFP+ input neurons in all conditions, but in substantially higher number in the cortices of APP/PS1 and aged mice (Figures 1B-D). Notably, the starter cell number did not systematically vary between the experimental groups (Figure S2C, D), despite the apparently prominent differences in the number of their pre-synaptic partners in the visual cortex. Thus, transplanted neurons survive and integrate into these brain environments in the absence of any prior loss of neurons.

Excessive local connectivity of neuronal grafts in amyloid plaque loaded cortex

Next, we quantified the brain-wide input connectivity traced by RABV expressed as connectivity ratio (CR) for each and all innervating regions as described before (Falkner *et al.*, 2016). The number of fluorescent input neurons in a given brain region was divided by the total number of starter neurons (RFP+/GFP+) within the graft, to calculate the CR and allow comparison between mice and conditions. A total of 26 innervating brain regions were mapped, with corresponding CRs color-coded (Figure 2A). Importantly, all regions containing input neurons are known to innervate V1 (Oh *et al.*, 2014; Zingg *et al.*, 2014) indicating the absence of aberrant connectivity in all these conditions. We consider this an important finding for cell-based therapy for AD patients and in elderly.

Most inputs derived from local visual cortex (Vis) neurons with the highest input ratio consistently for this region (Figure 2A), as is the case for a V1 neuron in the naïve mouse brain (Oh *et al.*, 2014; Zingg *et al.*, 2014; Falkner *et al.*, 2016). Interestingly, the CR from these local neurons in the control group was very similar to endogenous neuron connectivity as determined previously (Falkner *et al.*, 2016). In contrast, this local innervation was significantly elevated in APP/PS1 (Figures 2A, B), as indicated by a 3-fold increased CR. Interestingly, this was not the case for one of the main afferents of V1, the thalamic dorsal lateral geniculate nucleus (LGN, Oh *et*

al., 2014), with no significant increase in the LGN-to-Vis connectivity in APP/PS1 as compared to control brains (Figures 2A, C). We were curious to determine the CR for other afferent regions with high plaque deposition. However, also for other plaque-loaded cortical regions known to innervate V1, such as the RS, PtPa, SS, MO, Aud, ECT, ENT, Tea, and Orb (abbreviations in Figure 2A) the CR was well comparable between APP/PS1 and control brains of equal age (Figure 2D). This was also the case for the contralateral hemisphere (Figure 2E). Thus, in amyloid plaque-loaded cortex specifically the local input connectivity to the graft is enhanced. Intriguingly, this is not related to the plaque-load of the innervating region, as relatively normal connectivity from other cortical regions with similar plaque-load was observed.

Excessive cortical connectivity of neuronal grafts in the cortex of ageing mice

To determine, if the above findings were specific to the APP/PS1 condition, we examined the synaptic integration of the same donor cells in the cerebral cortex of 16-18 months old WT mice. Intravisual host-graft connectivity was significantly increased in the ageing cortex compared to that in 8 months old mice (Figure 2A, B). Indeed, the Vis CR was similar to the one observed in APP/PS1 mice, although with greater interindividual variability. In addition, we noted a significant increase in CR also from other cortical regions, only in the aged cortices (Figure 2D). Also, the mean CR of the input from LGN was 5x higher compared to controls but failed to reach statistical significance due to the large interindividual variation (Figure 2C). Conversely, interhemispheric connectivity was well comparable to the control and other experimental groups (Figure 2E). Taken together, in both host environments, transplanted neurons receive excessive inputs from the local or overall cortical circuitry, respectively. Surprisingly, the ageing cortex which is the model with milder gliosis (Figure S1) exhibited a more widespread effect in graft connectivity as compared to the 8 months old APP/PS1 mice.

Comprehensive proteome analysis of cortical environments inducing host-graft hyperconnectivity

Given the connectivity differences in the two conditions that also differ in the extent of gliosis we used unbiased proteomics to understand the composition of these environments causing hyperconnectivity. Tissue punches were collected from visual cortices of both brain hemispheres

of 5 APP/PS1 and 9 C57BL/6J mice (4 ageing WT, 5 WT 8 months old). State-of-the-art liquid chromatography-mass spectrometry (LC-MS/MS) allowed reproducible detection of 5368 proteins. About 7.2% i.e., 384 proteins were significantly enriched in APP/PS1 tissue samples relative to the WT age-matched samples ($p \leq 0.05$; Figure 3A, Table S1a). As expected, amyloid-beta A4 protein (APP) was significantly more abundant in the cortex of APP/PS1 mice (Figure 3A) consistent with its overexpression under the Thy1-promoter in these mice (Radde *et al.*, 2006). Further validating the amyloidogenic model, Beta-secretase 1 (Bace1), known to initiate the APP processing and later on to accumulate around plaques, was also significantly increased (Peters *et al.*, 2019; Table S1a). In line with the prominent reactive astrogliosis upon amyloid deposition in these mice (Figure S1; Pekny and Nilsson, 2005; Sirko *et al.*, 2013; Orre *et al.*, 2014; Osborn *et al.*, 2016), Vimentin (Vim) and glial fibrillary acidic (GFAP) protein were significantly increased (Figure 3A). Along with reactive gliosis, we detected differentially regulated components of the extracellular matrix (ECM), such as Vitronectin (Vtn) and several integrins like integrin beta 2 (Itgb2). Interestingly, members of the classical complement system, complement C4-B (C4b) and complement subcomponent C1q, subunits a, b, and c (C1qa, b, c) were significantly enriched in the APP/PS1 group (Figure 3A). The significant enrichment of “complement activation, classical pathway” was corroborated by Gene Ontology (GO) term analysis of significantly increased proteins along with “synapse pruning”, including immunoglobulin kappa constant (Igkc), immunoglobulin heavy constant gamma 2b (Ighg2b), integrin alpha (Itgam), besides C1qc, C1qb, and C1qa (Figure 3B, Table S1b). The most significant GO terms were all related to the immune system and inflammatory processes, such as “immune response” including C1qa, C1qb, C1qc, Clusterin (Clu), APP, signal transducer and activator of transcription (Stat1), or the immune-related GTPase family M protein 1 (Irgm1) amongst others. Thus, inflammation and immune response may contribute to activate microglia-mediated synapse removal via the complement system (Presumey, Bialas and Carroll, 2017). Consistent with the synaptic dysfunction and loss during amyloidosis, proteins related to neuronal activity and synapse function, such as Leucine-rich repeat and fibronectin type 3-domain containing protein 2 (Lrnf2) and glutamate ionotropic receptor kainate type subunit 5 (Grik5) were significantly downregulated in the APP/PS1 samples (Figure 3A).

When comparing the cortex samples from 17 months to those of 8 months old mice, 175 proteins differed significantly in their abundance (Figure 3C, Table S1c), thus contributing to only

half of the total number of significantly regulated proteins in the APP/PS1 cortex. Members of the ECM were significantly increased also in the aged cortex but differed from those in the APP/PS1 cortex e.g., the hyaluronan and proteoglycan link protein 2 (Hapln2), laminins and collagen. Interestingly also in the aged cortex the GO term “complement activation, classical pathway” was significantly enriched including proteins such as Igkc, Ighg2b (Figure 3C, D, Table S1d) and several others, such as Ig mu chain c region (Ighm) involved in the immune response (Song *et al.*, 2014). Other significant GO terms were mostly related to immune system-relevant biological processes again including proteins such as Igkc, Ighg2b and Ighm (Figure 3D). Moreover, synapse proteins like bassoon (BSN) or synaptopodin (Synpo) were found downregulated (Figure 3C), therefore altogether suggesting that immune regulation, complement activation, and synapse loss are common hallmarks of the environment in both ageing and amyloid-loaded cortex.

To identify factors relevant for excessive transplant connectivity, we assessed proteins regulated in both connectivity-promoting environments. We noted that the term “phagocytosis, recognition” consisting of the complement system-related proteins Igkc and Ighg2b appeared amongst the commonly enriched proteins (Figure 3E). These proteins further consisted of e.g., Ig gamma-2B chain C region (Igh-3) and Collagen alpha-1(II) chain (Col2a1; Table S1e, f). Interestingly, many of the commonly regulated proteins are related to synaptic function and the complement system like C1q and are involved in early synapse loss in AD mouse models (Hong *et al.*, 2016) and in the ageing brain (Stephan *et al.*, 2013; Luchena *et al.*, 2018). Immunostaining confirmed the increased levels of C1q in both the plaque-loaded and aged cerebral cortex (Figures 3F-H) with a patchy appearance in the latter reported before (Stephan *et al.*, 2013) and possibly relating to the higher variability of host-graft connectivity in the aged brains. In the APP/PS1 cortex, C1q was dramatically and homogeneously increased (Figure 3G). Taken together, this analysis implies the upregulation of C1q protein and a related loss of synapses as a possible cause for an increased number of free afferents in the recipient parenchyma ready to connect with fully competent transplanted neurons rather than diseased and compromised endogenous counterparts.

Discussion

Here we explored integration of transplanted fetal neurons into brain circuits affected by amyloidosis or altered in the course of healthy ageing. This revealed excessive innervation from local neurons in these conditions, spreading further in the brains of ageing mice – a notably different outcome compared to controls or young mice subjected to a cortical injury with mild inflammatory reaction, where new connectivity is quantitatively similar to the naive (Falkner *et al.*, 2016).

Our work elucidates several basic principles underlying the integration of new neurons into brain circuits demonstrating foremost that neuron loss is not a prerequisite. There is no neuron loss in the cerebral cortex of APP/PS1 mice at 8 months of age (Rupp *et al.*, 2011) and in ageing WT mice (Jucker *et al.*, 2000; Burke and Barnes, 2006), yet new neurons receive even more connections than in models with previous neuron loss (Falkner *et al.*, 2016). Intriguingly, this effect is restricted to local connectivity in APP/PS1 mice and hence not directly related to the amyloid plaque load throughout the cerebral cortex. Notably, however, the plaque load is highest in more posterior regions such as the hippocampus and the visual cortex (Whitesell *et al.*, 2019). In the ageing brain environment, excessive inputs derive also from other cortical regions, suggesting that the cause for hyperconnectivity is slightly more pronounced and widespread in this environment and hence not directly related to the plaque load.

To explore which factors may render these brain environments so conducive for excessive inputs onto graft neurons, we performed unbiased proteome analysis. Activation of the complement and immune system were the most pronounced common hallmarks in both these environments that may be key for the observed hyperconnectivity. Complement activation has a well-documented role on tagging synapses for elimination by microglia (Stevens *et al.*, 2007; Stephan, Barres and Stevens, 2012; Hong *et al.*, 2016), a process that may be enhanced in these immune activated environments (Hong *et al.*, 2016; Xiong, Ge and Ma, 2019; Györffy *et al.*, 2020). Interestingly, a knockout of C1q in APP/PS1 transgenic mice leads to a decrease in neuropathology, especially less activated glia cells, suggesting that C1q also contributes to gliosis (Fonseca *et al.*, 2004). Synapse loss is mediated through complement upregulation (Hong *et al.*, 2016) and C1q protein increase is associated with postsynaptic sites in prefrontal cortex of macaque in the course of ageing (Datta *et al.*, 2020). We therefore propose that synapse loss

mediated through inflammation and complement activation rather than loss of neurons fosters the excessive input connectome to grafted neurons in ageing and amyloid-loaded brains.

Neuronal activity plays a key role in synaptogenesis and synaptic plasticity and synaptic competition has been described as crucial mechanism for integration of adult generated neurons in the dentate gyrus (Tashiro *et al.*, 2006; Toni *et al.*, 2007; Toda and Gage, 2018). After transplantation, like in adult neurogenesis, new neurons that are typically particularly active and excitable compete with pre-existing neurons and synapses. Thus, synapses formed onto the transplanted neurons may be preferred due to higher postsynaptic activity compared to synapses on pre-existing neurons. Consequently, activity-dependent mechanisms favoring the transplanted neurons over neurons pre-existing in the pathological environment may contribute to the hyperinnervation of neurons transplanted into the amyloidosis and aged environment. Beyond the mechanisms mediating neuron integration, our work highlights further need to optimize connectivity of transplants for AD and elderly patients, suggesting that preceding synaptopathy conditions may need to be alleviated prior to implementing neuronal replacement strategies in such conditions.

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Author contributions

M.G. conceived and financed the project; M.G. and S.G. designed the study, M.G., S.G. and J.T. designed experiments, interpreted, and discussed the data. J.T. performed all experiments and analyzed the data. S.G. provided the expertise on transplantation, RABV-based tracing, and analysis. S.M.H. provided proteomics expertise, performed mass spectrometry, and directed the analysis of the proteome data. K.K.C. provided the expertise on RABV tracing and produced the RABV vectors. J.T., M.G. and S.G. wrote the manuscript and all authors read and commented on it.

Declaration of interests

The authors declare no competing interest.

STAR Methods

Key Resource Table

Antibodies	Source	Identifier
chicken anti-GFP (1:1000)	Aves Lab	Cat. # GFP-1020; RRID: AB_10000240
rabbit anit-RFP (1:1000)	Rockland	Cat. # 600-401-379; PRID: AB_2209751
rabbit anti-C1q (1:1000)	Abcam	Cat. # 182451
mouse anti-GFAP (1:500)	Sigma	Cat. # G3893
rabbit anti-Iba1 (1:1000)	Synaptic Systems	Cat. # 234013
Secondary antibodies		
anti-chicken Alexa Fluor 488	Thermo Fisher	Cat. # A11039; PRID: AB_2534096
anti-rabbit Cy3	Dianova	Cat. # 711-165-152
anti-mouse IgG Alexa Fluor 647	Dianova	Cat. # 115-606-072
anti-rabbit Alexa Fluor 488	Thermo Fisher	Cat. # A21206
Chemicals		
HEPES 1M	Life Technologies, Gibco	Cat. # 15630056
HBSS 1x	Life Technologies, Gibco	Cat. # 24020133
Trypsin-EDTA (0.05%)	Life Technologies	Cat. # 25300054
B27 Supplement	ThermoFisher Scientific	Cat. # 17504044
DMEM (1x) Glutamax high glucose	Life Technologies, Gibco	Cat. # 61965-026
Triton X-100	Sigma	Cat. # T9284
DAPI	Sigma	Cat. # 28718-90-3
poly-D lysine (PDL)	Sigma	Cat. # P0899
Fetal bovine serum (FBS)	Pan system	Cat. # P30-3302
Penicilin-streptomycin	Life Technologies, Gibco	Cat. # 15140-122
Bovine Serum Albumine (BSA)	Sigma	Cat. # A9418
Aqua Polymount	Polysciences	Cat. # 18606-5
Paraformaldehyde (PFA)	Roth	Cat. # 0335.4
Phosphate buffered saline (PBS) 10x	Life Technologies	Cat. # 14200083
Plasmids and Virus		
RV: CAG-DsRedExpress2-2A-Glyco-IRES2-TVA	in-house production	N/A
Rabies virus (ΔG-GFP or ΔG-mCherry)	provided from Prof. Conzelmann's Lab	N/A
Experimental organisms		
C57BL6/J mice	in-house breeding	N/A
APP/PS1 mice (Radde et al., 2006)	provided from Prof. Haass' Lab	N/A
Emx1Cre-G/TVA-GFP	in-house breeding	N/A
(Iwasato et al., 2000; Nakamura, Colbert and Robbins, 2006; Takatoh et al., 2013)		
Software		
Prism	Graphpad	Version 5.0
ZEN imaging software	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
ImageJ	ImageJ	https://imagej.net/Downloads PRID: SCR_003070
Brain Explorer 2	Allen institute for brain sciences	https://mouse.brain-map.org/static/brainexplorer

Experimental model and subject details

Animals

Animals were kept in the animal facility of the Biomedical Centre, LMU Munich and experiments were performed in compliance with German and European Union guidelines and approved by the Government of Upper Bavaria. Mice were kept in specific pathogen-free conditions and had housing conditions of 12:12 h light-dark cycle, food, and water *ad libitum*. Control C57BL/6J mice, APP/PS1 transgenic mice (Radde *et al.*, 2006), and APP/PS1 wild type littermates underwent surgery at 8 months of age, ageing C57Bl/6J mice at 16-18 months of age, and both female and male mice were used for all conditions. APP/PS1 transgenic mice express human amyloid precursor protein ($APP_{KM670/671NL}$) and mutated Presenilin-1 ($PS1_{L66P}$) under Thy1 promotor and were bred on a C57BL/6J background. This genotype leads to amyloid-ß deposition from 3 months of age on, accompanied by gliosis, microglia activation, and dystrophic synaptic boutons in the absence of detectable neuron loss (Radde *et al*., 2006).

Method details

Embryonic cortex collection, cell culture, and viral labelling

Neocortical tissue from embryonic day (E) 14 C57BL/6J embryos was dissociated using Hanks balanced salt solution (HBSS) buffered with HEPES (10mM; Life technologies). A density of 200000 cells/well were plated into a poly-D-lysine (PDL, Sigma-Aldrich) coated 24-well plate using neuronal plating medium consisting of DMEM Glutamax high glucose (4.5 g/l), penicillin-streptomycin (Life Technologies), and 10% fetal bovine serum (FBS; Pan Biotech). The cells were transduced 2-4 hours (h) later with 1.0-1.2µl of Moloney-murine leukaemia virus (MMuLV)-derived retrovirus (CAG-DsRedExpress2-2A-Glyco-IRES2-TVA) per well to provide the fluorescent protein DsRed, the G-protein, and TVA receptor to the donor cells which is needed for tracing of the input connections later on. The following 2 days the medium was gradually replaced with neuronal differentiation medium consisting of DMEM Glutamax high glucose (4.5 g/l), penicillin-streptomycin, and B27 (1:50; Life Technologies). Cells were cultured 3-5 days before transplantation. On the day of transplantation, cells were washed with 1x phosphate-buffered saline (PBS) to remove viral particles, then trypsinized (0.025% of trypsin-EDTA) and collected into a tube filled with FBS-containing medium (1:1) to inhibit the enzymatic reaction. After centrifugation, the remaining cell pellet was resuspended in differentiation medium, cells were counted, and a final cell suspension of 50000 cells/µl was prepared for subsequent transplantation (cells were kept on ice until surgery).

Alternatively, neocortical tissue from triple-transgenic E18 Emx1Cre-G/TVA-GFP embryos was dissociated without culturing and viral labelling. For this purpose, only GFP-fluorescent brains were picked for dissociation as these embryos express the G-protein and TVA receptor needed for later RABV tracing. Dissociation was performed as described above and embryonic cells were resuspended in neuronal differentiation medium and centrifuged. The cell pellet was

resuspended in differentiation medium, cells were counted, and a cell suspension was prepared in the same way as for the E14 cultured embryonic cells.

Surgeries

For all surgical procedures, the animals received intraperitoneally injected anesthesia consisting of fentanyl (0.05mg/kg; Janssen), midazolam (5mg/kg; Roche) and medetomidine (0.5mg/kg; Fort Dodge) and postoperative analgesia. After each surgical procedure, mice were awakened by subcutaneous administration of atipamezol (2.5mg/kg, Janssen), flumazenil (0.5mg/kg, Hexal), and buprenorphine (0.1mg/kg, Essex). On the day of surgery and the following 3 days, analgesic meloxicam (1mg/kg, Metacam) was given to the mice.

Transplantation

Mice (Control and aged C57BL/6J, APP/PS1 WT littermates, and double-transgenic APP/PS1) were transplanted with 1 µl of cell suspension (~ 50000 cells) into the visual cortex of the right hemisphere using the following coordinates from lambda: 2.5 ± 0.3 mm mediolateral, 0.0 ± 0.2 mm anteroposterior. Before injection of cells, a cranial window was drilled to open the skull. Subsequently, donor cells were transplanted using a ga33 mm Hamilton syringe and injected at a cortical depth from 0.6 to 0.2 mm (corresponding to cortical layers 1 – 4) at very low speed. The bone lid was carefully placed back onto the brain surface and the skin was sutured.

Rabies-virus injection for transsynaptic tracing

In order to analyze brain-wide synaptic input connections to the grafted cells, we injected a genetically modified rabies virus (RABV; EnvA-coated and ΔG-eGFP or ΔG-mCherry; Wickersham *et al*., 2007) 4 weeks after transplantation, as described before (Falkner *et al*., 2016). During the surgical procedure, RABV was injected around the graft area within the visual cortex. To do so, the cranial window was reopened and RABV was injected using a thin glass capillary inserted into an automated nanoinjector (Nanoliter; World Precision Instruments). Three injections around the graft area were performed (200nl per injection; injection speed of 1nl/second) to ensure an even distribution of RABV at the graft site.

Immunohistochemistry

One week after RABV injection, mice were transcardially perfused under Ketamine (100mg/kg) + Xylazine (10mg/kg) anesthesia. For perfusion procedure, 1x PBS was supplied for ~ 5min followed by 4% paraformaldehyde (PFA) for ~ 20 min. Afterwards brains were carefully dissected and subsequently stored in 4% PFA at 4°C overnight. For connectome analysis, the brain was cut serially into 70µm sagittal slices using a vibratome. Free-floating sections were washed and incubated in blocking solution (3% bovine serum albumin and 0.5% triton X-100 in 1x PBS) for 2h and the following antibodies were diluted in blocking solution: chicken anti-GFP (1:1000, Aves Labs) and rabbit anti-RFP (1:1000, Rockland). Sections were incubated for 48h at 4°C, washed, and incubated with following secondary antibodies for 3h at room temperature (RT): anti-

chicken Alexa Fluor 488 (1:1000, Invitrogen) and anti-rabbit Cy3 (1:1000, Dianova). Before mounting, 4.6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich) was applied to the sections for 15 minutes to label cell nuclei.

For C1q, GFAP, and Iba1 immunostainings, the same staining procedure applies except that 50µm sections were used to ensure better penetration of the antibody. Rabbit anti-C1q antibody (1:1000, Abcam), mouse anti-GFAP (1:500, Sigma), or rabbit anti-Iba1 (1:1000, SySy) were applied 1 or 2 overnights and secondary anti-rabbit or anti-mouse Alexa Fluor 647, or anti-rabbit or anti-mouse Alexa Fluor 488 were applied for 3h at room temperature followed by DAPI staining.

Quantification of input connections

Each brain section (in serial order) was checked for GFP-labelled input cells and whenever cells were detected, a 10x tile scan of the whole section was made with automated scanning, tile alignment and image stitching using an epifluorescent microscope (Zeiss, Axio Imager M2) in order to assign input cells to a certain anatomical brain area. Brain regions were assigned by overlapping the tile scan with the corresponding section in the Allen Brain Reference Atlas of the adult mouse brain. The very lateral brain sections are not available in the Reference atlas and thus, if GFP+ cells were found in very lateral sections, the tiles were aligned using the Brain Explorer 2 software (Allen Institute for Brain Science) to identify the correct anatomical region. Whenever a high number or closely assembled labelled cells were found, Z-stack scanning was performed with a laser-scanning confocal microscope (Zeiss, LSM 710) using a 40x objective. In sections that contained the graft area, all GFP+ cells with neuronal morphology and all GFP+/RFP+ cells with neuronal morphology (starter cells) were counted. The input connectivity ratio for a given brain region was calculated by dividing the number of total GFP+ input cells per anatomical brain region through the total number of GFP+/RFP+ neuronal starter cells within the graft in the primary visual cortex. Data is presented as mean ± SEM calculated between different mice for each condition ($n \geq 5$).

Image analysis

An epifluorescent microscope with a motorized stage and 10x objective (Zeiss, Axio ImagerM2) and a laser-scanning confocal microscope (Zeiss, LSM 710) with a 40x objective were used for imaging. To analyze whole brain input connections, the ZEN 2012 (Zeiss) and ImageJ 1.48p software were used. With the plug-in "Cell counter" in ImageJ and by carefully checking serial sections of the confocal Z-stacks, exact numbers of starter and input cells were counted.

Mass spectrometry

8 months old control C57BL/6J (n=10), 17 months aged C57BL/6J (n=4), and 8 months old APP/PS1 mice (n=5) were sacrificed through cervical dislocation, brains were removed and placed into cold 1x PBS. Biopsy punches of the visual cortex of both hemispheres were taken with a tissue puncher (2.5 mm diameter) and meninges and white matter were carefully

removed using forceps to have a tissue sample consisting of grey matter only. Each sample was put into a low-protein binding Eppendorf tube, frozen on dry ice, and stored at – 80°C until further processing.

Tissue samples were lysed in NP40 buffer (1% NP40 in 10mM Tris, pH 7.4, 150mM NaCl) in a Precellys homogenizator (VWR) and 10µg total protein per sample were proteolyzed with Lys-C and trypsin using a modified FASP procedure (Grosche *et al.*, 2016).

LC-MSMS analysis was performed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) online coupled to a nano-RSLC (Ultimate 3000 RSLC; Dionex). Tryptic peptides were accumulated on a nano trap column (Acclaim PepMap 100 C18, 5 µm, 100 Å, 300 µm inner diameter (i.d.) × 5mm; Thermo Fisher Scientific) at a flow rate of 30µl/min and then separated by reversed phase chromatography (µPAC™ column, 200cm length, with pillar array backbone at interpillar distance of 2.5µm, PharmaFluidics, Zwijnaarde, Belgium) using a non-linear gradient for 240 minutes from 3 to 42% buffer B (acetonitrile [v/v]/0.1% formic acid [v/v] in HPLC-grade water) in buffer A (2% acetonitrile [v/v]/0.1% formic acid [v/v] in HPLC-grade water) at a flow rate of 300nl/min. MS spectra were recorded at a resolution of 60,000 with an AGC target of 3 x 106 and a maximum injection time of 50ms at a range of 300 to 1500 m/z. From the MS scan, the 10 most abundant ions were selected for HCD fragmentation with a normalized collision energy of 27, an isolation window of 1.6 m/z, and a dynamic exclusion of 30 s. MS/MS spectra were recorded at a resolution of 15,000 with an AGC target of 105 and a maximum injection time of 50ms.

Proteome Discoverer 2.4 software (Thermo Fisher Scientific; version 2.4.1.15) was used for peptide and protein identification via a database search (Sequest HT search engine) against Swiss Prot database, taxonomy mouse (17038 sequences), considering full tryptic specificity, allowing for up to two missed tryptic cleavage sites, precursor mass tolerance 10ppm, and fragment mass tolerance 0.02 Da. Carbamidomethylation of Cys was set as a static modification. Dynamic modifications included deamidation of Asn and Gln, oxidation of Met, and a combination of Met loss with acetylation on protein N-terminus. Percolator (Käll *et al.*, 2007) was used for validating peptide spectrum matches and peptides, accepting only the top-scoring hit for each spectrum, and satisfying the cut-off values for FDR <1% (high confidence). Protein groups were additionally filtered for an identification FDR<5% (target/decoy concatenated search validation). The final list of proteins complied with the strict parsimony principle.

Data processing – Label-free quantification

Peak intensities (at RT apex) for top 3 unique peptides were used for pairwise ratio calculations. Abundance values were normalized to the total peptide amount to account for sample load errors. The protein abundances were calculated summing the abundance values for admissible peptides. The final protein ratio was calculated using median peptide ratios of at least 8 biological replicates each (8 replicates WT 17 months, 10 replicates APP/PS1 and WT 8 months). The statistical significance of the ratio change was ascertained employing the approach

described in (Navarro *et al.*, 2014) which is based on the presumption that we look for expression changes of proteins that are just a few in comparison to the number of total proteins being quantified. The quantification variability of the non-changing "background" proteins can be used to infer which proteins change their expression in a statistically significant manner. Data was filtered to ensure direct identifications (not based on match-between run) in at least 30% of samples within at least one experimental group. To visualize the data, volcano plots with log2 abundance ratios of sample replicates of each brain condition and the corresponding log10 p-values were created with Microsoft Excel. For gene ontology enrichment, significantly differentially expressed up- or downregulated proteins were run against a background list of all detected proteins using the webserver GO-rilla (http://cbl-gorilla.cs.technion.ac.il/; (Eden *et al.*, 2009).

Statistical analysis

Per experimental condition, input connectivity quantification was performed with at least 5 animals of different sets of experiments. Graphs and statistical analysis were done with Graphpad Prism 5.0 software. Values are reported as mean ± SEM calculated between different mice. Statistical significance was defined as $*p \leq 0.05$. For variables normally distributed statistical significance was calculated using one-way ANOVA followed by Bonferroni post-test or, if not normally distributed, Kruskal-Wallis test was performed.

Data and code availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD023407 and 10.6019/PXD023407.

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Main figure legends

Figure 1: Transplanted neurons integrate into the cortex of APP/PS1 and ageing mice

(A) Scheme of the experimental procedure. (B-D) Representative micrographs of transplantation site in control (B), amyloid plaque-loaded (APP/PS1; C), and aged (D) cortex at 5wpt (sagittal sections, confocal z-projection; CC corpus callosum; scale bar 200 µm). TVA expression in donor cells (RFP+) renders them susceptible to RABV (EnvA, GFP+) infection, resulting in RFP/GFP co-expressing starter neurons (insets shown in high magnification in B'-D', white arrowheads; scale bar 100 µm). Expression of the Glyco (G) protein (A) in transplanted cells allows RABV to propagate retrogradely across one synapse resulting in GFP-only labelling of first-order presynaptic neurons.

Figure 2: Excessive input connectivity to transplants in APP/PS1 and ageing cortex

(A) Colour-coded connectivity ratio (CR) for all brain-wide input neurons normalized to transplanted starter neurons per brain region at 5wpt in control, APP/PS1, and ageing cortex and list of abbreviations. (B-E) CRs of the different regions indicated on the y-axis in the brain environments indicated on the X-axis. In (D) cortical regions considered: RS, PtPa, SS, MO, Aud, ECT, ENT, Tea, Orb. Data are shown as mean ± SEM. One-way ANOVA followed by Bonferroni post-test, $*p \leq 0.05$.

Figure 3: Comprehensive proteome changes in APP/PS1 and ageing cortex compared to controls

(A, C) Volcano plots showing mean protein abundance ratios comparing APP/PS1 (A) or ageing (C) cortex with controls. Significantly different proteins (-log10 p-value, y-axis; log2 abundance ratio, x-axis) are indicated in colour (enriched in red, reduced in blue). (B, D) Most relevant enriched GO terms (BP biological process) in APP/PS1 and ageing brains vs. control. (E) Venn diagram showing the overlap of proteins enriched in APP/PS1 and ageing cortex vs. control. (F-H) Micrographs of C1q immunostaining in the visual cortex of animals indicated on top. Dashed line (H) shows C1q-high patch in the ageing cortex. (sagittal sections, confocal z-projection, scale bar 100 µm and 10 µm).

Supplemental figure legends

Figure S1: Different extent of reactive gliosis in the visual cortex of 17 months aged and 8 months old APP/PS1 mice

(A-D) Micrographs of sagittal sections from visual cortex (confocal z-projection) depict microglia (Iba1 in A, B) and reactive astrocyte (GFAP in C, D) immunostaining. Note the low activation of microglia and astrocytes in the aged (A, C) in contrast to strongly reactive Iba1+ microglia and GFAP+ astrocytes around amyloid plaques in the APP/PS1 cortex (B, D). Scale bars: 100 µm.

Figure S2: Similar graft size and starter cell numbers in different cortex environments

(A) Micrographs of 3 serial sagittal showing the graft (RFP+ cells) size in APP/PS1 cortex at 5wpt (70 µm thickness per section, 10x tiles-can and 10x confocal pictures). **(B, C)** Histograms depicting graft size (measured as number of serial sections covered by the graft, mean ± SEM, One-way ANOVA, ns, in **B**) and starter cell numbers (mean ± SEM, One-way ANOVA, ns) in **C)** at 5wpt in the different brain environments. Note that grafts size spans 3-6 sections in all conditions **(B)** and no systematic difference is detectable in the number of starter cells **(C)**. Importantly, similar local visual cortex (V1) connectivity ratios as shown in **(D)** for each condition are observed independent of the difference in starting cell numbers. Scale bars in A: 1000 µm and 200 µm.

Other Supplemental Items

Table S1: Protein list with GO term enrichment analysis (additional excel file that is not included in this thesis).

Figure 1

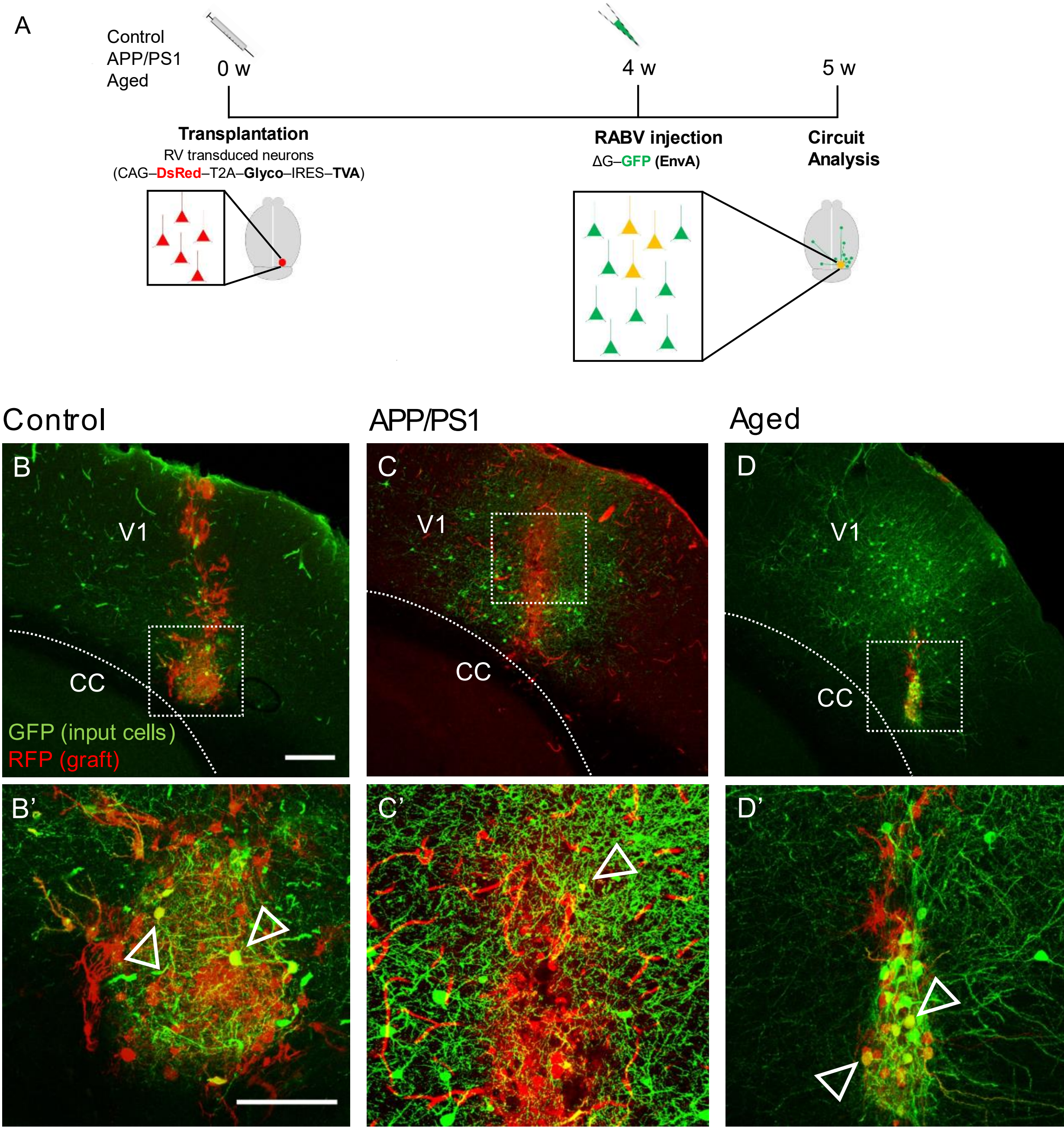
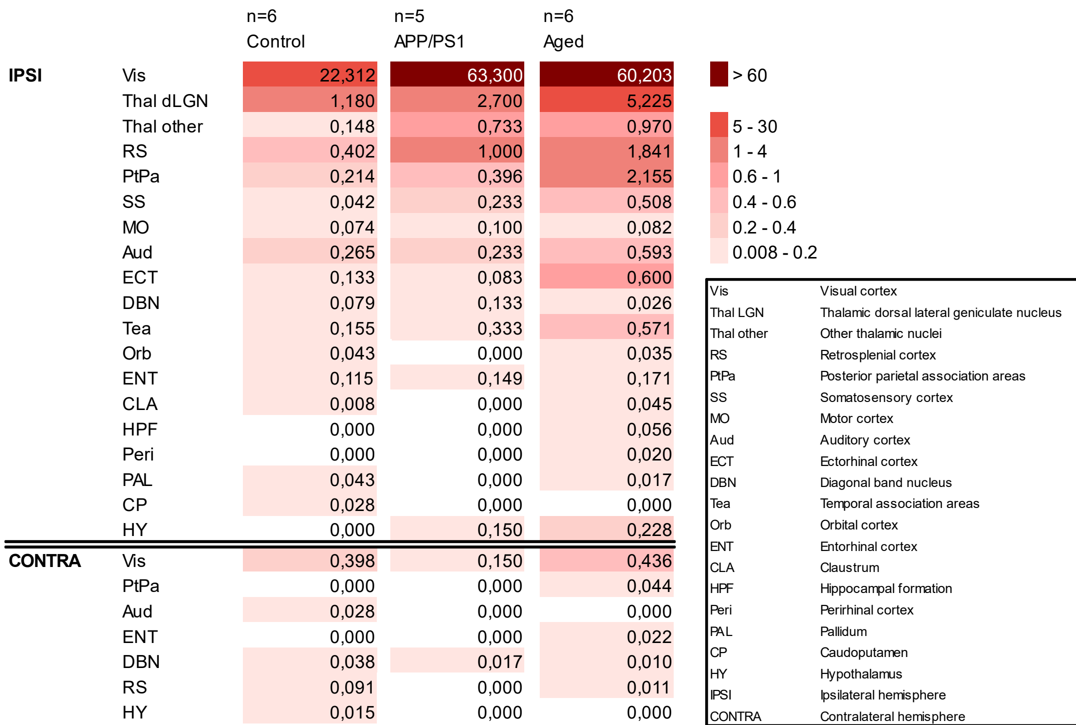


Figure 2

A



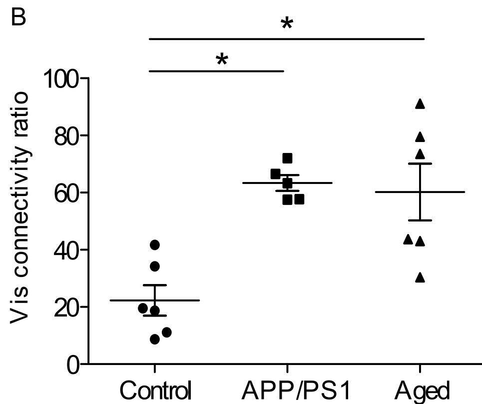
Input connectivity ratio

		n=6 Control	n=5 APP/PS1	n=6 Aged
IPSI	Vis	22,312	63,300	60,203
	Thal dLGN	1,180	2,700	5,225
	Thal other	0,148	0,733	0,970
	RS	0,402	1,000	1,841
	PtPa	0,214	0,396	2,155
	SS	0,042	0,233	0,508
	MO	0,074	0,100	0,082
	Aud	0,265	0,233	0,593
	ECT	0,133	0,083	0,600
	DBN	0,079	0,133	0,026
	Tea	0,155	0,333	0,571
	Orb	0,043	0,000	0,035
	ENT	0,115	0,149	0,171
	CLA	0,008	0,000	0,045
	HPF	0,000	0,000	0,056
	Peri	0,000	0,000	0,020
	PAL	0,043	0,000	0,017
	CP	0,028	0,000	0,000
	HY	0,000	0,150	0,228
CONTRA	Vis	0,398	0,150	0,436
	PtPa	0,000	0,000	0,044
	Aud	0,028	0,000	0,000
	ENT	0,000	0,000	0,022
	DBN	0,038	0,017	0,010
	RS	0,091	0,000	0,011
	HY	0,015	0,000	0,000

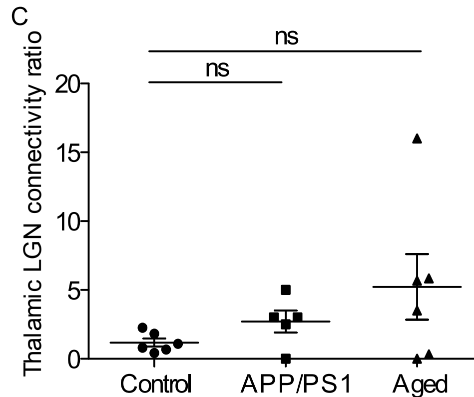
Colour scale
> 60
5 - 30
1 - 4
0.6 - 1
0.4 - 0.6
0.2 - 0.4
0.008 - 0.2

Abbreviation	Region
Vis	Visual cortex
Thal LGN	Thalamic dorsal lateral geniculate nucleus
Thal other	Other thalamic nuclei
RS	Retrosplenial cortex
PtPa	Posterior parietal association areas
SS	Somatosensory cortex
MO	Motor cortex
Aud	Auditory cortex
ECT	Ectorhinal cortex
DBN	Diagonal band nucleus
Tea	Temporal association areas
Orb	Orbital cortex
ENT	Entorhinal cortex
CLA	Claustrum
HPF	Hippocampal formation
Peri	Perirhinal cortex
PAL	Pallidum
CP	Caudoputamen
HY	Hypothalamus
IPSI	Ipsilateral hemisphere
CONTRA	Contralateral hemisphere

B



C



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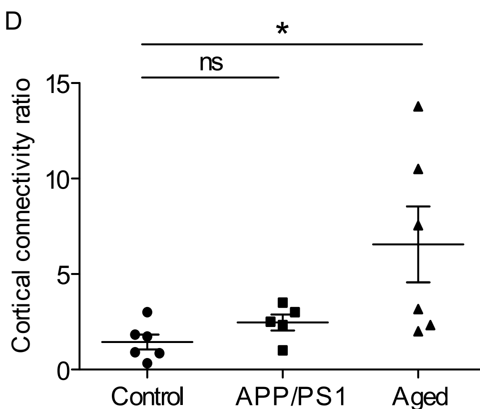

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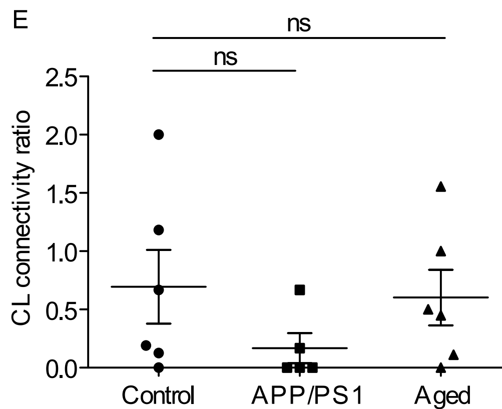


Figure 3

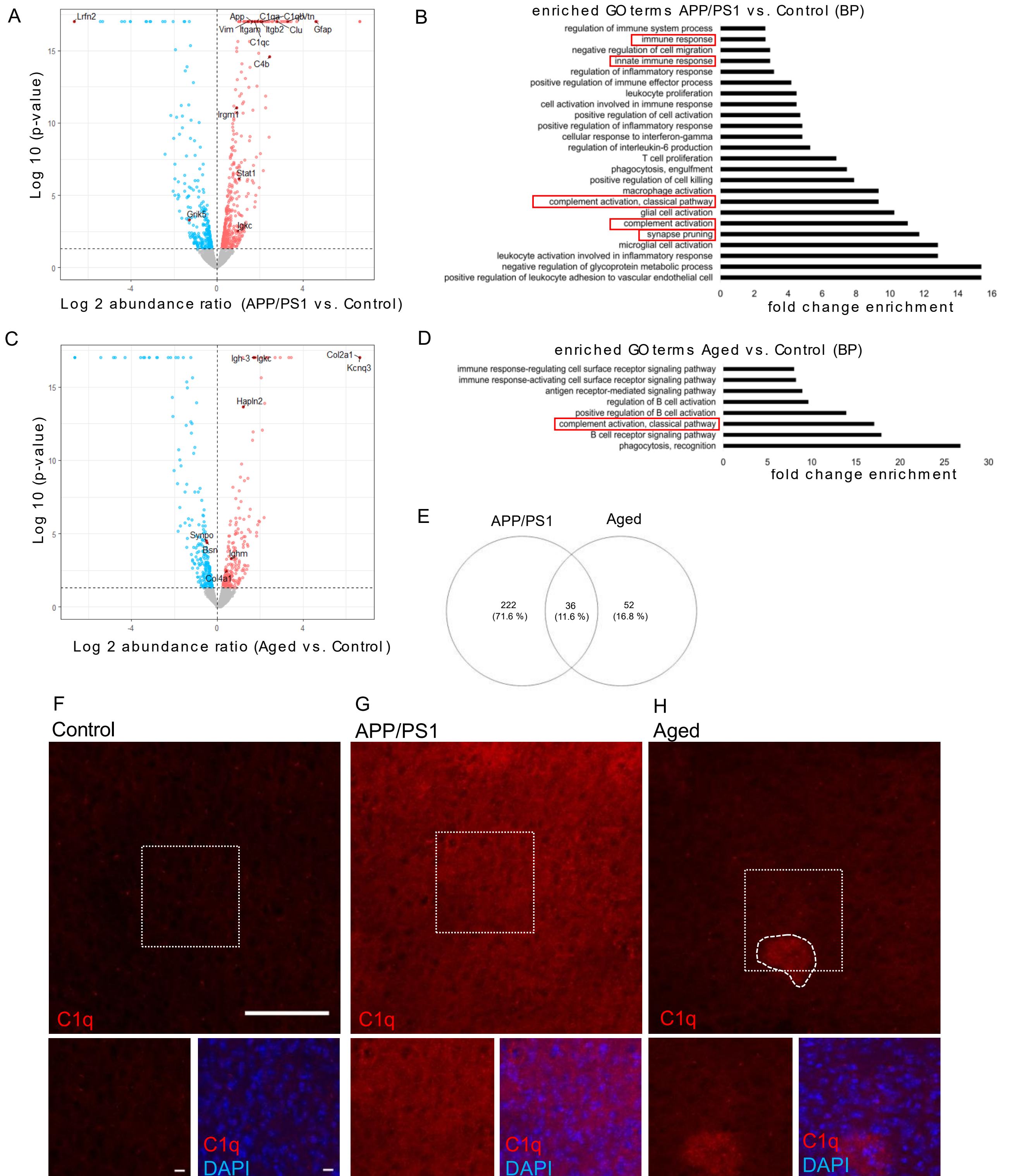


Figure S1

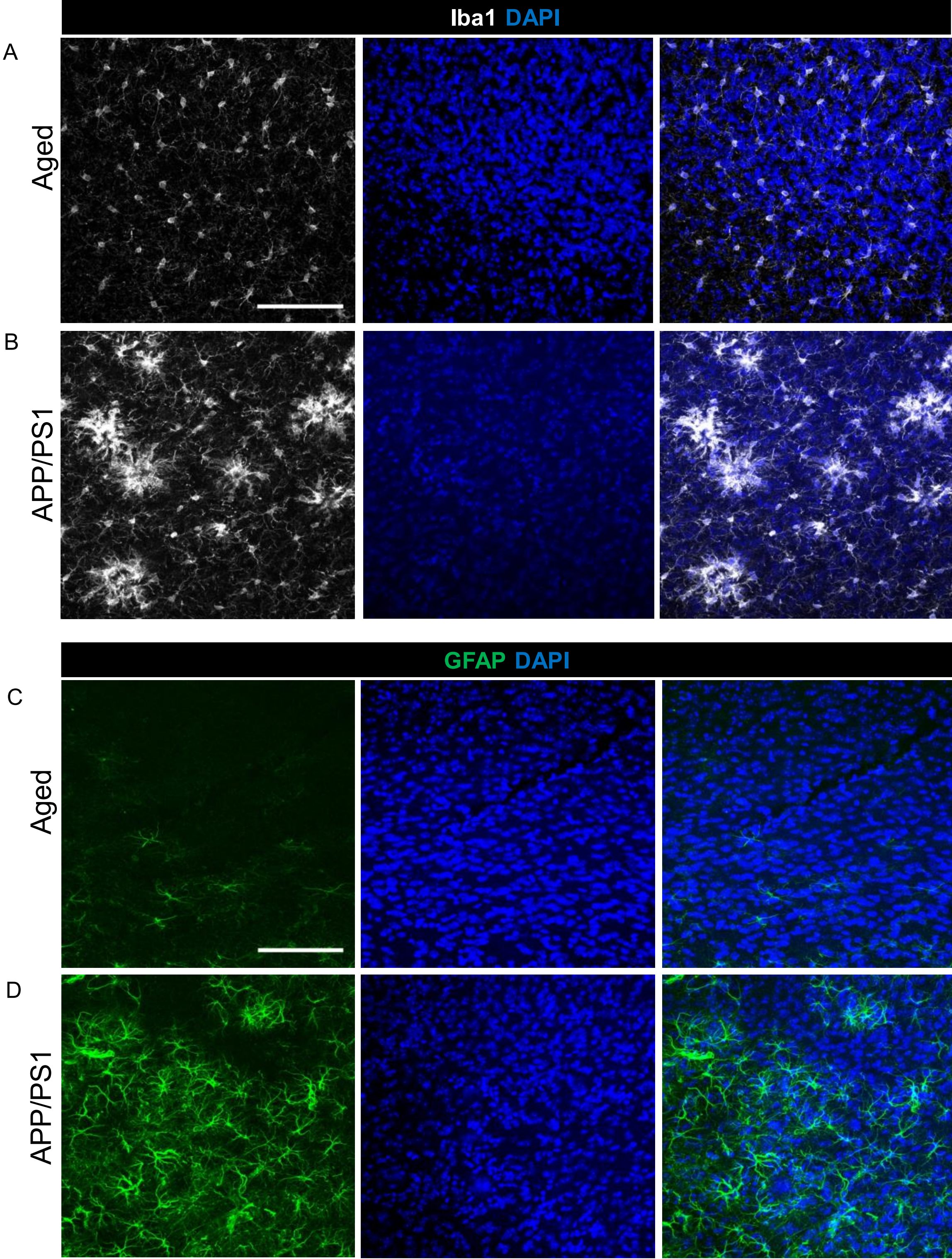
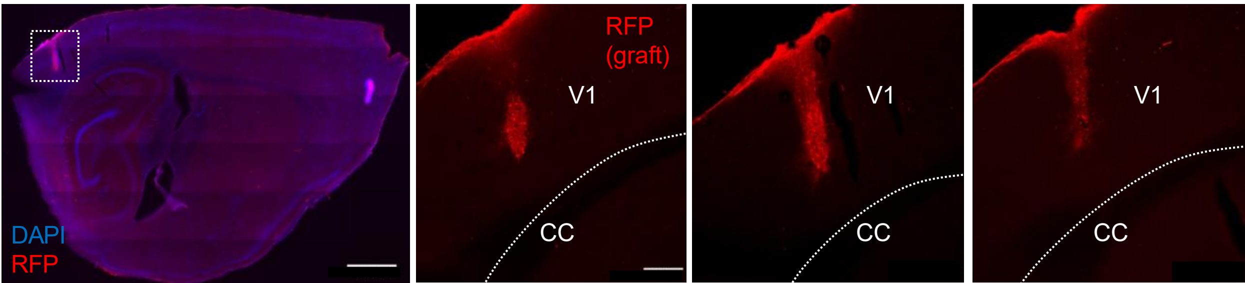


Figure S2

A



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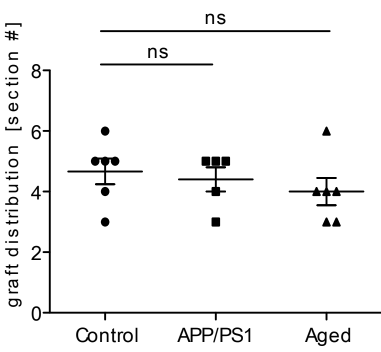

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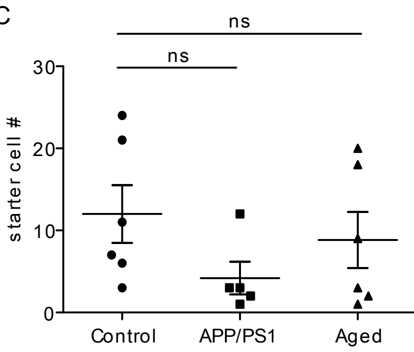

D

Control	starter cell #	V1 input ratio
	7	11.1
	6	19.5
	3	18.6
	24	41.7
	21	8.7
	11	34.2

APP/PS1	starter cell #	V1 input ratio
	12	66.5
	3	57.6
	3	63.3
	2	57.5
	1	72.0

Aged	starter cell #	V1 input ratio
	1	43.0
	20	79.5
	18	30.3
	9	91.1
	2	73.5
	3	43.7

DISCUSSION

3. Discussion

During my PhD studies, the data of Grade *et al.* and Thomas *et al.* proved that fetal cortex cells transplanted into the adult mouse visual cortex form input connections with the host brain that was either acutely injured (SW), solely inflamed (LPS), physiologically aged, or amyloid-plaque loaded. Firstly, transplanted neurons survive and differentiate equally well with comparable graft sizes within all of these different host conditions. However, the SW-injured host environment has a huge impact on the input connectivity of grafted cells, such that new neurons form massive local connections with the endogenous neurons. Interestingly, this effect is even more pronounced in the aged and amyloid-plaque loaded host brain environments. On the other side, the younger uninjured and untreated control as well as the uninjured but inflamed host cortices also allow new neuron integration but to a much lower extent (Figure 5), which is below endogenous input connectivity levels. These interesting findings indicate that a preceding neuronal loss is not necessary for new neurons to integrate and in addition highlight the importance of the host brain microenvironment into which a graft is placed. The formation of hypo- or hyper-connections with the host circuitry, depending on the existing condition of the brain parenchyma, could influence the therapeutic outcome of neuron transplantation for brain repair and should therefore be carefully considered for future clinical trials.

3.1 Acute injury and inflammation differentially affect synaptic integration

TBI causes neuron loss, disturbs synaptic connections, and leaves the brain tissue inflamed and scarred which eventually has a negative outcome on the patient's health. Brain injury has been studied intensively regarding transplantation (Weston and Sun 2018) and many studies showed that grafted neurons survive, differentiate, and mature, e.g. in a stroke environment (Kokaia, Tornero and Lindvall, 2017; Laterza *et al.*, 2018; Yu *et al.*, 2019) and also in other brain injury conditions (Falkner *et al.*, 2016; Haus *et al.*, 2016; Zhu, Eom and Hunt, 2019).

In this study we show that fetal neurons grafted into the acutely injured, inflamed, and young intact control brain environment survive and integrate. The quantitative connectivity data shows that the local visual cortex input connections are increased in the SW injury condition not only compared to the intact controls, but also compared to the endogenous visual cortex and the

reconstructed visual cortex connectivity analyzed in Falkner *et al*. This is an important finding for two reasons. First, it appears that a severely injured parenchyma promotes the formation of more synaptic connections that exceed the reconstructed circuitry after mild cortical insult, as well as the endogenous input connections (Falkner *et al.*, 2016). Second, although both the intact and injured cortex integrate new neurons, the disrupted and inflamed SW environment facilitates synaptic integration to a higher extent. One reason could be the strong glial reactivity and disrupted BBB observed in invasive injuries like the SW (Buffo *et al.*, 2008; Sirko *et al.*, 2013; Frik *et al.*, 2018; Mattugini *et al.*, 2018) as compared to noninvasive upper layer neuronal loss (Madison and Macklis, 1993; Falkner *et al.*, 2016). A consequential question is if the observed excessive local input connectivity would further increase along with more severe injury conditions or remain at a stable level. Repetitive cortical injury was recently shown to promote more astrocyte proliferation compared to a single cortical injury (Lange Canhos *et al.*, 2021). Therefore, transplantation experiments in conditions of bigger or multiple lesions may reveal if stronger reactive gliosis further increases the connectivity levels of transplanted cells. Additionally, it would be interesting to investigate host-graft connectivity levels in a stroke condition in which immune response and inflammation is significantly increased (Lambertsen, Finsen and Clausen, 2019). Neurons transplanted into a stroke-injured environment were shown to integrate into the host circuit but the level of connectivity was never quantitatively measured as in our SW injury model (Tornero *et al.*, 2017; Yu *et al.*, 2019; Palma-Tortosa *et al.*, 2020).

To elucidate if inflammation alone would be sufficient to cause increased synaptic input connectivity, we injected LPS intraperitoneal into otherwise young and healthy mice to cause cerebral inflammation. It is known that a peripheral inflammation leads to CNS inflammation and dysfunction (Biesmans *et al.*, 2013; Strehl *et al.*, 2014; Widmann and Heneka, 2014) and therefore, this enabled us to examine the effect of cerebral inflammation on new neuron integration. Surprisingly, we did not find a striking increase in the local connectivity ratio as in the injured cortex, and instead the connectivity ratios were closer to the input levels that we found in the young intact cortex. This is interesting because the LPS-triggered glial reactivity is very similar, yet not as pronounced, in the SW-injured cortex, as shown by reactive microglia and astrocyte immunostaining (see Grade *et al*., Figure 3). However, a severe cortical injury like a SW causes BBB disruption and elicits monocyte invasion (Ikeshima-Kataoka and Yasui, 2016; Frik *et al.*, 2018), which are additional aspects besides inflammation that may foster new neuron

integration. Thus, these results indicate that inflammation itself is not sufficient to cause high levels of synaptic integration of grafted neurons.

However, there is evidence that the cortical inflammation induced by LPS from different species affects synapse and neuronal function differently. LPS from *Escherichia coli,* which was also used in our study, did not change dendritic complexity in the hippocampus of adult mice at 3 months after stimulation. On the other hand, changes in neuronal and dendritic morphology occurred after *Salmonella tymphimurium* LPS injection (Beyer *et al.*, 2020). Potentially we did not observe a strong inflammation-induced increase of connectivity ratios because transplantation one week after LPS stimulation was too early to allow the generation of robust neuroinflammation. Alternatively, *E.coli*-derived LPS was not effective enough to generate high levels of inflammation and hence influence input connectivity. Therefore, we assume a rather minor influence of inflammation and the resulting reactive gliosis on new neuron integration. Although we show that prior neuron loss is not necessary for new cells to integrate, a certain damage to the brain parenchyma appears advantageous for cells to integrate to a high extent. However, the mechanisms behind our observation need to be elucidated.

Our comprehensive proteome data comparing cortical tissue from acute injury, LPS injection, and intact conditions shows many differentially expressed proteins between the hyper-connectivity (SW) and hypo-connectivity (intact and LPS-injected) environments. There is an elevated expression of inflammation-, ECM-, and complement system-related proteins in the SW as compared to the intact control condition. Especially complement factors involved in the classical as well as the alternative pathway are highly enriched one week after SW injury. The complement system is a known interactor of TBI propagating neuroinflammation and is implicated in secondary injury (Elvington *et al.*, 2012; Hammad, Westacott and Zaben, 2018; Roselli *et al.*, 2018). Moreover, the targeted inhibition of specific complement pathways or components was shown to improve functional recovery after brain injury (Ruseva *et al.*, 2015; Rich *et al.*, 2016; Alawieh *et al.*, 2018). Importantly, complement system factors are also involved in tagging synapses for their phagocytic removal by microglia e.g. (Stephan, Barres and Stevens, 2012; Presumey, Bialas and Carroll, 2017). The GO term analysis with the proteome dataset also revealed a decrease in synaptic plasticity-related proteins, which would match the known role of complement factors involved in synapse elimination.

Additionally, we found the blood coagulation protein fibrinogen to be upregulated in the SW-injured cortex. Fibrinogens are typically found in the brain parenchyma of conditions in which BBB disruption or leakage occurs (Petersen, Ryu and Akassoglou, 2018). Furthermore, it was shown that vascular damage can induce synapse elimination (Merlini *et al.*, 2019). Thus, we suggest that a loss of synapses rather than actual neuron loss is beneficial for the formation of new synapses with grafted neurons. Injury-induced upregulation of classical and alternative complement pathways along with fibrinogens could lead to increased synapse elimination, which in turn facilitates the formation of new synaptic contacts with transplanted neurons. This effect is probably further supported by inflammation, but as mentioned before, inflammation *per se* seems not to be sufficient to lead to excessive host-graft connectivity.

The role of the complement system in brain injury and in synapse tagging is intriguing as we found that the excessive levels of input connectivity are transient. Transplanted neurons connect excessively in the SW-injured environment at 4 wpt but this effect disappeared at 12 wpt. More precisely, the local connectivity ratio dropped to half the level of the endogenous visual input connectivity (Falkner *et al.*, 2016) and decreased almost 3 times from 1 to 3 months after transplantation in the SW injury paradigm. This suggests that mechanisms of the host cortical network led to excessive pruning of the overnumbered synaptic connections. This is particular interesting as this effect was not observed under the condition of neuronal ablation in which input connectivity levels remained constant between 1 and 3 months after transplantation (Falkner *et al.*, 2016). A transplantation study in a PD mouse model showed that host-graft input connections stayed very similar between 6 weeks and 6 months post grafting (Grealish *et al.*, 2015). RABV tracing of transplanted neurons in a rat model of stroke showed that the number and distribution of traced neurons was also stable between 2 and 6 months (Tornero *et al.*, 2017). On the other side, recent data of RABV-traced grafted human progenitors in a model of Huntington's disease revealed an increase of traced input connections (but also starter cells) from 1 to 2 months after transplantation, suggesting that additional synaptic connections are being formed after 1 month (Besusso *et al.*, 2020). Although connectivity seems to be stable in some cases, our data and Besusso *et al*. show that host-graft connections are still being modified 1 month after transplantation. However, this process may depend on the grafted brain region and the disease model. Regarding neuron transplantation after cortical SW injury, our data is the first to show such an evident quantitative decrease of input connections over time. It is known

that network connectivity may be altered after traumatic injury, which goes along with enhanced excitatory connections and such a hyperinnervation can eventually lead to epilepsy (Jacobs *et al.*, 2000). Our findings illustrate that the host cortical network adapts newly formed connections over time to a lower level. Whether this effect is beneficial or detrimental for the existing cortical circuitry remains to be determined.

Furthermore, our data suggest that transient input connections are a consequence of synapse pruning. Generally, synapse elimination is a physiological process that occurs during postnatal development and is mediated by microglia to shape the circuitry and guarantee network homeostasis (Paolicelli *et al.*, 2011). Synapse pruning has also been shown to occur after brain injury (Stephan, Barres and Stevens, 2012). However, if the observed transient input connectivity in the SW condition is indeed due to synapse engulfment needs to be proven. Quantification of synaptic contacts between host and graft cells or *in vivo* live imaging experiments of the dendrites and spines of transplanted neurons could provide a definite answer to our observation. Furthermore, it is crucial to understand the mechanisms behind such a decrease of newly formed connections. If synaptic connections will stabilize at some point or continue to decrease is important to know, because this could affect the behavioral outcome of such brain repair strategies.

3.2 The impact of aging and amyloid-plaque deposition on new neuron integration

The aging and degenerating brain parenchyma are characterized by a progressive increase of reactive glia cells and inflammation, as well as decreased synaptic plasticity, although these effects vary between brain areas and disease types. During my PhD project, I wanted to understand how age- or disease-related changes of the brain possibly influence new neuron integration. The impact of the aging CNS is not yet well studied regarding neuronal replacement strategies as most studies were performed in injury or in disease-specific conditions. For instance, NSCs were grafted into the stroke-injured striatum of young and aged rats and a beneficial outcome of the transplantation was shown even in the aged animals (Tang *et al.*, 2014). NSCs have been grafted into the hippocampi of young and aged rats and in both cases robust engraftment was observed (Shetty and Hattiangady, 2016). Moreover, transplantation of NSCs into 15-18 months old aged mice suffering from spinal cord injury led to functional recovery

and grafted cells displayed better survival rates in aged compared to young transplanted mice (Takano *et al.*, 2017). Thus, there is proof that the aging CNS is amenable for cell transplantation, but it is not understood to which extent the healthy aging neocortical parenchyma, unaffected by injury, is synaptically integrating new neurons. Neurodegenerative disease brain environments have been studied regarding new neuron transplantation e.g. in PD (Hallett *et al.*, 2015; Yasuhara *et al.*, 2017; Song *et al.*, 2020) or AD rodent models (Li, Yu and Cai, 2015; Li *et al.*, 2016; Espuny-Camacho *et al.*, 2017; Martinez-Losa *et al.*, 2018). However, the effect of a slowly degenerating cortical environment in which the structure and function of neurons and synaptic contacts, and hence network function is disturbed, e.g., through amyloid-plaque deposition in AD, has never been specifically investigated regarding host-graft connectivity.

My data shows that transplanted fetal neurons survive and integrate in the aging and amyloid-plaque enriched mouse cortex that did not experience any insult or neuronal death. Thus, a preceding neuronal loss is not required for grafted cells to integrate, which is also supported by the data of Grade *et al.* in which grafted cells survive and integrate in the young uninjured control condition (as discussed before). In the aging environment, the cells connected solely with appropriate input regions, but formed excessive local and cortical host-graft connections. It appears that the aging cortex is permissive for new neurons to integrate even though preceding neuronal loss did not occur. Similarly, we found a massive increase in local input connections in the amyloid-plaque loaded cortex of APP/PS1 transgenic mice although this hyper-connectivity was restricted to local visual connections. Neuron transplantation has been performed previously in AD mouse models (Tong *et al.*, 2014; McGinley *et al.*, 2018; Zhang *et al.*, 2019) but quantitative input connectivity to grafts placed into the amyloid-plaque loaded cortex has never been measured. Therefore, this transplantation study is the first to explicitly distinguish the healthy aging and the neurodegenerative brain environment and my data shows that the aging and amyloidosis-affected neocortex highly shape synaptic integration of new neurons.

Interestingly, the aging cortex differs a lot from the amyloidosis cortical environment. During aging, astrocytes and microglia gradually become more reactive, although only mildly as compared to the amyloid-plaque rich brain, which is characterized by a more pronounced inflammation and reactive gliosis (Radde *et al.*, 2006; Norden and Godbout, 2013; Sirko *et al.*, 2013; Heimann *et al.*, 2017; Lupo *et al.*, 2019). This effect was confirmed by immunostainings for reactive microglia and astrocytes, clearly showing more abundant and hypertrophic glia cells

accumulating around plaques but to a much lower level in the 17 months old, aged cortex. Since there is such a high input connectivity ratio in both environments, this suggests that reactive gliosis and inflammation alone are not directly causing this effect. Although known to be quite different in those environments, progressive inflammation and reactive gliosis could still support mechanisms that lead to excessive host-graft connectivity. To better understand the actual effect of inflammation and/or reactive gliosis in these environments, it would be interesting to investigate host-graft input connectivity at even older ages of the mice. Glial reactivity is known to increase with age (Niraula, Sheridan and Godbout, 2017) and growing amyloid-plaque deposition (Radde *et al.*, 2006; Denver, English and McClean, 2018; Webers, Heneka and Gleeson, 2020) and thus, more excessive connectivity ratios in older animals could support the idea that inflammation is indeed partially fostering new neuron integration.

Another aspect to keep in mind, is that the transgenic APP/PS1 mice do not directly model AD but rather they model an important hallmark of AD, known as cerebral amyloidosis (Radde *et al.*, 2006; Webers, Heneka and Gleeson, 2020). Tau pathology and the more robust neurodegeneration observed in AD are modeled through other mouse lines (see https://www.alzforum.org/research-models/alzheimers-disease; Myers and McGonigle, 2019) and it remains to be investigated if an environment of Tau pathology is equally influencing new neuron integration.

Since both clinically relevant conditions promote synaptic integration, their specific composition was examined more closely. We performed mass spectrometry along with comprehensive proteome analysis and found several candidates possibly influencing the observed effect. GO term analysis revealed the biological processes of classical complement system activation, as well as many immune system and inflammation-related proteins, to be highly enriched in both hyper-connectivity conditions. Importantly, as mentioned before, the complement system is well known to be involved in synapse elimination, which mainly involves factors like C1q (Presumey, Bialas and Carroll, 2017; Lee, Coulthard and Woodruff, 2019). For example, it was shown that the complement system mediates the loss of dendritic spines in mouse models of AD (Hong *et al.*, 2016; Shi *et al.*, 2017; Wu *et al.*, 2019) and C1q is progressively increasing in the course of normal brain aging (Stephan *et al.*, 2013; Krukowski *et al.*, 2018; Datta *et al.*, 2020). This suggests that the increasing expression of complement factors in these brain environments is implicated in tagging and elimination of synapses, which may in turn facilitate the synaptic

integration of transplanted neurons. A progressive loss of synapses in cortices of aged and APP/PS1 mice could be the mechanism eliciting the observed hyper-connectivity.

Intriguingly, only locally within the visual cortex do we observe this excessive host-graft connectivity (and in some other cortical regions of the aged brain). Complement activation or immune reactivity could be upregulated differently depending on the anatomical brain region and therefore lead to the observed visual cortex-specific hyper-connectivity. For instance, it is known that amyloid-plaque load appears first and most dense in the isocortex followed by hippocampal and other subcortical areas in APP/PS1 mice (Whitesell *et al.*, 2019), which could explain the lower levels of connectivity ratios present in other non-local brain regions. To investigate this option, the proteome composition of other input areas like the thalamic LGN should be analyzed as well. On the other side, we observed very little input connections from the contralateral visual cortex, although visual cortices from both hemispheres were considered for proteome analysis. Hence, it may take longer than 4 weeks for more distant brain regions to connect excessively with the grafted neurons (like the thalamus or contralateral hemisphere). Moreover, the transplantation procedure itself leads to a slight disruption of the BBB and triggers an immune reaction in the graft area that may support these massive host-graft input connections.

Studies that investigate human neuronal cells for transplantation are conducted in immunosuppressed animals to avoid graft rejection (Chen *et al.*, 2016; Mansour *et al.*, 2018; Linaro *et al.*, 2019). Such cross-species transplantations are useful to study how human neuronal cells behave after engraftment. However, our proteome data obviously showed many immune system and inflammation-related proteins being upregulated in all hyper-connectivity brain environments, which suggests that a certain level of immune reaction is supportive for synaptic integration. It should therefore not be underestimated that immune system suppression could theoretically elicit a whole different level of host-graft connections and eventually influence therapeutic success. Therefore, the aspect of cell reactivity of the host microenvironment should be considered more carefully for future preclinical studies that investigate the behavior of human grafts. Patients that receive neuronal grafts for brain repair often need immunosuppression (Barker *et al.*, 2013; Osborn *et al.*, 2020), thus the influence of the immune system, suppressed or not, needs to be studied to guarantee adequate synaptic integration of transplanted neurons.

Another important aspect and continuation of our study is to investigate if the increased local connectivity is transient over time as observed in the SW injury condition. If excessive local input connections are likewise pruned in the aged and amyloidosis environment, this indicates that these host environments are still plastic enough and capable of adapting host-graft connections. Long-term experiments to study the levels of connectivity in different brain environments are important to better understand graft synaptic integration because neuronal replacement is supposed to alleviate symptoms for the rest of a patient's life. If circuit integration is unstable, the therapeutic effect of the newly grafted neurons could be diminished. On the other hand, if the host network is not plastic enough to adapt synaptic connections, hypo- or hyper-connectivity could be detrimental to network function.

Aside from that, not only the host microenvironment influences graft integration but also the grafted cells themselves can affect cell integration into the host brain network. For example, a recent study showed that grafted embryonic precursors enhanced host neuronal activity and chemogenetic activation of grafted cells improved functional recovery (Andreoli *et al.*, 2020). Selective excitation of transplanted neural progenitors through photo-stimulation creates an activity-enriched environment that benefits the recovery of the stroke-injured mouse brain (Yu *et al.*, 2019). Moreover, a gene expression analysis of the grafted and optogenetically stimulated brain area revealed a downregulation of the inflammatory response (Daadi *et al.*, 2016). These studies suggest that grafted cells can likewise impact the host brain regarding synaptic integration and thus, possibly improve the therapeutic outcome.

Furthermore, one needs to investigate the mechanism behind synaptic integration in order to manipulate certain states of the host brain prior to transplantation. Our proteome results hint at a combination of an upregulated inflammatory profile and complement system activation as a main influence. Through experimental activation or inhibition of specific complement pathways one could learn more about the actual interplay of complement factors and graft integration. Since neuron loss is not a prerequisite for good graft integration, it remains to be investigated if synapse loss (e.g., mediated through complement) is required to promote synaptic integration. Experimentally induced synapse loss in healthy young mice prior to transplantation would uncover if this is indeed a necessity for new neurons to become highly innervated by host neurons.

Mostly elderly patients will need brain repair and circuit reconstruction, and therefore, the old and degenerating brain environment needs to be studied in more detail with regard to its capabilities to integrate new neurons. Our study is a first important step towards a better understanding of the host environment, which indeed hugely influences the integration of transplanted neurons. Future experiments are necessary to elicit the specific mechanisms underlying such hyper-connectivity to improve neuronal replacement therapies.

Cortical Environment	Local Connectivity	Inflammation	Gliosis	Synapse Loss	Neuron Loss
young intact	–	0	0	0	0
SW injury	++	+++	+++	+++	+++
LPS	–	++	++	0	0
8 months intact	+	–	–	0	0
APP/PS1	+++	++	++	++	0
Aged	+++	+	+	++	0

Figure 5: Simplified overview of the different brain environments depicting different levels of local connectivity ratio, inflammation, reactive gliosis, synapse loss, and neuron loss based on the findings of Grade et al., Thomas et al., and known literature. **0** = null change; **–** = low change; **+** = elevated level; **++** = high level; **+++** = very high level.

3.3 General conclusions

In summary, the data arising from my PhD project shed light on the important influence of the host brain environment on new neuron integration. Transplanted fetal neurons survive, integrate, and connect with the correct input brain regions independently of prior neuron loss. Not only do transplanted neurons integrate excessively into the host brain that suffered from acute injury, aging, or amyloidosis but this effect is almost exclusively restricted to the local visual cortex connectivity. Interestingly, the young intact and inflammation-triggered parenchyma integrate new neurons as well, but with much fewer host-graft connections (Figure 5). The comprehensive proteome data from both projects which compares hypo- and hyper-connectivity conditions indicates that a combination of inflammation and complement system activation are involved in forming excessive connections. One hypothesis is that the loss of synapses leads to free afferents in the injured, aging, and amyloid-plaque loaded conditions, and therefore grafted neurons can connect massively with endogenous neurons. However, to identify the mechanisms of high- or low-level synaptic integration, further experiments are needed. Through *in vivo* overexpression or knockout of complement system components, the actual influence of the complement system on transplanted neurons can be further evaluated. Moreover, experimentally decreasing synaptic connections in otherwise young and healthy brains would demonstrate if prior synapse loss is really a prerequisite to integrate new neurons.

Besides the need for more basic research, our data clearly shows that it is necessary to exercise caution when applying cell-based brain repair strategies in patients. Considering a patients' age, brain health status, and the affected brain region is highly relevant because we proved that the brain environment has a huge impact on synaptic integration, and thus, ultimately on the therapeutic success.

4. References

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List of publications

- Grade S., Thomas J., Conzelmann K.K., Hauck S.M., Götz M.

  Injury environment critically influences the brain-wide input connectome of transplanted neurons.

  This manuscript was submitted.

- Thomas J., Conzelmann K.K., Hauck S.M., Grade S., Götz M.

  Excessive local host-graft connectivity in ageing and amyloid-loaded brain.

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Declaration of author contribution

1. Grade S.*, **Thomas J.**, Conzelmann K.K., Hauck S.M.*, Götz M.*
   "The injury environment critically influences the brain-wide input connectome of transplanted neurons"

   *Corresponding authors
   M.G. initially conceived the idea. S.G. and M.G. conceived and coordinated the project. S.G. designed, performed, and analyzed all experiments except the proteomics. **J.T.** and S.M.H. conducted the proteome study. **J.T. performed injuries, collected samples and post-analyzed the data.** S.M.H. prepared the samples, performed measurements and quantitative analysis. K.K.C. provided the expertise and viral vectors for RABV tracing. S.G. and M.G. wrote the manuscript with input from all co-authors.

2. **Thomas J.**, Conzelmann K.K., Hauck S.M.*, Grade S.*+, Götz M.*+
   "Excessive local host-graft connectivity in ageing and amyloid-loaded brain"

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   M.G. conceived and financed the project. M.G. and S.G. designed the study. M.G., S.G., and J.T. designed experiments and discussed the data. **J.T. performed all experiments and analyzed the data.** S.G. provided the expertise on transplantation, RABV-based tracing, and analysis. S.M.H. provided proteomics expertise, performed mass spectrometry, and directed the analysis of the proteome data. K.K.C. provided the expertise on RABV tracing and produced the RABV vectors. J.T., M.G., and S.G. wrote the manuscript and all authors read and commented on it.

Affidavit

Eidesstaatliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation **„Synaptic integration of transplanted fetal neurons into different neocortical environments"** selbständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation **"Synaptic integration of transplanted fetal neurons into different neocortical environments"** is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, 29.01.2021 Judith Thomas