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Anatomy of Graft-induced Dyskinesias: Circuit Remodeling in the Parkinsonian Striatum

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Abstract

The goal of researchers and clinicians interested in re-instituting cell based therapies for PD is to develop an effective and safe surgical approach to replace dopamine (DA) in individuals suffering from Parkinson's disease (PD). Worldwide clinical trials involving transplantation of embryonic DA neurons into individuals with PD have been discontinued because of the often devastating post-surgical side-effect known as graft-induced dyskinesia (GID). There have been many review articles published in recent years on this subject. There has been a tendency to promote single factors in the cause of GID. In this review, we contrast the pros and cons of multiple factors that have been suggested from clinical and/or preclinical observations, as well as novel factors not yet studied that may be involved with GID. It is our intention to provide a platform that might be instrumental in examining how individual factors that correlate with GID and/or striatal pathology might interact to give rise to dysfunctional circuit remodeling and aberrant motor output.

Keywords

Grafting; Dyskinesia; Parkinson's disease; Plasticity; Synapses; Co-Neurotransmission

Grafting Offers Unrealized Hope to Those with PD

"It's not that I'm so smart, it's just that I stay with problems longer." -Albert Einstein

There has long been interest in whether cells of the nervous system that die due to trauma or disease could be replaced by new ones. While very early attempts at brain cell grafting were unsuccessful, Dr. Elizabeth Dunn [1], at the turn of the twentieth century, showed that brain tissue grafted from one newborn rat to another newborn rat could survive transplantation. Despite this landmark discovery, little progress was made in the area of neural transplantation over the next fifty years. However, resurgent interest in the 1970s, particularly in the field of Parkinson's disease, led to an explosion of preclinical research. Promise from animal studies in the field led to the initiation of clinical grafting trials. A

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number of small open-labeled clinical trials took place throughout the 1980s and 1990s. There was a lack of consensus over the optimal grafting paradigm to employ and, accordingly, these studies were quite variable in their outcomes. However, a significant number of studies were able to show unequivocal evidence of grafted cell survival, graft-derived neurite outgrowth and behavioral recovery (e.g.: [2–8]). These encouraging findings culminated in two large double-blind placebo controlled studies preformed in the late 1990s with the aim of confirming the efficacy of fetal tissue grafting for PD.

Unfortunately, both double-blind placebo control trials reported failure to find statistical significance in their primary behavioral endpoints at the end of the blinded phase of each study [9,10]. However, a recent follow-up report from the Denver/Columbia trial [9] showed that at 2 and 4 years post-transplantation there was continued and significant symptomatic improvement [11]. Despite the generalized disappointment from these double-blind studies, clinical trials involving transplantation of embryonic mesencephalic DA neurons have demonstrated that grafted neurons can survive long-term [11], innervate the denervated striatum [12–14], release DA [15], and become functionally integrated into host neural circuits [16]. Further, it is important to appreciate that clinical data indicate that a favorable therapeutic response to transplantation appears to be dependent on specific variable including the extent of loss of nigrostriatal dopaminergic terminals at pregraft baseline and age of the recipient [e.g.: 9, 11, 51]. Thus, the *clinical efficacy is perhaps best viewed as variable rather than a failure*, and a primary challenge to the employment of graft therapy for PD is in understanding how to develop strategies to allow all patients to respond favorably to engraftment of replacement DA neurons.

The Unexpected Side Effect: Graft-induced Dyskinesias

More disconcerting than a lack of consistent therapeutic efficacy of grafting is an ethical challenge related to a bothersome side-effect that was noted in a subpopulation of grafted patients in both placebo control trials, and one retrospective study. The side-effect has come to be known as graft-induced dyskinesias (GID), the cause is not known, and in contrast to levodopa-induced dyskinesias (LID), these behaviors cannot be ameliorated by lowering the dose of antiparkinsonian medications like levodopa. In the remainder of this manuscript, we detail the phenomenology that is GID, contrast it to LID, and examine possible mechanisms that may underlie GID etiology. The ethical dilemma of this unforeseen challenge relates to the Latin phrase, often attributed to the Hippocratic oath, “primum non nocere” (“above all else, do no harm”). Indeed, it was the intermittent expression of the sometimes devastating GID side-effect that was the principal reason for the discontinuation of clinical trials worldwide, although a lack of a consistent beneficial effect was a contributing factor.

The post-surgical side effect of GID was first described in the Denver/Columbia placebo control, double-blind trial [9] with an additional incidence subsequently reported in a retrospective clinical report out of Sweden [17] and the second placebo control, double blind trial in the United States [10]. In these trials, 15 to 50% of trial participants developed abnormal dyskinesia with characteristics largely unique from the common drug-induced dyskinesias. Indeed, information available suggests that GID may represent a distinct neurological entity from the common drug-induced, levodopa-induced dyskinesia (LID) [18,19].

The idea that GID and LID may represent distinct neurological phenomena comes from several lines of evidence. First, the temporal expression of these two behaviors is disparate (Figure 1). In both humans [9,10,17] and animal models [19–21] the specific post-graft dyskinetic profile develops as the graft matures, and as the typical pre-graft profile of LID disappear, thus giving an inverse time-course of expression of these two behavioral

phenotypes. Second, in contrast to the more widespread (e.g.: involving both upper and lower extremities), primarily dystonic and choreic appearance of drug-induced dyskinesias [22], GID bear some resemblance to biphasic drug-induced dyskinesias [18], often involving stereotypy and hyperkinesia, and localized to the upper or lower extremities [10, 17, 20]. Unlike LID, lowering the dose of levodopa does not provide relief from this troublesome side effect. In fact, GID in humans occurs primarily when plasma levodopa is low or absent. Interestingly, in both humans [9, 10, 17] and animal models [21], GID appear to be dopamine (DA) mediated despite the above mentioned paradox in patients. The pharmacology of GID will be detailed later in this review.

Modeling Graft-induced Dyskinesias

In order to delineate the mechanisms responsible for the expression of aberrant behaviors following fetal tissue grafting in PD, research laboratories have created a rodent model of experimental GID. By utilizing rats rendered unilaterally parkinsonian via 6-hydroxydopamine (6-OHDA) delivery to the nigrostriatal pathway, researchers have found that aberrant behaviors similar to those seen in the clinic can be elicited in experimental animals following the delivery of a fetal mesencephalic tissue graft to the parkinsonian striatum.

The post-graft dyskinetic profiles expressed by animals are phenotypically similar to the GID observed in human patients. Specifically, similar to grafted patients (see [18] for review), in one of the rodent models (e.g.: the model where levodopa rather than amphetamine is used to induce the expression of experimental GID [19, 20]), experimental GID is expressed as focal, stereotypic and repetitive movements. These behaviors typically affect the upper limbs, head, mouth and tongue of the animal, and as depicted in Figure 1, these focal levodopa-elicited post-graft dyskinetic behaviors emerge as the typical, widespread pre-graft LID profiles decrease [20]. This specificity of body region and focal localization of these behaviors in grafted parkinsonian rats is comparable to the expression of GID in patients in the Denver-Columbia trial [9], whose GID were primarily restricted to the upper body, but different from those reported in the Tampa-Mount Sinai trial [10] in which GID primarily effected the extremities of the lower body. This discrepancy is more likely due to a variation in graft placement than a difference in biological mechanisms. As referenced above, another similarity to *clinical GID* in patients is the timing of *experimental GID* expression in rats. Like with human patients, these behaviors are not seen prior to grafting and develop as the grafted cells mature in the parkinsonian striatum (e.g.: [19,20,21,23,24]).

Although experimental models of GID are invaluable to our understanding of clinical GID, there are several discrepancies between the human and rodent phenomena that warrant attention. Most notably, experimental GID is elicited in animal models when plasma DA levels are elevated by pharmacological agents like levodopa or amphetamine [19–21, 23–25], while these behaviors are seen in grafted patients when patients are ‘off’ DAergic medication and plasma levodopa levels are low [9, 10, 17]. Based on the temporal and spatial expression of GID, it has been hypothesized that post-graft ‘off-medication’ dyskinesias represent a form of prolonged biphasic dyskinesia, i.e.: those that occur at the beginning and end of the levodopa cycle and tend to be expressed as focal, stereotypic and repetitive movements [26]. In Figure 2 we pose the question of whether the difference in amount of tissue typically grafted into patients with PD versus rats with experimental parkinsonism might impact how pharmacological agents interact with the grafted striatum to produce clinical versus experimental GID behaviors, respectively. Briefly, because of the relative small amount of tissue that has generally been grafted in the rat brain, it is possible that there is need for a DA agonist to “push” the extracellular levels of DA up into the

concentration range that would induce a biphasic-like dyskinesia and give rise to expression of the experimental GID phenotype. In contrast, in PD patients, the relatively larger quantity of tissue that has generally been grafted may, as levodopa concentrations are waning or absent, reduce striatal DA concentrations into the concentration range that provokes a biphasic-like dyskinesia phenotype (more detail is given in Figure 2). Importantly, experimental GID can be observed in grafted rats while off levodopa, however, they occur at such random intervals as to make their systematic evaluation impossible.

While there are these discrepancies in post-graft dyskinesias between patients and rodent models, in this review, we will refer to dyskinetic behaviors in the rat that fulfill the characteristics of “not seen prior to grafting, or worsening/emerging in the presence of a mesencephalic graft” as “experimental GID”. As appropriate, we will further define whether we are referring to these post-graft dyskinesias occurring in patients, or an animal model that requires induction by a DA agonist. It is important to point out that in the model where experimental GID is elicited by levodopa (e.g.: [19,20,25]), it is inappropriate to refer to these as LID without additional qualifiers. Specifically, the time course of expression is inverse for expression of these post-graft experimental GID phenotypes elicited by levodopa compared to the typical pre-graft LID. Further, these levodopa-elicited experimental GID behaviors show differential regulation of the transcription factor fosB/ Δ fosB from the pre-graft LID profiles, and the outward phenotype is different [20].

Solving the Mystery of Graft-induced Dyskinesias: Theories and Hypotheses

It has been 10 years since GID were first reported in the literature. In the past decade numerous theories have been proposed and hypotheses tested in an attempt to better understand possible mechanisms of GID. Broadly speaking, there are two sources of factors that could underlie GID development: 1) the graft itself, and 2) the host brain. Within these two categories, multiple factors have been considered to be of interest based principally on correlation with clinical findings and/or strong biological rationale.

- “The Graft”:
 - Pattern of Reinnervation/Degree of DA Replacement
 - Preparation of Tissue (stored versus fresh; solid versus suspension)
 - Non-DA cells/Serotonin cells
- “The Host”:
 - Immune Response
 - Age of Graft Recipient
 - Disease Severity
 - Pre-graft Levodopa History (e.g.: “priming”):
 - Abnormal Target Plasticity

Indeed, several of these as well as other factors have been evaluated in clinical studies and/or tested in animal models, with some appearing to be more promising for their involvement in GID than others. However, it is likely that no single factor in isolation will be found to be responsible for GID development. It is beyond the scope of this review to do an extensive recitation of what is known about all factors considered of interest in GID, and numerous reviews have already provided such discussion. In this review, we attempt to provide comparison of studies exploring some of the factors that we consider the most provocative

of those bulleted above. We also introduce new ideas regarding how these mechanisms may interact to alter neuronal circuitry in the parkinsonian, grafted striatum to produce the unwanted behavioral output known as GID. Significantly, clinical grafting in PD patients is now being re-visited in a European trial entitled “Transeuro” (<http://www.transeuro.org.uk>, December 2011). “Transeuro” is hoping to develop an effective and safe method to treat individuals with PD using fetal cell based therapy, guided by what is known about potential factors underlying the lack of favorable outcome. So what do we know about potential causes of GID that can help guide future trials of this still promising therapy?

Pattern of Reinnervation and Graft-induced Dyskinesias

One initial hypothesis proposed that GID were the result of an excessive reinnervation of the striatum [9]. However, post-mortem analyses failed to report overgrowth of dopaminergic tyrosine hydroxylase positive (TH+) fiber reinnervation or supranormal levels of DA function measured with 18fluorodopa (18F-DOPA) positron emission tomography (PET) [10,12,13,27]. More probable is the suggestion that GID is the result of concentrated ‘hot spots’ of DA activity against a background of significant DA depletion. Evidence of focal areas of increased 18F-DOPA uptake was reported in grafted patients expressing clinical GID [27]. This hypothesis is supported by findings in the parkinsonian rat. Specifically, more focal grafts consisting of an estimated $\frac{3}{4}$ of an embryonic ventral mesencephalon placed into 2 separate striatal sites [21] or one full ventral mesencephalon placed into a single striatal site [20] induced experimental GID in rats. In contrast, when this same amount of tissue (i.e.: one ventral mesencephalon) was placed into 6 sites distributed across the striatum, resulting in uniform widespread DA cell distribution, experimental GID was significantly reduced [20].

Clinical data also supports the hypothesis that incomplete or patchy reinnervation seems to play a role in GID, and that the *degree* of partial reinnervation may be a critical variable. Specifically, it is interesting that in the Denver/Columbia clinical trial, the increase in overall putamen PET 18F-DOPA signal in the GID-expressing patient group was twice that of patients not expressing GID at 12 months ($P < 0.03$) and almost three times larger at 24 months ($P < 0.005$) [27]. Despite the 18F-DOPA signal being 3 times higher in the GID expressing patients, it was still below the level of the normal striatum by about 20% [27]. Thus, it has been suggested that, in the patients expressing GID behaviors, the levels of DA replacement by the grafted cells, while incomplete, is perhaps in the range of that required to induce biphasic dyskinesia (e.g.: [26,27]). Again, the interaction of graft size and relative restoration of striatal DA concentration as it relates to the various behavioral outputs including biphasic dyskinesias or GID is presented in Figure 2.

Beyond the fact that grafted cells are releasing DA into the parkinsonian striatum at levels which are below normal and in patchy areas, there are more complex considerations of how and whether these grafted cells are able to re-establish physiological circuits in an adult, diseased brain. In the following paragraphs we consider how the cellular composition of a graft and the environment into which it is placed might impact the establishment of graft-host circuits.

Cellular Composition and Graft-induced Dyskinesias

The cellular composition of fetal tissue grafts has been suggested as a possible contributing factor to the appearance of GID as well as the limited benefit observed in grafted patients. For example, depending on the dissection protocol employed, fetal mesencephalic grafts can contain numerous cell types including glial cells, DAergic (A8, A9 and A10 cell groups), γ -aminobutyric acid (GABA)ergic, and serotonergic neurons. Recent studies have suggested that the proportion of A9 DAergic progenitors (destined for a substantia nigra (SN) fate)

grafted is positively correlated with motor recovery benefit in a rodent model [28]. This is likely due to the fact that these neurons, in contrast to A10 cells (destined to become ventral tegmental area (VTA) neurons), have the unique ability to innervate and establish functional synapses with neurons native to the target structure in which they are grafted, i.e.: the striatum. The impact of differential proportions of A9 and A10 neurons on GID-type behaviors has not been directly investigated. While it could be hypothesized that the reversal of motor disability might benefit more from grafting A9 cells, this cell type may be more prone to producing post-graft dyskinetic behaviors. Rationale for this concept is expanded in the section below entitled “Dopamine Depletion as a Modifier of Graft Outcome” but in brief, the attempted formation of synaptic contacts between A9 neurons and striatal target neurons (i.e.: medium spiny neurons (MSNs)) that have an abnormal morphology, which is known to occur in the PD brain, could result in abnormal circuitry and the expression of GID.

An additional cellular component that has been proposed to impact graft functional outcome, specifically GID behavior, is the proportion of serotonergic to dopaminergic progenitors within the graft. Further, sprouting of *host* 5-hydroxytryptamine (5HT, serotonin) neurons into the grafted, parkinsonian striatum is also considered a risk factor that may influence this side effect. An abundance of recent data suggests a mechanistic role for 5HT neurons in the expression of typical LID in non-grafted subjects, although not without caveats (e.g.: see [29] for conflicting results). However, data suggesting a role of 5HT in post-graft GID is less conclusive and strongly mixed. Recent findings supporting or refuting the involvement of 5HT terminals in GID behaviors from both clinical and preclinical studies are contrasted in Table 1.

Serotonin Neural Circuits and Graft-induced Dyskinesias

While the “jury is still out” on the importance of 5HT neurons in GID, it is worth considering that if these neurons do promote this behavior, how might this occur? Both nigral DA and raphe 5HT neurons contain aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter 2 (VMAT2) and thus both of these neuronal populations can produce and release DA. According to the current dogma, the mechanism by which 5HT neurons produce dyskinesias is related to their capacity to synthesize, store and release DA from systemically administered levodopa treatment (reviewed in [30]). While 5HT neurons can release DA as a so-called “false transmitter,” the release is non-regulated because of a lack of DA autoreceptors. Thus, DA release from 5HT terminals is non-regulated and non-physiological, which has long been associated with dyskinetic side effects of long-term levodopa [31–33]. Interestingly, two recent reports reveal that in the non-grafted parkinsonian rat [34] and non-human primate [35] dyskinogenic levodopa treatment results in increased numbers of serotonergic varicosities beyond that noted after the destruction of the nigrostriatal pathway. Thus, it is reasonable to assume that an increased density of 5HT fibers, which are releasing increased amounts of non-regulated DA, could lead to dyskinetic behaviors.

However, there is an additional mechanism by which 5HT neurons might be influencing striatal circuitry and expression of dyskinetic behavior, perhaps both LID and GID. Interestingly, and perhaps of greater significance than the appearance of increased axonal sprouting, is the recent report of a significant increase in 5HT synapses *per se* in the dyskinetic rat brain [34]. This is of interest because under normal conditions 5HT terminals within the striatum have a very low incidence ($\pm 6 - 18\%$) of actual synapse formation [34*, 36–38, 39†]. Thus, most of the neurotransmitter release in the striatum from 5HT varicosities presumably occurs through autocrine mechanisms. The finding of an increase from 18% to 46% incidence of 5HT terminals forming synaptic specializations in

parkinsonian rats expressing LID [34] is striking and potentially of significance in understanding the role of the 5HT system in dyskinesias.

A New Layer of Complexity: Serotonin/Glutamate Co-Transmission

While the role of 5HT hyperinnervation producing excess DA makes intuitive sense with regard to the production of dyskinetic behavior, the involvement of striatal 5HT axons in this behavior may be more complex than the relatively simple notion of enhanced non-regulated DA release. Specifically, an unexpected observation was made in recent years that a majority of central 5HT neurons express the vesicular glutamate transporter 3 (vGLUT3) [40–44]. Interestingly, vGLUT3 is expressed primarily in neurons thought to release other conventional neurotransmitters, such as 5HT, acetylcholine, and GABA, in a classical manner [45,46]. Indeed, in the adult rat or mouse raphe nuclei, approximately 80% of 5HT neurons, which are the neurons that hyperinnervate the parkinsonian striatum, express *vglut3* mRNA and axonal vGLUT3 protein [42,47]. Interestingly, triple label immunofluorescence studies have shown that axon terminals that are positive for vGLUT3 and 5HT are generally not positive for the serotonin transporter (SERT, the 5HT reuptake transporter protein).

Little is yet known about the functional significance of vGLUT3 co-expression in 5HT neurons and it is certainly not clear whether it plays a role in GID (or LID). However, there is compelling evidence that suggests that it may warrant investigation. First, vGLUT proteins are unambiguous markers of glutamate neurotransmission [45,46]. Accordingly, both *in vitro*, using electrophysiological cell culture technique [48], and *in vivo*, using an optogenetic murine model [49], it has been demonstrated that the co-release of glutamate from 5HT neurons can result in fast α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-receptor mediated depolarizing currents. This colocalization of 5HT and glutamate in the axons that hyperinnervate the parkinsonian striatum implies that there would be an enhanced fast excitatory signaling component at these synapses viewed as being strictly modulatory. Because of the important role of glutamatergic signaling in striatal plasticity, these findings add a new layer of complexity that has untapped potential in understanding the dynamics of 5HT hyperinnervation in dyskinesia development and expression.

In addition to vesicular packaging of glutamate that is involved with fast excitatory currents, vGLUT3 in 5HT terminals has another important role. As reviewed by [46], a major functional property of the glutamatergic co-phenotype is increased filling of synaptic vesicles with the ‘primary’ neurotransmitter, in this case 5HT. This phenomenon is known as “vesicular synergy”. However, if vesicular synergy is to be considered important in the development of dyskinesias, it would be necessary for DA to be included in the enhanced vesicular loading in 5HT neurotransmitter vesicles. Current data supports the idea that in 5HT neurons the same pre-synaptic vesicles contain both vGLUT and VMAT2 [45], suggesting that vesicular synergy would likely involve enhanced DA loading in striatal 5HT terminals within the parkinsonian striatum in individuals treated with levodopa. However, this hypothesis remains untested.

In addition, both excitatory post-synaptic signaling (from glutamate co-release) and the size of the readily releasable pool of transmitter (e.g.: DA and/ or 5HT) are heavily dependent on the number of synapses formed by a neuron [45]. Keeping in mind that in the dyskinogenic parkinsonian rat striatum there is a 2.5-fold increase in the number of synapses formed by 5HT terminals [34], it would be reasonable to suggest that dyskinesias might be linked to enhanced post-synaptic glutamatergic signaling as well as enhanced DA release, with both events coming from the 5HT terminal fields. The incidence of 5HT synapses from grafted 5HT cells and their potential correlation to GID has not been examined.

A recent report by Dupre and colleagues [50] presents an intriguing, potential bridge in the gap between serotonergic and glutamatergic mechanisms in the expression of LID [30]. Their study demonstrates that striatal vesicular glutamate release is directly tied to 5-hydroxytryptamine 1A receptor (5HT1AR)-mediated suppression of LID. Specifically, these investigators found that stimulation of striatal 5HT1AR reduces local levodopa-induced glutamate efflux in the parkinsonian striatum while concomitantly diminishing the expression of LID [50]. The assumption in this study is that the reduced glutamate efflux is tied to cortical glutamate axons, which may or may not be the case. It is interesting to consider that accompanying striatal 5HT hyperinnervation and hyper-synapse formation in the vGLUT3 expressing raphe 5HT terminals there may be enhanced packaging and subsequent non-regulated release of DA and enhanced glutamate co-release. Such phenomena would be expected to exert powerful modulation of circuit activity within the grafted striatum.

Target Environment and Graft Dysfunction

Regardless of the cellular composition of grafts, the status of the host target neurons will undoubtedly impact the ability of grafted cells to reconnect and remodel the diseased brain. This assumption is supported by findings from clinical trials showing graft efficacy to be dependent on the patient's age [9] and/or disease severity [10], and to vary amongst grafted individuals despite similar levels of graft survival and 18F-DOPA uptake [10]. Recent studies have attempted to determine how the PD modified striatal environment may be impacting graft efficacy and the development of GID. In the following paragraph, we will examine how disease severity as it relates to pathology of striatal target neurons and the host immune environment might interact with grafted embryonic neurons to alter synaptic circuitry and produce post-graft dyskinesia behaviors. Table 2 summarizes clinical and preclinical observations related to various factors involved in striatal pathology and how they might impact graft dysfunction. We provide additional detail for some of these factors in the following 4 sections.

Dopamine Depletion as a Modifier of Graft Outcome

One potentially important finding from clinical trials has been the observation that GID seems to correlate strongly with a patient's pre-surgical extent of DA depletion [17,51]. Similarly, animal studies show that a DA graft in the context of less severe DA depletion is also more therapeutically effective [52]. This finding seems to implicate the severity of striatal DA loss as a modifier of graft integration. It has been known for several decades that the loss of striatal DA results in significant morphological alterations of MSNs, the primary target neuron for DA axons. Specifically, in advanced PD, there is a marked atrophy of dendrites and spines of these neurons [53–55]. A similar pathology is observed in mice, rats, and non-human primates with severe DA depletion (e.g.: [25,56–58]). In the striatum, dendritic spines of MSNs are the primary site of afferent input for nigral DA neurons, and glutamate input from the cortex and thalamus. The majority of nigral DA neurons primarily synapse onto the *neck* of dendritic spines where DA is thought to modulate glutamate signaling, which is transmitted to the MSN through excitatory synapses onto the *head* of the same dendritic spines [59] (Figure 3a). This morphological arrangement of synaptic inputs between DA and glutamate in the striatum is critical for normal motor behavior and is disrupted in the parkinsonian brain. It is reasonable to suggest that the loss of MSN spines that accompanies severe DA depletion is creating an environment that fosters inappropriate connectivity between host MSNs and grafted cells due to lack of normal contact sites.

Indeed, ultrastructural analyses have documented aberrant synaptology in the parkinsonian brain between graft and host cells with the grafted DA neurons making more atypical

contacts onto dendritic shafts and fewer normal contacts onto spines themselves [60–64]. This shift in post-synaptic target and alterations in other synaptic features has recently been shown to correlate with the abnormal behaviors in the rat model, including drug-induced experimental GID and the suboptimal behavioral efficacy observed following grafting [19]. While such data is a good first step toward understanding the functional impact of remodeled circuits in the parkinsonian striatum, there is currently very limited data on this topic. In addition, there is very limited understanding of the multitude of changes that likely occur and/or specific factors that influence the remodeling of circuits within the parkinsonian and grafted striatum. Without such understanding, scientists will not know how to achieve physiological remodeling within the PD brain, or whether it will ever be possible to do so.

An Additional Layer of Complexity: Dopamine/Glutamate Co-Transmission

There have been several reports suggesting that the dynamic changes of the DAergic *pre*-synaptic ‘compartment’ determines susceptibility to dyskinetic behaviors in rats, particularly LID (e.g.: [65–67]). However, we suggest here that the complexity of pre-synaptic DA release and its involvement with dyskinesias, either LID or GID, has perhaps been underestimated, and that there is an important interplay between pre- and post-synaptic compartments. Similar to the discussion above about the co-localization of the vGLUT3 glutamate transporter in 5HT neurons in the section entitled “A New Layer of Complexity: Serotonin/Glutamate Co-Transmission”, midbrain DA neurons can also use fast glutamatergic transmission to communicate with their post-synaptic targets. Indeed, molecular and biochemical evidence demonstrate that DA neurons also co-localize a glutamate transporter, specifically vGLUT2 [68–74]. Similar to 5HT neurons, there are several studies that provide unequivocal evidence that midbrain DA neurons can co-release glutamate with DA and that this co-release of vesicular glutamate from DA terminals results in the generation of fast excitatory current via ionotropic glutamate receptors (e.g.: [71,75–78]). This contrasts the slower modulatory action of synaptic DA acting on G-protein coupled DA receptors. These findings suggest the interesting possibility that such co-release could lead to the encoding of parallel signals that operate on different timelines (e.g.: fast excitatory transmission via ionotropic glutamate receptors versus slower G-protein coupled modulatory transmission via DA receptors).

Whether there is any functional significance of the glutamatergic co-localization in DA neurons in the development of dyskinetic behaviors is unknown. However, there is accumulating evidence that suggests a critical assessment of the possible involvement of glutamate release from DA terminals in the pathophysiology of dyskinesias is warranted. It is important to point out that most evidence for the co-release of glutamate with DA, and the generation of fast excitatory current has been found in the normal adult rat in the mesolimbic DA system, but not the dorsal striatal DA system [71,78]. That being said, there importantly appears to be a dynamic regulation of vGLUT2 expression and function in DA neurons of the striatum with PD-relevant factors including age and DA itself being apparent dynamic regulators of this co-transmission. Specifically, Mendez and colleagues [70], using single-cell reverse transcriptase polymerase chain reaction (RT-PCR), showed that VGLUT2 is the only vGLUT expressed in VTA/SN DA neurons *in vivo*, that its expression decreases with age and is negatively regulated through a contact-dependent signal provided by GABA and DA neurons. Similar findings of an age-associated decrease in the amplitude of fast excitatory currents in cultured midbrain DA neurons also has been reported [79]. Of relevance to PD, a recent study revealed that there is an induction of vGLUT2 expression in TH+ neurons of the striatum under pathological conditions (i.e.: following striatal DA depletion) [68]. Moreover, the functional properties of midbrain DA-glutamate co-release can be regulated by chronic application of glial cell-line derived neurotrophic factor

(GDNF) [79]. Thus, the glutamatergic phenotype of striatal mesencephalic DA neurons is dynamically regulated by age, pathology and trophic factors; these are all relevant factors for consideration in PD research.

Target Neuron Pathology Alters Dopamine Circuitry in the Grafted Brain

On the basis of ultrastructural parameters, a concept was formulated that symmetric (Gray Type II) synapses are inhibitory and asymmetric (Gray Type I) synapses are excitatory (reviewed in [80]). In the normal adult striatum, nigral DA afferent axons that preferentially synapse onto the neck of dendritic spines of MSNs establish symmetric (presumed inhibitory) synaptic connections 97% of the time [19]. Work in our lab using immunoelectron microscopic methods has revealed that when embryonic DA neurons are grafted into the striatum of parkinsonian rats and they develop the experimental GID phenotype, the TH+ terminals show an increased incidence of asymmetric (presumed excitatory) post-synaptic specializations. The incidence of asymmetric (Gray Type I) TH+ profiles in the striatum of rats expressing experimental GID phenotype increases approximately 10-fold from 3% to 38% [19]. It remains to be determined whether the increase in asymmetric profiles, which are traditionally associated with the excitatory neurotransmitter glutamate, are created by reintroduction of vGLUT2 that occurs in the parkinsonian striatum [68], or reflects the fact that the grafted DA neurons, being embryonic likely express vGLUT2 [70]. Further, it is hypothesized that the loss of dendritic spines on MSNs predisposes the new DA terminals from grafts to make contacts onto the dendritic shaft (e.g.: [19,60]; discussed above in “Dopamine Depletion as a Modifier of Graft Outcome”), which further predisposes the new synapses to establish a new functional phenotype (i.e.: change from symmetric to asymmetric per [19]).

While recent optogenetic data demonstrates that the activation of DA axon terminals that originate in the VTA and co-localize vGLUT2 induces AMPA receptor mediated fast synaptic responses of MSNs of the nucleus accumbens [71,78], it remains to be determined whether similar glutamatergic receptor activation occurs in the striatum of a parkinsonian subject in which there is re-introduction of vGLUT2 co-expression [68]. Similarly, it remains to be determined whether the asymmetric TH+ synapses within the grafted striatum [19] mediate any functional excitatory response or contain AMPA or other glutamatergic receptors in the post-synaptic density. Because striatal AMPA receptors are strongly implicated in the pathophysiology of LID [81–83], elucidating whether such phenomenon occur in the parkinsonian striatum could be highly instructive. Whereas it is clear that AMPA receptor signaling in the striatum is linked to LID, it has been assumed that the source of aberrant glutamatergic signaling derives exclusively from corticostriatal or thalamostriatal axons. Indeed this may be the case. However, understanding whether there is contribution from the nigrostriatal DA and glutamate co-phenotype would provide a new level of insight into the pathophysiology of LID and/or GID. While the pharmacological characterization of LID is well established for clinical and experimental LID, there is a large paucity of pharmacological data in experimental GID in rats and in clinical GID in PD patients (Table 3).

Finally, in addition to potential post-synaptic excitatory receptor plasticity, the potential role of vGLUT2 proteins in grafted DA neurons would also be anticipated to result in “vesicular synergy” ([46]; introduced above in “A New Layer of Complexity: Serotonin/Glutamate Co-Transmission”) and enhanced local release of the primary transmitter (i.e.: DA). If vesicular synergy does occur in grafted DA neurons, this could contribute to focal “hot-spot” activation of striatal circuits, which is considered as a potential causative factor in GID behaviors. Additionally, as demonstrated by Soderstrom and colleagues [19], in parkinsonian rats that express experimental GID, there is a complex array of excitatory

circuits in the grafted striatum [19] (Figure 3c). Specifically, in the vicinity of the graft, there is an increase in the density of perforated, asymmetric, non-TH+ synapses contacting dendritic shafts (rather than spine heads) of local target neurons. Perforated synapses are ultrastructural landmarks of long-term potentiation (LTP) [84], which is characterized by a long-lasting enhancement of synaptic efficacy. There is also the presence of asymmetric, excitatory synapses contacting the grafted TH+ cells, which presumably would increase the excitatory synaptic drive of these cells. In addition, the grafted TH+ cells themselves form asymmetric (presumably excitatory) synapses onto dendrites of local target neurons. Thus, we hypothesize that this dramatic synaptic reorganization could produce experimental GID by producing significant alterations in the functional output of striatal circuitry. Interestingly, this 'rewiring' of striatal circuitry correlates significantly with the severity of experimental GID in rats [19].

Neuroplasticity, the Immune System, and Graft-induced Dyskinesias

Although factor(s) that could lead to dysfunctional circuit remodeling and aberrant motor output are likely numerous, insight into some of these may be garnered from examining the timing of GID expression in clinical trials. For example, in one study [10] patients showed improvements in parkinsonian motor deficits with no abnormal behaviors reported for 6 months followed by the appearance of clinical GID and a cessation of behavioral improvement. It is possible that in this 6-month period something adverse occurred to the grafts themselves or to their synaptic contacts with the host neurons. One potential factor that could explain this delay is the host immune response to grafting. Despite historically being regarded as an immuno-privileged site, there is a moderate degree of immune activation that occurs in the central nervous system following grafting. This occurs primarily because of the necessity of using non-genetically identical donor tissue, or allografts.

While animal studies suggest an extremely low immune response when genetically identical donor tissue is used, this is not feasible in human grafting studies and, therefore, allogeneic grafts are often used in conjunction with immunosuppression regimes. In two clinical grafting trials for PD, the appearance of GID appeared to coincide with the withdrawal of immunosuppression [10,17]. This observation, coupled with our recent finding that graft-induced dyskinesias fail to develop in grafted rats receiving donor tissue from rats of the same inbred strain [19] but occur with increased frequency in allografted rats with elevated immune responses, suggests a possible role for the host immune response in the development of dyskinesias following grafting.

Two parallel preclinical studies [19,85] have used varying experimental protocols in attempts to determine whether the host immune response might play a role in experimental GID expression. In Figure 4 we put forth rationale for the possible involvement of immune factors in modulating striatal circuitry and compare the results of these two aforementioned studies. The collective data from these 2 studies in allografted rats with experimental GID induced by pharmacological agents that elevate local DA (i.e.: levodopa or amphetamine) seems to suggest that: 1) these behaviors develop when there is mild immune activation; 2) they increase in severity with elevated immune activation; and 3) they are dependent on the presence of DA grafted cells, presumably to act as the source of the formation of aberrant synaptic connections. Indeed, when grafted DA neurons are completely rejected, experimental GID in those rats disappear [85].

In contrast to the long held belief that glial cells in the brain play passive roles in structural support or immune surveillance, there is a large body of evidence that demonstrates an active role of glial cells in synaptic plasticity in health and disease (e.g.: [86,87]). Recent data suggests that even in the healthy brain, quiescent microglia appear to regulate the

dynamics of plasticity at individual synapses as they explore the local environment to pursue immune surveillance roles [87]. In addition, the concept of tripartite synapse (presynapse, postsynapse, and astrocytes) was introduced approximately a decade ago in which synaptically associated astrocytes were proposed to exchange information with neuronal synaptic elements at glutamatergic synapses, even impacting synaptic strength (reviewed in (e.g.: [86]). Recent ultrastructural data has revealed that there is a significant expansion of the astrocytic presence in striatal tripartite synapses in the parkinsonian brain, providing further evidence that both neuronal and glial elements are involved in the pathophysiology of PD [86]. Thus, continued examination of host immune cell function in post-graft motor dysfunction in PD patients should be considered a high priority. Understanding whether dysfunctional remodeling and/or experimental GID can be eliminated by immunosuppressive therapies will be an important next step in preclinical studies. It would also be useful to understand which specific components (microglia, complement factors, specific cytokines, etc.) of the immune system play an important role in the dynamic regulation of the phenotype of synapses in the parkinsonian, grafted striatum. Based on what is known about some immune/glial factors and what has been hypothesized as mechanisms underlying GID, it is possible that some glial factors may enhance synaptic remodeling that leads to GID (e.g.: GDNF, [79]) while others may play a role in preventing this phenomenon (e.g.: interleukin-2 (IL-2), [88]).

Validating Circuit Components of Graft-Induced Dyskinesias with Pharmacology

Primarily what has been studied in both experimental models of GID in rats and in clinical GID in PD patients are the responses of these behavioral phenotypes to drugs that impact synaptic DA and/or 5HT content. To assess the role of DA and 5HT in the expression of experimental GID induced by amphetamine (AMPH), the selective DA or 5HT uptake inhibitors, GBR 12909 and fluvoxamine, respectively, were administered either alone or in combination to rats that previously received intra-striatal graft tissue derived from the embryonic ventral mesencephalon and the effects compared to AMPH administration [21]. Although systemic administration of fluvoxamine alone did not produce experimental GID, systemic administration GBR 12909 alone produced low levels of limb and orolingual experimental GID. Co-administration of GBR 12909 and fluvoxamine produced levels of limb and orolingual experimental GID comparable to that observed following AMPH administration. In contrast, axial and locomotor experimental GID induced by administration of the GBR 12909 alone or by co-administration of the DA uptake inhibitor plus the 5HT inhibitor did not differ qualitatively or quantitatively from that induced by systemic AMPH administration [21]. These findings suggest a role for an increase in extracellular DA concentration in the expression of experimental GID. With regard to the role of 5HT in the expression of GID, the findings suggest an interaction between the 5HT and DA neurotransmitter systems since 5HT uptake inhibition increased GID only when extracellular DA levels were increased. Moreover, these findings are consistent with the notion of the release of DA from 5HT neurons as a so-called “false neurotransmitter” discussed above in the section entitled “Serotonin Neural Circuits and Graft-induced Dyskinesias”. Unfortunately, despite the fact that AMPH, which stimulates the release of DA, 5HT, and norepinephrine (NE), is often used to evoke abnormal involuntary movements in experimental models of GID, the role of NE in the expression of GID has not yet been directly assessed (e.g.: via the administration of the potent and selective inhibitor of NE reuptake, nisoxetine [89]).

In grafted PD patients, the role of 5HT has also been examined to test the hypothesis that clinical GID are caused by dysregulated DA release from graft-derived striatal serotonergic terminals, discussed above in the section “Serotonin Neural Circuits and Graft-induced

Dyskinesias”. In recent reports by Politis and colleagues [90,91] three PD patients who had received intra-striatal implants of DA-rich fetal mesencephalic tissue received repeated systemic administration of buspirone (Buspar), a 5HT_{1A}R partial agonist (IC₅₀ = 35 nm) that displays dopamine D₂ receptor (D₂R) antagonist (IC₅₀ = 250 nm) properties [92] and is metabolized to the potent α_2 -adrenergic receptor antagonist, 1-(2-pyrimidinyl-piperazine) [93–95]. Repeated administration of buspirone attenuated clinical GID severity in these patients [90,91]. Although several other possibilities exist including the activation of postsynaptic 5HT_{1A}R, it was proposed that the buspirone alleviated GID behaviors by blocking the release of DA from serotonergic neurons by activating inhibitory 5HT autoreceptors (per mechanisms described in [96,97]). This clinical finding suggests that excess DA release from ectopic 5HT terminals may indeed contribute to GID; however, it is counter to what seems to be evidenced from preclinical studies [19,29,98].

In addition to the aforementioned studies that have examined the contribution of pre-synaptic monoamine release, the role of DA and 5HT receptors in the expression of experimental GID in rats has been examined. Lane and colleagues [98] reported that systemic administration of the selective D₂R antagonist, raclopride [99], and the selective dopamine D₁ receptor (D₁R) antagonist, SCH23390 [100], suppressed AMPH-induced experimental GID. This finding suggests that activation of D₁R and D₂R play a role in the induction of experimental GID in the rat model [98]. Moreover, these authors found that systemic administration of the selective 5HT_{1A}R agonist, 8-OH-DPAT, reduced the severity of AMPH-induced experimental GID. However, rats that received a 6-OHDA-lesion plus a DA graft and were subjected to 5, 7-dihydroxytryptamine (5, 7 DHT) lesions to dramatically decrease the serotonergic innervation of the striatum, exhibited a level of AMPH-induced experimental GID comparable to those animals without the 5, 7 DHT lesion [98]. This finding indicates that host raphe-striatal 5HT system does not appear to play a role in the expression of experimental GID.

It is important to appreciate that continued pharmacological studies in subjects with an altered striatal synaptology will advance our understanding of GID and how to effectively treat them. Future pharmacological studies that rely on procedures that provide for better spatial and temporal control of drug administration have the ability to directly test current hypotheses regarding the neural substrates of GID and may allow for the development of new hypotheses. One rational approach is to use selective compounds to test the hypothesis that the pattern of synaptic connections formed between transplanted neurons and host neurons, although dynamic and in a state of flux, may underlie both behavioral recovery as well as GID [19,25,60,62]. For instance, as first introduced above in the sections “Dopamine Depletion as a Modifier of Graft Outcome” and “Target Neuron Pathology Alters Dopamine Circuitry in the Grafted Brain”, in animals that receive rat embryonic ventral mesencephalic grafts following a 6-OHDA lesion of the nigrostriatal pathway and showed complete behavioral recovery, the axons of the grafted neurons formed symmetric (Gray Type II, presumably inhibitory) synapses onto spine necks and dendritic shafts of host MSNs [60], a pattern of synaptic input observed in untreated rats [59]. However, in these same animals DAergic fibers of the transplanted neurons formed dense “baskets” of fibers with frequent symmetric synaptic contacts that surrounded the perikarya of the host large aspiny cholinergic interneurons, a phenomenon never seen in the normal striatum [60]. Since DA has an inhibitory effect on striatal cholinergic interneurons [101] and striatal cholinergic interneurons have an excitatory effect on MSNs [102], this pattern of abnormal synaptic connectivity suggests a mechanism by which DA could potentially dampen the activity of MSNs. Studies that explore the role of activation and/or blockade of striatal muscarinic M₁-like and/or M₂-like receptors, which are expressed on the cell surface of both MSN and the large aspiny cholinergic interneurons [103–105] in GID would be useful in determining

whether or not this pattern of abnormal synaptic connectivity is related to the induction or expression of GID.

As detailed in the section above “Target Neuron Pathology Alters Dopamine Circuitry in the Grafted Brain”, Soderstrom and colleagues [19] demonstrated that in DA grafted rats expressing experimental levodopa-induced GID, GID severity was positively correlated with the proportion of asymmetric (Gray Type I, presumably excitatory) synapses contacting grafted cells. Importantly, most, but not all, asymmetric synapses are glutamatergic and, thus, in most, but not all cases, the terminal bouton exhibits immunoreactivity for glutamate [106–108]. In addition, the post-synaptic density, one of the defining features of the asymmetric (Gray Type I) synapse, is best viewed as a complex assembly of hundreds of structural and signaling proteins associated with the post-synaptic membrane including the ionotropic glutamate receptors (i.e.: AMPA and N-methyl-D-aspartate (NMDA)) [109,110]. Thus, studies that explore the role of activation and/or blockade of striatal AMPA and/or NMDA receptors in the induction and expression of GID would be of great interest.

Future Directions: What Pieces of the Puzzle are Missing?

There is good correlative data now available to suggest that several specific mechanisms are likely to play a role in the development of abnormal motor movements following engraftment of embryonic DA neurons into the parkinsonian striatum. As detailed in this review, these mechanistic factors include (but are likely not limited to) striatal 5HT fibers, co-localization of vesicular glutamate transporters in monoaminergic neurons, unknown component(s) of the immune system, and striatal target neuron pathology. In the above paragraphs, we presented a discussion of some of the primary data that is suggestive of particular mechanisms and what is still missing to determine the unequivocal role of these factors in clinical expression of GID. However, there are numerous other factors that have not yet been considered. In this final section of the manuscript, we present a few pieces of the scientific puzzle that are still missing and may warrant further investigation in the quest for understanding why abnormal behavioral profiles develop upon attempted brain remodeling during embryonic DA neuron grafting in PD.

So what is missing? One important piece of the puzzle that is missing is a more extensive characterization of the pharmacology of GID. As can be appreciated in the section “Validating Circuit Components of Graft-Induced Dyskinesias with Pharmacology” there have been some important initial studies done on the pharmacology of GID in the rat model and in PD patients. However, the extent of pharmacological classification of this phenomenon is extremely limited compared to what has been accomplished for non-graft associated drug-induced/levodopa-induced dyskinesias. A comparison of what is known about the pharmacology of GID versus what is known about the pharmacology of LID is presented in Table 3.

One important entity that is missing in the quest for understanding causative mechanisms of GID is a non-human primate (NHP) model of this side effect. There are currently no published reports of GID in a NHP model of parkinsonism. Doubtless, the establishment of off-medication dyskinesias following DA terminal replacement in a NHP model would be valuable. While the rodent model of drug-induced LID has strong face and predictive validity relative to the corresponding disorder in primates, the rodent model of experimental GID has the notable weakness that these behaviors are reliably quantifiable only after administration of drugs that elevate the striatal DA concentration. While this may or may not negate its validity (see Figure 2), an animal model of off-time GID would be ideal. Based on size and phylogenetic similarities between NHP and humans, it could be predicted that the NHP may be a more suitable preclinical model. Comparisons between a NHP model and the

rodent models of experimental GID would additionally allow for validation studies between these models and ultimately patients. Validation of the rodent model of GID, in spite of the fact that these experimental GID behaviors need to be elicited by drugs like AMPH or levodopa, would provide a less expensive, more widely available model for preclinical testing.

Another piece of the puzzle that is missing is the examination of post-mortem striatum from patients that had suffered with GID. Post-mortem analyses detailing the patterns of integration of DA fibers, and a characterization at the ultrastructural level of the extent of DA and non-DA synaptic remodeling that occurs would be instructive not only for understanding mechanisms of circuit plasticity in GID but for validating experimental findings in models of GID. While there is indication that specific structural modifications of post-synaptic specializations correlate with dyskinetic behaviors in rodent models [19,111], examination of this type of synaptic reorganization in striatal circuitry in the patients expressing GID are lacking.

Examinations of extrastriatal pathology are another missing puzzle piece. To date all mechanisms that have been described as relevant to GID have been principally restricted to the striatum (caudate/putamen). It is well known in parallel research (i.e.: drug abuse) that behavioral sensitization involving midbrain DA systems involve functional and molecular alterations throughout basal ganglia nuclei (reviewed in [112]). Specifically, it is known that ‘initiation’ of mesolimbic DA sensitization phenomena during chronic psychomotor stimulant administration involves temporally and anatomically distinct processes from ‘expression’, which is defined as the enduring neural alterations arising from the initiation process that directly mediate the augmented behavioral response [112]. It is conceivable that, similar to the mesolimbic DA system, the site of the dyskinesia ‘initiation’ process within nigrostriatal DA system might be the locus of the DA cells, specifically the SN pars compacta. If these systems are similar, it is further conceivable that neuronal events associated with ‘expression’ of dyskinesia may be distributed among the interconnected nuclei that are part of, or in communication with the basal ganglia motor system including globus pallidus (internal or external segments), subthalamic nuclei, prefrontal cortex, or supplementary motor cortex. Thus, to comprehensively impact our understanding of dyskinetic DA sensitization behaviors in PD, it will likely be necessary to examine alterations not only in the striatum, but also outside of this location.

Along these same lines, additional neurotransmitter systems such as cholinergic and noradrenergic systems have not been considered as factors contributing to GID. For example, in addition to 5HT neurons expressing vGLUT3, striatal cholinergic interneurons have also recently been shown to mediate fast vGLUT3-dependent glutamate transmission via NMDA-type receptor activation [113]. Despite representing a small percentage of total neurons in the striatum (1–3%), cholinergic interneurons have been shown to significantly influence the level of cocaine-induced sensitization [114]. This suggests another level of complexity of co-transmitter release regulating the excitability of striatal MSN activity. Whether cholinergic-vGLUT3 system influences striatal circuits causing the sensitization phenomena of dyskinesias, in general, and GID, specifically, is unclear.

Finally, we propose that one important aspect of GID research is the lack of a cohesive account of how the various phenomenological events interact to produce abnormal signaling and GID behaviors. As with drug-induced, non-graft associated dyskinesias (e.g.: typical LID) there is a large variety of phenomena that are documented to be associated with LID (reviewed in [115]). However, there remains no general consensus of which mechanism is “the mechanism” that “causes” expression of LID. This statement is supported by the fact that no effective clinical treatment is currently available to prevent or significantly abate

LID expression in PD patients. Thus, ultimately it will be important as investigations worldwide proceed to consider that GID (and probably LID) are centered in a collection of interconnected biochemical, molecular and structural changes that occur throughout the basal ganglia.

Summary

While there is valid disappointment that DA neuron grafting in PD is not yet successful, ‘surprise’ at the fact that it has not worked is perhaps less valid. Perhaps it would have been more surprising if functional restoration had been found following engraftment of new embryonic cells into the adult, aged brain; one lacking molecular signals for re-establishment appropriate synaptic connections and plagued with neurochemical and neuroanatomical remodeling that are hallmarks of PD pathology. We suggest that it is instructive to consider that we have largely uncovered conditions in which grafting of embryonic DA neurons do not work. There are, of course, exceptions to the idea that grafting does not work in PD. Thus, as the field moves forward, the focus of basic and clinical researchers will be to figure out under what circumstances the parkinsonian brain can be successfully remodeled with grafts of new cells.

In closing, we also would like to provide the perspective that transplantation of DA-producing cells into the brain to treat PD is still in a period of relative infancy. Indeed, the path to clinical success for grafting in PD is not unlike the path taken during the development of other organ transplant protocols. As one example, the first functional kidney transplant in a dog took place in 1902, with active research and lack of clinical use for 52 years, from 1902 thru 1954. It was not until 1954 at Brigham Hospital in Boston, that Dr. Joseph Murray performed the first successful human kidney transplant when a living donor gave a kidney to his identical twin ([116]; December 2011, US Department of Health and Human Services: <http://www.organdonor.gov>). While this was considered the first successful human kidney transplant, it was not until 1976 that real success was gained by the introduction of the immunosuppressant cyclosporine. Further modifications have continued to improve efficacy and survival over the past 30+ years. For DA neuron transplantation in PD, the first functional grafts in parkinsonian rats were reported in 1979 [117]. The first report of DA neuron grafting into patients with PD was in 1989 [118]. In the 22 years since the first DA neuron graft in a patient was reported, the field has made astonishing progress in understanding what works and what does not. Given the progress and promise of grafting for PD, it would be tragic to consider that we may be giving up too soon.

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Figure 1. Differential time course of expression of levodopa-induced dyskinesias (LIDs) versus graft-induced dyskinesias (GID)

The arrow demarks the time of graft surgery. As the graft matures, the incidence of LID lessens and there is an emergence of GID behaviors, e.g.: [9,10,17,20,21].

Figure 2. Modeling Graft-induced Dyskinesia: Does Graft Size Matter?

This diagram is used to consider whether it is possible that the unique post-graft dyskinetic behaviors noted in either parkinsonian rats or humans are differentially responsive to dopamine replacement therapy by virtue of graft size. In considering this, it is noteworthy that in the Denver/Columbia clinical trial after transplantation, the increase in overall putamenal PET 18F-DOPA signal in the GID-expressing patient group was twice that of the GID-negative group at 12 months ($P < 0.03$) and almost three times larger at 24 months ($P < 0.005$) [27]. Despite the 18F-DOPA signal being 3 times higher in the GID expressing patients, it was still below the level of the normal striatum by about 20% [27]. It is interesting to speculate whether the GID-expressing patient group could be represented by Graft 'X' and the GID-negative group represented by Graft 'Y' (both Graft 'X' and 'Y' appear in the right hand panel of graph). If one compares the amount of tissue that has been grafted into the parkinsonian rat in models examining GID-like behavior, there is substantially less tissue proportionately grafted in the rodent models compared to most human trials. **Rat Model:** [20]: 1 ventral mesencephalon (VM) with postmortem TH+ cell number = 2,800; [21]: < 1 VM with postmortem TH+ cell number = 280 (small grafts) or 17,408 (large grafts). **Human Grafting:** [9]: noodles; postmortem TH+ cell number = 62,507; [10]: 1–4 VM per patient with 70,000–120,000 TH+ cells per side; [12,13]: 6 VM per patient; postmortem TH+ cell number = 210,067; [17]: 6.3 ± 2.8 VM per patient, TH+ number not available. Thus, is it possible that the smaller number of grafted DA neurons in the rat brain requires a DA agonist to “push” the levels of DA into the biphasic dyskinesia range to elicit expression of GID? The case presented here is undoubtedly an oversimplification in that it does not account for potential differences in DA neuron biology between rodents and humans, the degree of DA neuron survival or neurite outgrowth, and the relative striatal volume influenced by grafts in the two situations. If one just looks at the number of DA neurons grafted per volume of the striatum between rat and human, such calculations remain equivocal in answering this question. For example, the human putamen (common clinical grafting site) can be estimated to be approximately 100× larger than the rat striatum [169–171, 174–175], and there are approximately 10× more DA neurons in the human SN compared to the rat SN [166–168, 172–173]. Based on such estimates grafting 1 ventral mesencephalon into a rat striatum would result in approximately 10,000 DA cells / 0.06 cm^3 or 166,666 DA cells/ cm^3 , which was the paradigm done by [20, 21] and one in which LD or amphetamine is apparently necessary to “push” striatal DA to levels that induce reliable experimental GID. In contrast, grafting 6 VM into the human striatum/

putamen as was done by [17] would result in approximately 600,000 DA cells/ 6.25 cm³ or 96,000 DA cells/cm³, a scenario where clinical GID are expressed “OFF” LD. In these two example comparisons the density of DA neurons/cm³ in the human striatum/putamen would be approximately 60% of that found in the rat striatum (assuming equal degree of DA neuron survival). However, this comparison would be accurate only *if* the grafted cells spread uniformly through the entire “striatum”. It is well documented that grafted DA neurons remain largely confined within and immediately surrounding the injection site [e.g.; 20,21,10,13], with the bolus of grafted neurons giving rise to neurites that extend into the target striatum, varying in density and distance. Thus, factors including neurite outgrowth and degree of regional DA replacement need to be considered when pondering the question above, which is suggested by the behavioral and pharmacological differences associated with the model and the patient. *Abbreviations:* 1) “ON” and “OFF” refer to the relationship with the intake of levodopa medication, i.e.; on-medication and off-medication. 2) The Y-axis label “Plasma LD/Striatal DA” refers to the plasma concentration of levodopa (LD) and the likely corresponding striatal concentration of DA, with lowest levels represented at the X–Y axis intercept, and higher levels at the top of the Y-axis. 3) +LD or +LD/amphet refers to administration of the drug LD or amphetamine to grafted subjects.

Figure 3. Schematic Depiction of the Connectivity in the (a) Healthy, (b) Parkinsonian and (c) Dopamine-grafted Parkinsonian Striatum

These diagrams are oversimplifications of particular aspects of striatal circuitry that may be relevant to understanding GID behaviors. Studies on which these diagrams are based are detailed in the body of this review. Numbers in parenthesis ‘()’ in the legend refer to numbers in the figures; numbers in brackets ‘[]’ refer to citations. **(a) In the healthy striatum**, descending glutamate fibers (purple) from the cerebral cortex and thalamus make asymmetric contacts (2) preferentially onto the head of numerous dendritic spines (1) of the resident MSNs. This input is modulated by ascending DA fibers (blue) from the substantia nigra pars compacta (3) that make symmetric *en passant* contacts preferentially onto the necks of those same spines. Additionally there are a small number of 5HT fibers from the raphe nucleus (not depicted) that project to the striatum, although these typically do not form synapses with the MSNs. **(b) In the parkinsonian striatum, following severe dopamine depletion**, there is a significant loss of dendritic spines (1) of the MSNs of the indirect pathway in rodents [56,57], and direct and indirect pathways in non-human primates [58]. With the loss of these critical input sites both glutamatergic (purple) (2) and remaining DAergic (blue) (3) fibers show an increase in the number of atypical contacts onto dendrites rather than onto their normal spinous targets [19]. Additionally, there is an increase in the number of ectopic serotonergic fibers (orange) (4) [e.g.: 34]; and in parkinsonian rats treated with dyskinogenic levodopa there is a larger proportion of these making actual synaptic contacts onto the MSN (not depicted) [e.g.: 34]. Finally, there is an increase in inflammation in the parkinsonian striatum depicted here with the presence of activated microglia (yellow) (5). **(c) In the dopamine-grafted parkinsonian striatum**, despite some restoration of DAergic tone, current data suggests that there remains a significant loss of dendritic spines (1) on MSNs. In addition to making atypical contacts onto dendrites, glutamate (purple) (2) and DA (blue) (3) fibers show additional changes in their specialization and symmetry, with glutamate fibers making more perforated contacts (2) [19], which is generally associated with synaptic plasticity, and DA fibers making more asymmetrical contacts (3), which are atypical for DA fibers, and is classically associated with excitatory synapses. There is also presence of excitatory synapses onto the grafted cells themselves (6) [19]. There continues to be an increase in the presence of 5HT fibers (orange), potentially from both the raphe nucleus as well as from serotonergic cells within the grafts themselves (4). Since all grafted PD patients to date have undergone long-term levodopa treatment, there should be a marked increased incidence of synapses (darkened orange lines) between these ectopic 5HT fibers and MSNs [34]. Additionally, pre-synaptic vesicles in 5HT terminal boutons co-express vGLUT3 protein (7) [40–44], indicative of co-transmission of glutamate at these monoaminergic synapses. Similarly, following striatal DA depletion in the rat, there is re-expression of vGLUT2 protein (7) [68]; however, whether this occurs in *grafted* DA neurons is unknown. Research evidence suggests that much of this atypical connectivity may be modulated by the increased inflammatory response observed in the grafted striatum, depicted here to be influenced by activated microglia (yellow) (5). ‘Perforated synapses’ are

depicted with the dashed, thickened post-synaptic arcs opposite the glutamate terminals; 'Asymmetric synapses' are depicted with thickened post-synaptic arcs opposite glutamate, DA and 5HT terminals. (7) While VMAT2 and vGLUT proteins likely occur in the same synaptic vesicles of monoaminergic neurons the occurrence of glutamate as a minor vesicular transmitter in the DA and 5HT terminals is depicted in this schematic as a single dark glutamate vesicle, indicating its relative minor presence.

In the clinics, GID occur when there is: 1) evidence of graft survival, and 2) following the withdrawal, or in the absence of immunosuppression [9,10,17], giving rise to a *hypothesis* that *DA neurons are grafted into an environment of excess immune activation which contributes to GID.*

Rationale/Mechanisms

- Brain immune cells, specifically microglia, play an active, constant role in the creation and elimination of synapses [87].
- Models of inflammation have shown elevated expression of genes involved in synaptogenesis [119] and microglial activation associated with synaptic stripping [120].
- Synaptic remodeling in the striatum is a key feature underlying the development and persistence of neuroleptic-induced dyskinesias [111], indicating a direct link between structural changes at the synaptic level and dyskinetic syndromes
- Inflammatory cytokines have been linked to increased induction of activation associated genes, including components of the activator protein-1 (AP-1) family of transcription factors [121]; within this family of transcription factors, FosB/ΔFosB is upregulated in animal models of dyskinesias [20,122].

Testing the Hypothesis: Preclinical Models

Rat Model A. [19]

- Allografting; Host: Sprague Dawley rats; Donor: Lewis rats
- Focal, novel (not seen prior to grafting) post-graft experimental GID induced by levodopa appear in all DA grafted rats
- Subcutaneous inoculation with Lewis rat spleen cell induce a moderate level of immune activation throughout the striatum and a *high level of immune activation* in region of graft; DA graft survival was maintained in the presence of immune activation; *experimental GID were elevated* in rats with escalating immune activation
- Ultrastructural evidence shows aberrant synapses correlate with experimental GID and elevated immune activation

Rat Model B. [85]

- Allografting; Host: Sprague Dawley rats; Donor: Wistar rats
- Post-graft experimental GID induced by amphetamine appear in all DA grafted rats
- Experimental GID disappear when DA graft is rejected following skin graft
- 10 day intrastriatal minipump delivery of interleukin-2 (IL-2) or saline results in *mild level of immune activation* in region of graft; *experimental GID were unchanged* from pre-minipump levels and did not differ between the two groups

Agreement?

In allografted rats, post-graft dyskinesias (PGD) induced by pharmacological agents that elevate local DA (i.e.: levodopa or amphetamine) develop when there is mild immune activation; PGD increase with elevated immune activation; and they are dependent on the presence of DA grafted cells.

Figure 4.

Immune Activation and Graft-induced Dyskinesias.

Footnote; Model A: Experimental GID occurred only in dopamine-grafted rats, and not in cell-free, sham-grafted rats, despite the presence of equal numbers of MHC class II+ cells in sham and G21 rats, suggesting that the impact of host immune factors is more likely to be related to graft–host synaptic organization than to a direct action on host signaling pathways.

Table 1
Serotonin and Graft-induced Dyskinesias (GID)

Striatal 5HT fibers, derived from brainstem host projections and/or donor graft tissue, have been postulated to play a role in GID. Evidence supporting or negating this hypothesis is presented in this table. While there is a great deal of data to suggest that following levodopa administration there is non-regulated DA overflow produced from the striatal serotonergic hyperinnervation within the parkinsonian striatum that causes typical **LID** behavior in non-grafted subjects, data for the role for 5HT in **GID** is less certain. However, if we are to accept that 5HT neurons are a causative factor in off-time GID in patients, the question that needs to be considered is: “When the patient is off levodopa, what is the source of excess DA production from serotonin neurons?” Abbreviations: positron emission tomography, PET; single photon emission computed tomography, SPECT; 11C-labeled 3-amino-4-(2-dimethylaminomethylphenyl)sulfanyl benzonitrile, 11C-DASB; ventral mesencephalon, VM.

Evidence Supporting a Role for 5HT neurons	Citation	Evidence Against a Role for 5HT neurons	Citation
PET and SPECT evidence shows elevated 5HT/DA transporter ratio in one PD patient that developed GID; the 5-HT _{1A} agonist/DA antagonist molecule, given at small, repeated doses suppressed this behavior	[90]	In a rat model, 5-HT cells within a graft were shown to be neither detrimental nor beneficial for functional effects of DA-rich transplants; however, in absence of sufficient numbers of DA neurons, transplanted 5-HT neurons were able to induce dyskinetic behaviors following levodopa (i.e.: LIDs)	[123]
11C-DASB PET imaging of serotonergic innervation shows evidence of excessive serotonergic innervation in the grafted striatum of two patients that exhibited major motor recovery and later developed GID; the 5-HT _{1A} agonist/DA antagonist molecule, given at small, repeated doses suppressed this behavior	[91]	Evidence demonstrates that in each of 5 PD patients grafted with human fetal VM tissue there were abundant serotonin neurons but that none of them were observed to have GID	[124,125]
		In a rat model, transplant-induced AIMs noted following amphetamine administration were seen to primarily depend on a pharmacological activation of dopamine receptors; and serotonin neurons within either the grafts or the host brain played a negligible role	[24]

Table 2
Target Pathology and Graft Dysfunction

It is not an unexpected finding that grafting new DA neurons into a target wrought with pathology will result in suboptimal therapeutic outcome. Examples of how specific pathologies associated with PD have been shown to influence DA graft efficacy are outlined in this table.

Evidence	Citation	Potential Mechanism
Preventing loss of dendritic spines on striatal medium spiny neurons (MSNs) moderates GID in parkinsonian rats	[25]	Striatal DA depletion results in loss of dendritic spines on striatal MSNs* (e.g.: [5356]), which are normal input sites for nigral DA afferents and glutamatergic inputs originating in the cerebral cortex and thalamus. In parkinsonian striatum, grafted nigral DA neurons make atypical contacts on dendritic shafts and the loss of typical spinous contacts correlates with abnormal dyskinetic behaviors post-grafting [19]
Preventing loss of dendritic spines on striatal medium spiny neurons (MSNs) enhances reversal of motor dysfunction in parkinsonian rats	[25]	Per the above described pathology*, enhancing normal input sites is predicted to enable the formation of a typical pattern of synaptic connectivity giving rise to enhanced behavioral output following DA terminal replacement
Long-term levodopa in parkinsonian rats decreases the ability of grafted DA neurons to reverse motor deficits	[126]	Chronic levodopa results in a vast array of neurochemical and electrophysiological alterations within the parkinsonian striatum† e.g.: [29,35,133,134]
Long-term levodopa increases amphetamine-induced post-graft dyskinesias in grafted parkinsonian rats	[127]	Per the above described pathology†
PD patients with the best functional outcome after DA neuron grafting exhibited significantly less DA denervation in areas outside the grafted areas preoperatively compared to patients that showed lesser functional recovery	[17,51]	Less severe DA depletion coincides with less severe striatal pathology ††; e.g.: [53, 128]
Survival, axonal outgrowth and functional benefit of grafted DA neurons are critically dependent on the severity of damage to the host DA system	[52,129]	Per the above described pathology††; also, sparing pathology of non-striatal forebrain areas innervated by midbrain DA systems may provide additional benefit to striatal grafting paradigms
Advanced age decreases efficacy of grafted DA neurons in patients with PD and parkinsonian rats	[9,130]	Advancing age is a definitive risk factor for pathology within the striatum; e.g.: [131,132]

Table 3
The Pharmacology of LID and GID

In this table, what is known about the pharmacology of LID (i.e.: the widespread typical dyskinesias noted in the absence of DA cell grafting) is contrasted with what is known about the pharmacology of GID (i.e. the largely focal dyskinesias that develop in the presence of a DA cell graft). + indicates that there is evidence that the neurotransmitter, receptor, or second messenger system plays a role in LID or GID; Nt = not tested and indicates that there is no evidence that the neurotransmitter, receptor, or second messenger system plays a role in LID or GID. This table contains examples of citations for each system listed; because it is beyond the scope of this article, it is not meant to serve as a comprehensive source.

Neurotransmitter/ Receptor	Levodopa Dyskinesia	Example Citations	Post-Graft Dyskinesia	Example Citations
D1R	+	[136–138]	+	[89]
D2R	+	[138,139]	+	[89–91]
D3R	+	[138,140–142]	Nt	-
Second messengers:	+	[144]	Nt	-
Ras	+	[143]	Nt	-
MEK 1/2	+	[144]	Nt	-
ERK1	+	[145]	Nt	-
mTOR				
Opioid Receptors	+	[146–148]	Nt	-
Endocannabinoids	+	[149–151]	Nt	-
5-HT	+	[135,152–155]	+	[89, 90,91,98]
Adenosine Receptors	+	[156, 157]	Nt	-
Nicotinic Receptors	+	[158–160]	Nt	-
Muscarinic Receptors	+	[161]	Nt	-
NMDA Receptors	+	[162,163]	Nt	-
AMPA Receptors	+	[162,164,165]	Nt	-