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Neuron-neuron attraction shapes morphology and activity of tissue engineered brain constructs

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The propensity of neuronal stem cells to aggregate is well established. Aggregation of differentiated neurons, particularly those of the brain regions such as the cortex, has been reported more recently (Hasan et al., 2019; Ming et al., 2020). However, the tendency of these cells to aggregate may play a significant role in the brain's response to injury, and may also be important in developing regenerative therapies to treat brain injury. Some types of injury, including stroke and trauma, result in formation of liquid-filled cavities in the brain (Kazim et al., 2011; Moreau et al., 2012). Cavities are also produced by resection surgery in patients suffering from epilepsy or by surgical brain tumor removal. Brain cavitation represents a loss of neural circuitry and therefore leads to deficits in function and behavior. Cell and tissue transplants and matrix implantation have been suggested as regenerative therapies to improve patient outcomes. The understanding of the processes occurring on cavity walls, which represent an abrupt transition between brain parenchyma and the liquid-filled interior of the cavity, is not complete. The size of the cavity can change dynamically in patients (Jarvis et al., 2012; Patel et al., 2018). This has been attributed to ongoing neuron loss after injury or cell proliferation in cancer patients. Cavity walls are characterized by gliosis, or formation of a glial cell-rich "scar" that separates neurons from liquid-filled cavity interior. This process has been attributed to injury-triggered inflammation and activation of wound healing response.

A recently published work suggests that the ability of postnatal neurons to aggregate may play a role in the processes affecting brain injury cavity (Hasan et al., 2021). In this work, a dense suspension of differentiated neurons (postnatal rat cortical cells and differentiated human pluripotent stem cell-derived neurons) was placed into a long rectangular microwell. Cells aggregated into a dense construct, with contraction maximized when astrocytes were absent or depleted. When astrocytes were added, they were found in the superficial layer of the contracted construct, surrounding lobules or clusters of neurons (**Figure 1A** and **B**). These results suggest that aggregation and contraction were driven primarily by neuron-neuron attraction. This attraction was stronger than neuron-astrocyte or astrocyte-astrocyte attraction, which led to the sorting of neurons and astrocytes into an aggregate with a neuron-rich center and astrocyte-rich sheath. A computational model was developed that simulated the process of neuronal and astrocytic aggregation and contraction with strong neuron-neuron attraction, weaker neuron-astrocyte attraction, and weakest astrocyte-astrocyte attraction. The model replicated experimental results in a rectangular well and was able to predict the morphology of neuron-astrocyte aggregates in disk-shaped wells. These results suggest an alternative mechanism for the processes occurring on cavity walls in brain's gray matter. The wall represents an abrupt border of the mixed neuronal-glial population. The strong attraction between neurons may result in pulling of neurons away from the border, while relatively weak neuron-astrocyte and astrocyte-astrocyte attraction would leave an astrocyte-rich layer at the border surface. This may contribute to dynamic changes in cavity size and formation of glial "scar" through a non-inflammatory, non-degenerative mechanism.

In vivo evidence shows that neuronal aggregation and concurrent glial scar formation are not limited to cultures. Transplantation of fetal cortical tissue has been examined as a potential treatment in an animal model of brain cavitation after stroke. Transplanted neurons aggregated into lobular and "rosette" like structures, which appear morphologically similar to neuronal clustering observed *in vitro* (**Figure 1C**) (Grabowski et al., 1994). A glial sheath separated the implant from the native brain tissue, similar to astrocyte-rich superficial layer observed *in vitro* (**Figure 1D**) (Zeng et al., 1999). This suggests that the processes of aggregation and sorting observed *in vitro* are determining graft morphology *in vivo*. In a more recent study, matrix-embedded neural precursors were implanted into a post-stroke cavity, with the goal of formation of brain parenchyma-like tissue after neuron differentiation and matrix degradation (Bible et al., 2009). This process left dense clusters of neurons attached to cavity walls, suggesting an important role for neuron-neuron attraction and aggregation. The presence of neuronal aggregation *in vivo* suggests that a thorough understanding of this process may be necessary to ensure long-term success of brain regenerative therapies.

Neuronal aggregation on cavity walls also suggests that attractive forces between neurons, and between neurons and astrocytes, may play an important role in shaping the cavity and determining the morphology of the surrounding tissue. Tissue at edge of the cavity may be unstable due to an imbalance of attractive forces: neurons will be attracted by other neurons and move deeper into the tissue, leaving a glial-rich layer at the surface of the cavity. While this movement of cells occurs at microscale (*in vitro* studies showed movement of neurons in the range of a few hundreds of micrometers), it may create a permanent gliotic layer lining the cavity wall. Elastic modulus of the glial "scar" tissue was found to be significantly lower than modulus of healthy cortex in a rat injury model (Moeendarbary et al., 2017). This finding may be partially explained by the same forces that result in sorting behavior of neurons and astrocytes: cells possessing higher contractility tend to sort to the inside of tissue (Heer and Martin, 2017). This depletes the border region of the more contractile cells (neurons), possibly lowering tissue tension and making the "scar" region softer. Evidence from an animal model of spinal cord injury with relatively small lesion size suggests that cellular grafts that filled the entire lesion cavity had reduced glial sheath formation (Kadoya et al., 2016). This may have contributed to functional integration of the graft, although axon growth into the graft was tract- and cell-type specific. Implants that fill the entire cavity volume may be able to balance the attractive forces and prevent segregation of the grafted and native tissues.

In vitro, the edge of 3D aggregate that has experienced the most neuron migration and contraction was characterized by a higher level of baseline [Ca^{2+}] in neurons. Elevations in [Ca^{2+}] may be associated with apoptotic or inflammatory processes driven by activated glial cells. Activity-dependent changes in [Ca^{2+}] were also higher at the contracting edges, suggesting higher excitability of neurons experiencing attractive force imbalance and tissue contraction. Severe brain injury such as penetrating head trauma is strongly associated with development of epilepsy, with incidence levels of up to 50% (Raymont et al., 2010). Loss of brain tissue due to the injury itself or subsequent surgery may lead to formation of a cavity. Increased neuron excitability at cavity edges, as described above, may contribute to posttraumatic epileptogenesis. Brain resection surgery is used to treat some cases of intractable epilepsy. In such surgery, an epileptic focus is identified by electroencephalography and electrocorticography, and removed. Resection leads to a reduction or elimination of seizures in patients. However, seizures frequently recur after several years. The cavity created by resection surgery may experience the processes described above: imbalance of attractive forces on neurons and astrocytes potentially leading to neuron hyperexcitability and then seizures. Increased stroke severity is also associated with cavity formation and increased risk of epilepsy. It is intriguing to speculate that prevention of the hyperexcitability of neurons on cavity walls may in turn prevent the development of epilepsy. This may be accomplished by mechanically stabilizing cavity walls to balance the attractive forces on neurons and astrocytes, potentially through matrix implantation strategies.

Maintenance of cerebral neurons in artificial or non-native extracellular matrices has been demonstrated *in vitro* and *in vivo*. Neuron densities in these constructs are significantly lower than those found in the brain, which

may limit their therapeutic value. Increase in neuronal density in soft matrices that mimic elastic modulus of brain's extracellular matrix leads to aggregation, contraction, and loss of control over construct geometry. This may be due to neuron-neuron attraction and the mechanical forces that it generates. Neurons in matrix-free constructs self-aggregate at densities similar to or exceeding that of the densest cortical layer, with a center-to-center neuronal spacing of < 100 µm (**Figure 1E**). Considering that average neuronal soma has a diameter of ~20 µm (rat cortical neurons), neurons in matrix-free aggregates do not appear to be close-packed. Space between neuronal soma may be filled with axons and dendrites sprouted by these neurons, and by extracellular matrix secreted by both neurons and glial cells. Ultrastructural studies of the cortex showed that most of the neuropil (volume between neuronal soma) is filled with dendrites, axons, and synapses. Relatively dense packing of neurons in matrix-free constructs may be due to the lower volume of dendrites and axons relative to the intact cortex. This may be a result of lower length of dendrites and axons sprouted by neurons in constructs, or may be due to absence of axons from distant neurons from other parts of the cortex. These morphological considerations, which are unique to the brain with its massive and long-distance interconnections between neurons, suggest that cortex-like constructs should contain neurons at sufficient numbers to generate cortex-like connectivity. This may necessitate placing neurons at high enough density such that connections between thousands of partners can be made, but with a lower limit on density that allows inter-soma spacing for the axons and dendrites to grow. Development of biomanufacturing strategies to accomplish this may lead to creation of therapeutic constructs and realistic brain tissue models for basic science and drug screening.

In summary, neuron-neuron attractive forces play an important role in determining the morphology and activity of tissue-engineered 3D constructs. They may also play a role in the response of brain tissue to injury. Regenerative approaches to treatment of brain injury may benefit from exploiting these forces.

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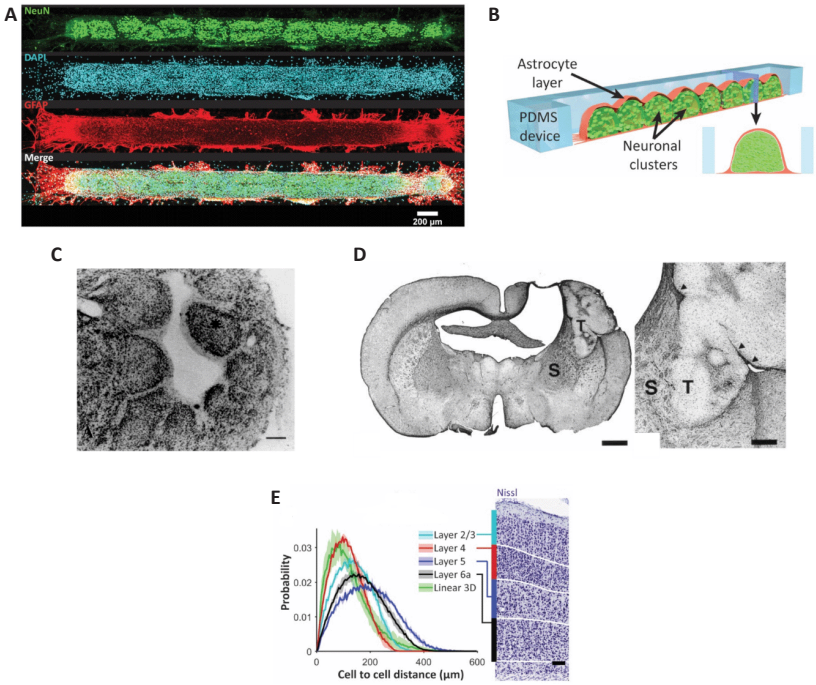

Figure 1 | **Neuron and astrocyte aggregation.**
(A, B) Suspension of postnatal rat cortical cells was placed into a rectangular polydimethylsiloxane (PDMS) well and allowed to aggregate and sort *in vitro* for 2 weeks. Resulting constructs were stained with antibodies for NeuN (marker of neuronal nuclei and soma, green), glial fibrillary acidic protein (GFAP) (astrocyte marker, red), and a nuclear stain 4′,6-diamidino-2-phenylindole (blue). Scale bar is 200 µm. Adapted from Hasan et al., 2021 under CC BY license. (C) Rats which have undergone arterial occlusion (stroke model) had cell suspension of fetal neocortical tissue implanted into the infarct cavity. Grafted tissue was made up of multiple lobules or rosettes. Cresyl violet stain. Scale bar is 200 µm. Reprinted from Grabowski et al., 1994 with permission from Elsevier. (D) GFAP stain of the fetal graft in rat model of stroke reveals gliotic infarct border between transplant (T) and striatum (S). Scale bars: 1430 µm for left, 480 µm for right. Reprinted from Zeng et al., 1999 with permission from Elsevier. (E) Cell to cell distance profiles obtained from different layers of Nissl-stained postnatal rat cortex (image courtesy: BrainMaps: An Interactive Multiresolution Brain Atlas; http://brainmaps.org) compared to profile of a linear 3D *in vitro* construct (A, B). Adapted from Hasan et al., 2021 under CC BY license.

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