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Review

Methods to Construct Biological Neural Circuits

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Abstract: Recent biotechnological innovations make feasible the new paradigm of creating biological neural circuits *de novo*. With advances in protein, cell and tissue engineering techniques, as well as cellular reprogramming methods, we are entering an era where the construction of neural circuits can open completely new ways for studying nervous systems and for treating nervous system disorders. I explore here three technologies, namely cellular engraftment, neuronal reprogramming and transsynaptic molecule engineering, and delineate how they are being used in a variety of basic research and translational medicine contexts. In basic neuroscience, neural circuit construction methods are enabling ways to study causality in neural development (e.g. neural precursor differentiation and migration) and circuit function (e.g. excitation/inhibition balance, neural population dynamics). In translational neuroscience, they are providing opportunities for the targeted correction of circuit malfunction in brain disorders, both psychiatric (e.g. schizophrenia) and neurological (e.g. Parkinson's, Huntington's and Alzheimer's disease, as well as epilepsy). I discuss the challenges that these methods currently face, such as targeting specificity and cell survival, and outline future paths and opportunities to realize the full potential of technologies for creating new biological neural circuits.

Keywords: neural; circuit; synthetic; biology; brain; neuron; psychiatry; neurology; development; connectome; synapse; engineering

1. Introduction

The current neurobiology and translational neuroscience toolsets mainly work with the principles of modification and reduction. We can functionally modify the activity of existing neural networks via optogenetics, pharmacological compounds and gene expression changes and we can ablate individual components of neuronal networks through lesioning or genetically encoded expression of disrupting molecules. Yet, this leaves open one fundamental way of testing hypotheses and impacting nervous systems, which is the construction of new neural circuits and new connections in the brain. In order to meaningfully test hypotheses of how neuronal computation works in the brain and how it is influenced by concrete circuit parameters, we need the ability to create fundamentally new neuronal connections, especially *de novo* long-range projections and multi-component neural circuits. Apart from addressing several basic science questions, synthetic neural circuit construction is also enabling new treatment approaches for psychiatric diseases, many of which have been termed „connectopathies" due to the absence of gross morphological changes and the involvement of more subtle abnormalities in connection patterns, as well as for neurological disorders such as epilepsy, stroke, injury and neurodegeneration.

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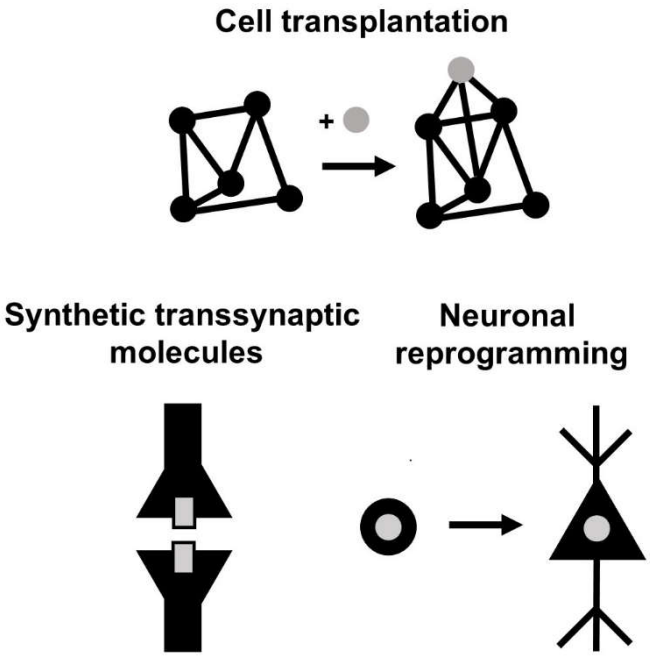

Figure 1. Methods for constructing synthetic neural circuits. Cell transplantation approaches enable the transfer of new neurons or neural precursors to build new neuronal connections and circuits. Neuronal reprogramming refers to the controlled transdifferentiation of non-neuronal cells (e.g. glia cells) into neurons. Transsynaptic molecule engineering allows for targeted connection of neurons through a key-lock principle of pre- to post-synapse protein brinding.

2. Methods for creating neural circuits

2.1. Cell transplantation

The arguably oldest technique for modifying brain circuits by construction *in vivo* is cell transplantation, in this case neuronal engraftment. Some of the earliest studies reporting engraftment of adult CNS tissue to the brain date back to the late 19th century, with W.G. Thompson reporting intra- and interspecies engraftment of brain tissue in different mammals (Thompson, 1890). In 1940, Clark reported in rabbits that fetal cortical tissue integrates into postnatal host brains and that it develops the typical laminated structure of adult cortex (Clark, 1940). Since then, the field has made steady progress, as reviewed in (Björklund and Stenevi, 1985). Lindvall et al reported the successful transplantation of fetal human dopamine neurons into the brain of a Parkinson's patient with subsequent improvements in motor function (Lindvall et al., 1990). In the decades since then, several studies have demonstrated that different variations of engraftment with regard to species (cross-species vs. intra-species; mouse, rat, monkey, human), individual (autologous vs. heterologous) and maturation stage (stem-cells, neural precursors or mature neurons), enable transplanted neurons to survive long-term and functionally integrate into host brain circuits with profound effects on overall circuit function and behavior. Wichterle et al. showed that neuronal precursors from the embryonic mouse brain can migrate long-distance into several brain regions after allogenic transplantation into adult hosts (Wichterle et al., 1999), demonstrating that this crucial capacity is preserved in transplanted cells, something which is of principle interest in tissue engineering as it will allow potentially brain-wide impact of locally injected cells via stereotactic means. Extending these findings, subsequent studies showed that immature mouse neurons from late-stage embryonic or early postnatal donors form long-range projections to contralateral hemispheres in host brains and display markers of mature neurons with histological evidence for afferent synapse formation (Fricker-Gates et al., 2002), and that mouse isochronic interneuron transplants migrated in a layer-

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specific manner (Valcanis and Tan, 2003). Regarding impact on circuit function, mouse embryonic medial ganglionic eminence cells develop into functionally mature GABA-interneurons after transplantation and increase inhibition onto pyramidal neurons (Alvarez-Dolado et al., 2006). Similarly, inhibitory neurons transplanted into the visual cortex of mice receive afferent synaptic inputs, produce efferent synapses onto target neurons and can induce profound ocular dominance plasticity, even outside of critical periods (Southwell et al., 2010). Falkner et al performed an extensive study of how embryonic mouse neurons integrate into the adult visual cortex of mice after transplantation, including detailed connectomic mappings and a demonstration of specifically tuned responses to environmental stimuli (Falkner et al., 2016). Extending the above findings to interspecies transplantation of neurons, human neuroepithelial stem cells transplanted into adult mouse brains develop into neuron-like cells with long-range projection patterns closely matching those of endogenous host neurons (Steinbeck et al., 2012) and pyramidal neurons derived *in vitro*, either from human embryonic stem cells or human induced pluripotent stem cells, functionally integrate *in vivo* into mouse brain circuits after transplantation (Espuny-Camacho et al., 2013). Similarly, different types of human iPSC-derived neurons functionally integrate into mouse host brain circuits after transplantation, such as forebrain interneurons (Nicholas et al., 2013) and striatal neurons (Victor et al., 2014). With regard to primates, it was reported that stem-cell derived neurons functionally integrate into the inferior colliculus brain circuits of rhesus monkeys and respond to environmental stimuli (Wei et al., 2016) and that human iPSC-derived dopamine neurons functionally integrate into the macaque brain (Kikuchi et al., 2017). In humans, Li et al have revealed that dopaminergic neurons, transplanted into the striatum of Parkinson's disease patients, survive for up to 24 years (Li et al., 2016) and similar studies report survival up to 14 years with successful tissue integration and healthy cellular marker expression and cell morphology (Hallett et al., 2014; Mendez et al., 2008). Human PSC-derived cortical neurons were also shown to synaptically integrate into human *ex vivo* cortical circuits (Gronning Hansen et al., 2020). Cell transplantation hence offers many exciting possibilities to insert neurons with completely new functions, for instance created through *ex vivo* genomic engineering approaches (Lissek, 2017), into networks and makes feasible the deliberate engineering of completely new networks inside the living brain. This prospect is especially exciting since the supporting technologies around human cell engineering (e.g. in T-cell engineering) are rapidly maturing with their transition into the clinic (Wang et al., 2021b).

2.2. In vivo neuronal reprogramming

Cellular reprogramming refers to changing cell identity through expression level changes of intracellular molecules or application of defined factors. The prototypical example is Yamanaka factor reprogramming through expression of Oct3/4, Sox2, Klf4 and c-Myc to obtain induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Direct cellular reprogramming is the targeted transition from one cell lineage to another, without intentional intermediate conversion to stem cells for instance, and by now direct reprogramming has been shown to reliably produce different target cells from a variety of starting cell types (Wang et al., 2021a). Direct reprogramming to obtain neurons, termed neuronal reprogramming, usually happens via the expression of pioneer transcription factors such as Neurog2, Ascl1 and Neurod1, that coordinate molecular programs to induce a neuronal phenotype and, depending on the combination of neurogenic factors, several distinct neuronal cell types can be reliably produced. The remarkable progress in this field has been reviewed recently by (Barker et al., 2018; Bocchi et al., 2022). A large amount of studies has now demonstrated neuronal reprogramming *in vivo* in mice (Bocchi et al., 2022) and we will focus here on studies that have demonstrated that resulting neurons can functionally integrate into brain circuits *in vivo*. Niu et al showed that astrocyte-derived neurons in the striatum display neuron-characteristic electrophysiological properties and receive synaptic input (Niu et al., 2013). Similarly,

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Heinrich et al showed that NG2-derived neurons in the cortex display functional synapses and electrophysiological responses (Heinrich et al., 2014) and Liu et al demonstrated robust functional synaptic network integration of midbrain astrocyte-derived neurons into existing neural networks with functional inputs and outputs (Liu et al., 2015). Subsequent studies extended this approach by producing more specific cell types with corresponding characteristic electrophysiologic properties, such as NG2 glia-derived neurons that closely resemble parvalbumin-positive interneurons, both with regard to physiology (e.g. exhibiting fast-spiking action-potential patterns) and cell markers (e.g. expressing parvalbumin) (Pereira et al., 2017). Focusing on different cell types, two studies produced functionally integrated dopaminergic neurons by conversion from astrocytes in the striatum of mice (Rivetti di Val Cervo et al., 2017)(Zhou et al., 2020). Qian et al demonstrated a more physiological approach by converting midbrain astrocytes to dopamine neurons to then show that these neurons functionally reinnervate the striatum and that they are able to rescue motor deficits in a mouse Parkinson's disease model (Qian et al., 2020). Another cell type, namely glia-derived GABAergic striatal neuron, was produced *in vivo* by two studies (Wu et al., 2020)(Giehrl-Schwab et al., 2022), with many electrophysiological parameters bearing close similarity to endogenous neurons. Interesting with regard to synthetic neural circuit approaches and reprogramming is a study by Lentini et al, in which the authors demonstrated that hippocampal glial cell-derived interneurons synaptically integrate into local networks in a highly specific manner and are able to normalize network function in epileptic mice (Lentini et al., 2021). The above studies hence show that direct neuronal reprogramming allows targeted modification of neuronal network function through the addition of novel cells, sometimes even cell types that are not normally present in the respective brain regions (e.g. dopaminergic cells in the striatum).

2.3. Transsynaptic molecule engineering

One way of modifying a neural circuit is by changing the interaction patterns of transsynaptic proteins (Fig. 1). Transsynaptic proteins, such as neurexins (presynaptic) and neuroligins (postsynaptic), work according to a key-lock principle and organize neural circuits along specific patterns of pre- to post-synapse matching. This guides the correct formation of excitatory, inhibitory and neuromodulatory synapses and the correct connection patterns between brain regions (Sudhof, 2017). Suzuki et al have reported the design and creation of CPTX, a synthetic protein combining elements of cerebellin-1 and neuronal pentraxin-1, which acts as an adapter between neurexins and AMPA-receptors to induce excitatory synapse formation *in vivo*. Through targeted expression of CPTX they were able to rescue synaptic function and behaviors such as memory, motor coordination and locomotion in mouse models for various CNS diseases (Suzuki et al., 2020). The transsynaptic molecule toolkit could be expanded much further with adapter proteins being developed for different transsynaptic proteins and different protein isoforms. It seems also feasible to engineer entirely new transsynaptic proteins that are functionally completely orthogonal to endogenous molecules. A similar approach has been taken in the field of immunology with the development of chimeric antigen receptors (Lim and June, 2017), thus demonstrating that it is possible to engineer transmembrane signaling molecules in a goal-directed manner with unique signaling properties. Another synthetic neural circuit approach is the introduction of electrical synapses to connect different neurons, as has been performed in previous studies via the expression of the innexin Panx1 in molluscs (Kelmanson et al., 2002) or ectopic expression of the connexin Cx36 in C. elegans (Rabinowitch et al., 2014). In both cases, the authors showed that it is possible to profoundly alter neuronal communication via this strategy, with the latter study also demonstrating an effect on whole organism behavior.

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3. Applications of synthetic neural circuits

Having reviewed the three major ways of creating synthetic biological neural circuits, the important question becomes what they can be used for.

3.1. Creating and modifying local and long-range circuits

The first set of capabilities that synthetic neural circuits enable is to construct new neuronal pathways and to modify existing ones in concrete ways. For instance, a critical property of many neuronal networks is their excitation/inhibition (E/I) balance, an important parameter in physiological information processing (Bhatia et al., 2019) and a central aspect in psychiatric diseases such as autism (Nelson and Valakh, 2015) and schizophrenia (Sohal and Rubenstein, 2019), as well as neurological diseases such as epilepsy (Fritschy, 2008). In order to manipulate E/I balance in local circuits *in situ*, one can transplant additional inhibitory or excitatory neurons into the network. Previous studies have used this approach to increase the number of inhibitory neurons in the mouse hippocampus, thereby reducing seizure frequency in epileptic mice (Hunt et al., 2013). Acting on the same principle of correcting seizure activity through increasing the number of inhibitory neurons, another study used neuronal reprogramming to transform glial cells into GABAergic interneurons in the mouse hippocampus and reported a reduced seizure frequency in epileptic mice (Lentini et al., 2021). Another critical local circuit property is neural synchrony and previous research has implicated increased local synchrony in the transition from wakefulness to the anesthetized state during general anesthesia (Lissek et al., 2016), motor behavior (van Wijk et al., 2012) and various brain disorders (Mathalon and Sohal, 2015). Importantly, research has shown that synchronization depends critically on certain neuronal cell types such as inhibitory interneurons (Bartos et al., 2007), and modifying local cell type numbers through cell engraftment or cellular reprogramming could give major insight into how cellular properties correlate to overall network function. In addition to these local circuit strategies, experimental results imply that one can also create novel long-range projections. Several studies demonstrated that transplanted neural precursors or neurons develop functional long-range projections from and into various brain regions (Falkner et al., 2016; Victor et al., 2014)(Fricker-Gates et al., 2002; Steinbeck et al., 2012). Establishing and profoundly modifying long-range projections patterns in the brain can hence enable major insight into brain-wide information integration in various behavioral contexts such as long-range synchrony during various behaviors (Harris and Gordon, 2015), especially with regard to coordination between different brain areas (Leong et al., 2016), as well as wakefulness and consciousness (Uhlhaas et al., 2009). Another area of future research can be the modification of cortical circuit motifs (e.g. see (Braganza and Beck, 2018)) to causally investigate the functional and structural building blocks of brain circuits and to test our concepts of how neurons in different layers functionally connect to each other.

3.2. New types of synaptic connections

Another interesting opportunity is the targeted involvement of new cell types in synaptic transmission. So far, most synaptic communication in the central nervous system happens via neuron-neuron and neuron-glia connections. Yet, research also demonstrates that it is possible to induce synapse formation from neurons onto non-neuronal targets cells through postsynaptic expression of synapse clustering molecules. For instance, transfection of HEK293 cells in neuron/HEK cell co-cultures with the gene for neuroligin triggers synapse formation in neurons (Scheiffele et al., 2000), and transfection of neuroligin, PSD95 and AMPA or NMDA receptor subunits results in functional synapses between neurons and HEK cells (Fu et al., 2003). Several studies confirm these findings with different postsynaptic proteins, resulting in different types of synapses (Biederer et al., 2002; Graf et al., 2004). It might hence become feasible to induce synapse formation onto non-neuronal target cells for refined and perhaps even voluntary control of target cell membrane potential. A fundamentally new approach in this regard could be the

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innervation of almost all bodily cell types to leverage bioelectrical signaling mechanisms for voluntary tissue control or regeneration. Bioelectric signaling, mostly via membrane potential changes, is emerging as a critical mechanism in many biological areas such as embryonic development and cancer (Levin, 2021). Bioelectric signals seem to be especially important for morphogenesis, organ patterning and crucially involved in tissue regeneration (McLaughlin and Levin, 2018). Previous research has hence explored the possibility of leveraging membrane voltage manipulations to induce profound tissue regeneration, such as limb regrowth after amputation and demonstrated the viability of this approach (Tseng et al., 2010). It would thus perhaps become feasible to leverage endogenous neurons or engrafted neurons to connect to wound areas after tissue damage and thereby induce more profound and coordinated regeneration.

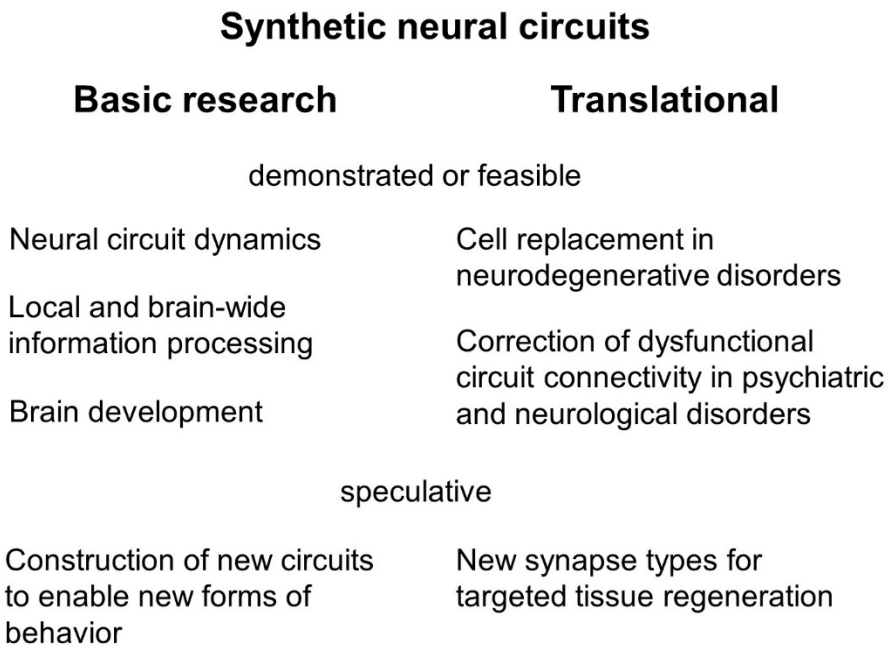

Figure 2. Applications of synthetic neural circuits. In basic research, synthetic neural circuits can be used to study neural circuit dynamics (e.g. oscillation behavior), local and brain-wide information processing (e.g. brain-wide synchrony) and brain development (e.g. neural precursor movement within cortical areas). A more speculative application is the construction of new neural circuits to enable entirely new forms of behavior. For translational applications, synthetic circuits can replace ones that are lost during neurodegeneration in Parkinson's, Alzheimer's or Huntington's disease and they can correct aberrant circuit function in psychiatric diseases such as schizophrenia. A speculative translational application is the creation of new synapse types to control tissue regeneration.

3.3. Treating psychiatric diseases

A major impact of synthetic neural circuit construction methods will be the treatment of brain disorders, particularly psychiatric diseases. As mentioned above, many purely psychiatric diseases are characterized by connectomic phenotypes, i.e. patients generally do not display gross morphological brain changes but more subtle changes in synaptic weights and cell type distributions. Such aberrations have been described or proposed for autism (Minshew and Williams, 2007), schizophrenia (Ross et al., 2006), addiction (Luscher and Malenka, 2011), OCD (Ting and Feng, 2011) and depression (Nestler et al., 2002) among others. Synthetic neural circuits could be used to correct for circuit function abnormalities. As an example, addiction could possibly be treated by transplantation of certain master control neurons, as has been previously suggested (Lissek, 2017). Another example is correction of local circuit malfunction in schizophrenia, as has been performed with GABAergic progenitor transplantation in schizophrenia mouse models (Donegan et

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al., 2017; Perez and Lodge, 2013). *In situ* transsynaptic protein engineering is also becoming plausible, as in recent times there has been steady progress in gene transfer technologies in human patients. Two examples are the clinical readiness of AAV-based therapies (Wang et al., 2019) and transfer of mRNAs via lipid nanoparticles (Hou et al., 2021). These technologies could be used to deliver pre- and postsynaptic proteins into respective brain regions or cell types and hence drive targeted connectivity changes to correct for psychiatric circuit malfunction.

3.4. Repairing the central nervous system in neurological disorders

One of the primary focus points of investigation with regard to synthetic neural circuits so far has been the treatment of neurological disorders. In many cases, the goal is the replacement of cells that were lost due to neurodegeneration and injury or to correct cell number imbalances or dysfunctions due to developmental disorders (e.g. certain types of epilepsy). So far, the most prominent disease entity for cell replacement is Parkinson's disease with very strong evidence from numerous studies in animals models and human patients for the method of cell engraftment. Early studies in a human patient reported that transplantation of human fetal mesencephalic dopaminergic neurons into the striatum produced significant clinical improvement of motor function (Lindvall et al., 1990). Histological studies suggested that transplanted dopamine neurons can survive for over a decade (Hallett et al., 2014; Mendez et al., 2008)(Li et al., 2016). Recently, Schweitzer et al reported the implantation of autologous iPSC-derived midbrain dopaminergic precursor cells into the striatum of a patient and subsequent clinical improvements (Schweitzer et al., 2020). In cellular reprogramming, studies in Parkinson's animal models have demonstrated the *in vivo* conversion of glial cells into dopaminergic neurons in the midbrain with subsequent improvement in motor function (Qian et al., 2020) and the conversion of striatal astrocytes into dopaminergic neurons with subsequent motor improvements as well (Rivetti di Val Cervo et al., 2017). Cell engraftment and cellular reprogramming have also been used to treat various other neurological diseases, such as in animal models and human patients Huntington's disease (Bachoud-Levi et al., 2006; Bachoud-Levi et al., 2000; Wu et al., 2020), and in animal models Alzheimer's disease (Ager et al., 2015; Guo et al., 2014), epilepsy (Hunt et al., 2013; Lentini et al., 2021) and stroke and brain injury (Andreoli et al., 2020; Ge et al., 2020; Palma-Tortosa et al., 2020; Zhu et al., 2019).

3.5. New ways to study brain development

A major question in neurobiology is how the brain organizes itself during development. All vertebrate development starts with a single fertilized egg cell and a limited repertoire of genes. It is remarkable that from such simple initial conditions, the brain essentially builds itself into a highly regular and complex structure. As synaptic signaling is a crucial part of this development process, synthetic neural circuit approaches can allow for targeted hypothesis testing. In fact, one major rationale behind using cell engraftment in animal experiments is to study how neuronal precursors migrate and functionally integrate into brain networks (Valcanis and Tan, 2003; Wichterle et al., 1999). As another example we can look at the already mentioned transsynaptic signaling molecules. These molecules, including neurexins, neuroligins, cadherins and protocadherins, contribute to transforming a linear code (base sequence in the genome) into a three dimensional construct (the brain) (Martinez-Garay, 2020; Sudhof, 2017; Wu and Jia, 2021). Expressing synthetic transsynaptic organizers during distinct times and locations in the developing brain could enable the testing of new hypotheses such as how alternative splicing dynamics in certain cell types regulate neuronal wiring during cortical development. Similarly, transplantation of neural progenitors with defined molecular properties can enable causal investigation of how certain cell properties influence overall brain development.

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3.6. Open challenges and future directions

There are several important challenges and opportunities for bioengineers to address in the future with regard to synthetic neural circuits. The first one relates to targeting specificity of synaptic connections. The majority of the above mentioned approaches do not specify which type of synapse (e.g. glutamatergic, GABAergic, dopaminergic) should be made onto which type of target neuron (e.g. pyramidal neuron, PV-interneuron, medium spiny neuron). Instead, transplanted or reprogrammed cells often seem to follow the endogenous connection patterns that are characteristic for their cell type. In order to fully make use of synthetic neural circuits, we need a high targeting specificity with controlled circuit formation. Here, cellular engineering approaches that restrict or change synaptic protein expression, as well as enable the expression of newly engineered proteins will be tremendously helpful. Previous work has explored the many ways in which neurons could be engineered through genome engineering technologies (Lissek, 2017). Target proteins for engineering efforts can be transsynaptic membrane molecules (e.g. Neurexins, Neuroligins) or ion channels (e.g. voltage-dependent calcium or sodium channels). Martinz-Losa et al for instance demonstrated the viability of this approach by overexpressing the sodium channel subunit Nav1.1 in transplant interneurons, which changed spiking dynamics in these cells, and showed that these neurons, but not wild-type ones, were able to rescue cognitive function in an Alzheimer's mouse model (Martinez-Losa et al., 2018). Another set of challenges relates to differentiation into concrete subtypes with regard to neuronal reprogramming and cell development after precursor transplantation. While, so far, many promising results show that specific neuronal subtypes can be obtained (Bocchi et al., 2022), it is unclear how these would behave in different contexts over the long-term and whether they would keep their differentiation, especially in human patients. Relatedly, in cell engraftment of immature cells and neuronal reprogramming of glial cells, there is the potential for oncogenic progression and development of these cells into tumors, as has been discussed in the context of stem-cell based therapies in several fields (Lee et al., 2013). As with many bioengineering efforts, there is also the challenge of cellular survival after transplantation, reprogramming or synthetic protein expression. All of these treatments can disrupt endogenous metabolism and induce cell death. For neuronal engraftment in particular, these challenges have been addressed in previous work (Anderson et al., 2011; Harrower and Barker, 2004; Rosenstein, 1995). Hence, it might be necessary to engineer additional tissue factors, such as vascularization (Newman Frisch et al., 2022) and extracellular matrix components (Yang et al., 2021), to ensure optimal cell survival and function.

4. Conclusion

Methods to construct synthetic neural circuits, namely neuronal engraftment, neuronal reprogramming and transsynaptic molecule engineering, introduce a profound new way to concretely modify brain function. Directing neural circuit wiring this way will enable the testing of old and new hypotheses in neural development and give profound insight into the self-organizational aspects of animal brains. In parallel, synthetic neural circuits are enabling the creation of powerful new approaches in psychiatric and neurological therapy development that treat brain malfunction through targeted correction of aberrations in neuronal function. In the end, due to their versatility, synthetic neural circuits could emerge as a universal platform to treat a whole variety of brain disorders. The extension and refinement of methods for constructing neural circuits *de novo* through more precise protein and cellular engineering methods and more complex circuit assemblies might open new doors to understand the brain and treat some of humanity's most challenging diseases.

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