Transplantation of autologous neural cell ecosystems as therapy for Parkinson’s disease: a preclinical study

Simon Borgognon

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Master thesis supervised by Simon Badoud & Professor Eric M. Rouiller
In collaboration with Dr. Jean-François Brunet & Dr. Jocelyne Bloch

Unit of Neurophysiology, Department of Medicine, University of Fribourg, Switzerland
Abstract

Nowadays, one of the biggest challenges of neuroscientists is to repair the adult central nervous system. Parkinson’s disease (PD) is the second most common neurodegenerative disease after Alzheimer’s disease and is characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc). Since the 70s, many laboratories explore DA neurons replacement as a therapeutic strategy in animal model of PD based on stem cell transplantations. However, major limitations such as lack of fetal donor, ethical controversies, tumor formation and/or immune rejections encourage turning towards other approaches. A recent technique called autologous neural cell ecosystems (ANCE) transplantation has emerged and abolishes limitations of stem cell therapies. It consists to cultivate adult neural precursor cells taken from a cortical biopsy of the same subject (who is his/her own donor) to subsequently re-implant them into brain regions of interest.

The aim of the project was to assess ANCE transplantation in four non-human primate models of PD (MPTP model), namely macaque monkeys. Behavioral follow-up (assessment of fine manual dexterity as well as spontaneous motor activity) was performed during the entire experiment. Moreover, investigation with $^{18}$F-dopa Positrion Emission Tomography (PET) scans allowed an in-vivo quantification of the dopaminergic system state. Taken together, the results showed an overall improvement of both motor symptoms and $^{18}$F-dopa uptake after the ANCE transplantation as compared to post-lesion phase.

This project may contribute to a better understanding of PD. Furthermore, this promising technique might add new therapeutic strategies to further lead to clinical applications.
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I. Introduction

Context

Nowadays, one of the biggest challenges of neuroscientists is to repair the central nervous system (CNS) following an injury. Indeed, the CNS cannot self-regenerate in the adult. Consequently, lesions occurring in CNS are permanent and, depending of the lesioned site and extent, could be very invalidating and even fatal for patients. Researchers try to find many therapeutic strategies in order to recover the CNS. Many studies have investigated in animal model (rodents and primates) different approach in different fields. Promising techniques have emerged such as recovery after spinal cord injury, cortex lesion and even neurodegenerative diseases (Alzheimer’s disease as well as Parkinson’s disease). Sometimes, therapeutic strategies lead to clinical trials. This project tries to investigate the potential benefit effect of cellular therapy based on autologous cells implantations in non-human primate model of Parkinson’s disease. This project may extend knowledge about Parkinson’s disease and about regenerative process after a brain dysfunction. Furthermore, it could lead onto clinical applications for Parkinson’s disease (e.g. for review: Olson, 1997; Santos Benito & Ramon–Cueto, 2003; Courtine et al., 2007; Okano et al., 2007; Freund et al., 2009; Kaeser et al., 2011; Lu et al., 2014).

Motor system generalities

In 19th century, Fritsch and Hitzig proposed the concept of motor cortex by electrically stimulating dog’s cerebral cortex and observing muscle contractions. Following this work, Hughlings Jackson practiced autopsies in syphilitic epileptic patients, in whom symptoms are limited to one side of the body. Then, he suggested an organization of motor cortex following a somatotopic arrangement. Later, David Ferrier confirmed this suggestion by conducting experiments (electric stimulations) in non-human primates (Figure 1A) (Fritsch and Hitzig, 1870; Jackson, 1873; Ferrier, 1873; Bennett and Hacker; 2001). At the end of the 19th century and beginning of the 20th century, Charles Scott Sherrington claimed the role of the spinal cord in stepping and standing as well in the flexion-reflexes (protective reflex). He proposed a lot of principles, among them the “relation between the brain and the final common pathway”. Moreover, he was the first to give a detail somatotopic representation of the primate motor cortex (Figure 1B) (Grünbaum and Sherrington, 1902; Bennet and Hacker, 2001; Burke, 2006). With recent studies, these works have been validated. The motor cortex includes the primary motor cortex (M1 or F1 in monkey), corresponding to Brodmann area 4, plus the supplementary motor area (SMA) as well as the premotor area (PM), both corresponding to Brodmann area 6. Within the cingulate sulcus, a cingulate motor area (CMA) has been identified (Figure 1C). Their functions are variable. For example, SMA is involved in initiating voluntary movement, whereas the premotor area is responsible for planning a movement. These motor cortices (especially M1) code the final motor (movement) information and sent it (directly in primates) to motoneurons through the

1 “The final common pathway” referred to motoneurons by which nerve impulses from central nervous system reach a muscle at the periphery.
corticospinal tract (CST) (**Figure 1D**). The direct CST projection to motoneurons in primates (corresponding to the cortico-motoneuronal system = CM) confers them a fine manual dexterity (precision grip: opposition thumb and index). These three motor cortical areas are interconnected and cooperate in order to generate an appropriate movement. Because of their reciprocal connections with many other brain structures (e.g. parietal and prefrontal areas as well as cerebellum and basal ganglia), the motor cortical areas are not sole responsible to create appropriate movements (e.g. Lawrence and Kuypers, 1968; Brinkman, 1981; Georgopoulos et al., 1982; Wiesendanger, 1986; Rizzolatti et al., 1996; Rizzolatti et al., 1998; Rizzolatti and Luppino, 2001, Dum et al., 2002; Lemon, 2008; Mendoza and Merchant, 2014).

**Figure 1**: (A) Drawing of Ferrier showing macaque brain regions, in which electrical stimulation leads to movements (Ferrier, 1886). (B) Brain of a chimpanzee with the somatotopic organization (Gruenbaum & Sherrington, 1902). (C) Motor areas in the frontal lobes of a macaque monkey. Dashed lines represent location of corticospinal neurons. ArS = arcuate sulcus; CC = corpus callosum; CgS = cingulate sulcus; CMAr = rostral cingulate motor area; CMAv = ventral cingulate motor area; CMAd = dorsal cingulate motor area; CS = central sulcus; IPS = intraparietal sulcus; M1 = primary motor cortex; PMd = dorsal premotor area; PMv = ventral premotor area; PS = principal sulcus; SMA = supplementary motor area (Dum & Strick, 2002). (D) Corticospinal tract from motor areas to motoneurons. The lateral corticospinal tract crosses the midline (decussation pyramidal), whereas the ventral corticospinal tract does not. Emergence of cranial nerves (III to XII) is shown in grey and do not belong to corticospinal tract (http://www2.fiu.edu/~condon/pathway.htm).
Basal ganglia

Generalities

As mentioned above, basal ganglia (BG) receive projections from the cerebral cortex, including motor cortical areas. This suggests a role in motricity. Actually, virtually all regions of the cerebral cortex, some thalamic nuclei and specific mesencephalic structures send projections to BG, then BG project back (in the form of a loop) to the cerebral cortex via the thalamus. Consequently, BG process highly associated cortical information leading to their contribution in movement production (planning and selecting) and in behavior. There are two output projection components from BG: (1) the «ascending» component, which consists of the basal ganglia-thalamo-cortical circuits and, (2) the «descending» component, which projects directly to mesencephalon, which in turn sends projection to motor structures (brainstem and spinal cord). The «ascending» component is the neural basis of their influence on motor, cognitive and executive behavior. Whereas, the «descending» component reflects the neural basis for their influence on posture and balance as well as on muscle tone. Mechanisms underlying their functions are through a selection of adequate and inadequate responses. Inadequate selection of motor and/or cognitive responses might underlie unappropriated connectivity or disturbance occurring in BG (as seen in Parkinson’s disease, Huntington’s disease, obsessive-compulsive disorder, addiction, mood disorder as well as schizophrenia) (e.g. for review: Nauta & Domesick, 1984; Rouiller et al., 1994; Parent & Hazrati, 1995; Haber, 2003; Wolters & Baumann, 2014).

Anatomy

Anatomically, the BG are composed by several nuclei located in the forebrain and the midbrain. Four main components encompass BG: (1) striatum (subdivided in three structure: (i) the putamen (Put), (ii) the caudate nucleus (CN) and, (iii) the nucleus accumbens (Acb)); (2) substantia nigra (subdivided in two parts: (i) the substantia nigra pars compacta (SNc) and, (ii) the substantia nigra pars reticulata (SNr)); (3) subthalamic nucleus (STN) and, (4) globus pallidus (including: (i) the external segment of the globus pallidus (GPe) and, (ii) the internal segment of the globus pallidus (GPi)) (Figure 2). All these BG structures are strongly interconnected (intrinsic connections). Consequently, the output and the input connections can be composed by many BG nuclei (eg. for review: Haber, 2003; Lanciego & Vázquez, 2011; Wolters & Baumann, 2014).
Figure 2: Sagittal histological slice (acetylcholinesterase staining) of a macaque monkey. Dashed line represents the thalamus (Th), which does not belong to basal ganglia but receive input from them. Acb = nucleus accumbens; CN = caudate nucleus; GPe = external segment of globus pallidus; GPi = internal segment of globus pallidus; Put = putamen; Th = thalamus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus (adapted from Lanciego & Vázquez, 2011).

Topography of projections and loops

The striatum receives inputs from the cerebral cortex (corticostriatal projection), thalamus and SNC. All cerebral cortex (except for the primary visual and auditory cortices) project topographically to the striatum (Figure 3A). In contrast of prefrontal and parietal cortices, only the sensorimotor cortex sends projection bilaterally to the striatum through the corpus callosum. The corticostriatal projection follows a dorsolateral to ventromedial topography gradient. Indeed, the dorsolateral part of the striatum receives inputs from motor, PM and somatosensory cortices. Moreover, associative areas (prefrontal cortices) project to the ventromedial part of the striatum. Finally, the limbic system reaches the striatal ventral parts (mainly the nucleus accumbens). Thus, the striatum can be functionally divided in three parts: (1) the sensorimotor circuit, (2) the associative (cognitive) circuit and (3) the limbic (emotional) circuit (Figure 3B). Following the same topography, the lateral SNC sends dopamine neurons to the sensorimotor part; the mediolateral SNC to the associative (cognitive) part and, finally, the limbic (emotional) part receives dopaminergic fibers from the medial part of SNC (and from the ventral tegmental area (VTA)) (e.g. for review: Carman et al., 1965; Kemp & Powell, 1970; Künzle, 1975; Nauta & Domesick, 1984; Parent & Hazrati, 1995; Haber et al., 2000; Haber, 2003; Leimer, 2003; Obeso et al., 2014; Wolters & Baumann, 2014).
Figure 3: (A) Topography projections from the cerebral cortex to basal ganglia. The gradual color change show overlapping projections (Heimer, 2003). (B) Corticostriatal projection segregations following the posterior, anterior and ventral striatum leading to three main loops: (1) sensorimotor circuit, (2) associative circuit and (3) limbic circuit. CN = caudate nucleus; GPe = external segment of globus pallidus; GPI = internal segment of globus pallidus; Put = putamen; Th = thalamus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus (adapted from Obeso et al., 2014).
**Direct and indirect pathways**

The efferent striatal system (to pallidum and SNr) is based on two levels of organization: (1) as for the afferent system, the efferent system is organized following the three topographies and, (2) the gamma-aminobutyric acid (GABA)ergic neurons subtypes, which form the entire striatal projection neurons. There are two subtypes of GABAergic neurons, those expressing D1 receptor subtype. These neurons project to the GPi and SNr. These two regions close the loop via inhibiting inputs to the thalamus. This pathway is called the direct striatal output pathway. The second type of GABAergic neurons express D2 receptor subtype and constitute the indirect striatal output pathway. First, the projection reaches the GPe, then the STN to finally project excitatory fibers to GPi and SNr. As the direct pathway, the loop closes via the thalamus. The anatomical distinction between the direct and indirect pathways has functional consequences. Indeed, the activation of the direct pathway lead to a facilitation of the movement (because GPi and SNr sent inhibition projections to thalamus). On the contrary, the activation of the indirect pathway has to opposite effect (movement suppression) by inhibiting the projection from GPe to STN, subsequently, STN can send exciting projection to GPi and SNr, which inhibits the thalamus projection to the cerebral cortex (Figure 4). These two systems are well orchestrated in order to have a good balance between them (e.g. for review: Anden et al., 1964; Albin et al., 1989; Nauta & Domesick, 1984; DeLong, 1990; Parent & Hazrati, 1995; Haber et al., 2000; Haber, 2003; DeLong & Wichmann, 2007; Braak & Tredici, 2008; Gerfen & Surmeier, 2011; Wichmann et al., 2011; Wolters & Baumann, 2014).
**Figure 4**: Motor circuit anatomy within basal ganglia. Red arrows represent inhibitory projections (GABAergic, except for SNc to putamen (dopamine) projection). Green arrows represent excitatory projections (glutamatergic, except for SNc to putamen (dopamine) projection). CM = centromedian nucleus of thalamus; CMAr = rostral cingulate motor area; CMAd = dorsal cingulate motor area; CMAv = ventral cingulate motor area; GPe = external segment of the globus pallidus; GPi = internal segment of the globus pallidus; M1 = primary motor cortex; Pf = parafascicular nucleus of the thalamus; PMd = dorsal premotor cortex; PMv = ventral premotor cortex; PPN = pedunculopontine nucleus; SMA = supplementary motor area; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus; VApc = ventral anterior nucleus of thalamus pars parvocellularis; VLm = ventrolateral nucleus of thalamus pars medialis; VLo = ventrolateral nucleus of thalamus pars oralis; VLc = ventrolateral nucleus of thalamus rostral pars caudalis; c = caudal; cl = caudolateral; and d = dorsal (DeLong & Wichmann, 2007).

**Disorders of Basal ganglia**

As mentioned above, a dysregulation of this well organized BG system can lead to many disorders. Here, several movement disorders will be considered. In a case of a hypokinetic disorder (as in Parkinson’s disease), the direct pathway becomes, by a lack of excitatory D1-receptors cells, less active leading to less striatal GPe/SNr inhibition. Moreover, in the indirect pathway, the loss of D2-receptor cells hyperinhibit the GPe, leading to a hyperexcitability of the efferent STN fibers to the GPi/SNR. Both mechanisms result on a higher activity of the GPi/SNR neurons outputs to thalamus (stronger inhibition of the thalamocortical neurons) (Figure 5B). Contrary to this pathophysiological system, a hyperkinetic disorder (as in Huntington’s disease and hemiballism) may be explained by a decreased activity of the GPi/SNR neurons outputs to thalamus. In Huntington’s disease (HD), the striatal neurons, which express D2-receptors, degenerate. This leads to an overinhibition of the GPe outputs to STN, giving rise to a decrease excitatory outputs to thalamocortical neurons (Figure 5D). In case of hemiballism, a large part of STN degenerates with the same consequence on the thalamocortical fibers as HD (Figure 5C). Hemiballism is characterized by unwanted movements in the contralateral part regarding the STN lesion whereas in HD, both sides of the body are affected by unwanted movements (e.g. for review: Nauta & Domesick, 1984; Bergman et al., 1990; DeLong, 1990; Parent & Hazrati, 1995; Richfield & Zeiss, 1995; Haber, 2003; DeLong & Wichmann, 2007; Braak & Tredici, 2008; Gerfen & Surmeier, 2011; Wichmann et al., 2011; Wolters & Baumann, 2014).
Figure 5: Simplified motor circuit derived from Figure 4. (A) Normal circuitry in where balance between direct and indirect pathway is maintained. (B) In hypokinesia disorder, the indirect pathway is more solicited than the direct pathway. (C) and (D) In hyperkinesia disorders, the direct pathway is more solicited than the indirect pathway. Attenuated grey represents a lesion. CN = caudate nucleus; GPe = external segment of globus pallidus; GPi = internal segment of globus pallidus; Put = putamen; Th = thalamus; SNC = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus.
Parkinson’s disease

Generalities

In 1817, James Parkinson described the clinical features of the second most common neurodegenerative disease in his book “An Essay on the Shaking Palsy". Later, Jean-Martin Charcot (French neurologist) paid tribute to James Parkinson by giving its name to the disease. After more than 100 years, Parkinson’s disease (PD) is described to be a neurodegenerative disease by the loss of neurons in the substantia nigra pars compacta (SNc). In 1957, Carlsson and colleagues showed that dopamine (DA) is a neurotransmitter (Nobel Prize in 2000). Three years later, Hornykiewicz discovered that DA concentration is dramatically decreased in PD patients suggesting the primordial role of DA in motricity. Actually, the SNc, located in the mesencephalon, contains a dense population of neurons producing the DA neurotransmitter. By the time, the SNc becomes pigmented because of an accumulation of neuromelanin (metabolite of DA). The manifestation of SNc cell loss is characterized by a hypokinetic motor impairment, although motor symptoms are often accompanied with non-motor symptoms (cognitive, cardiovascular, urogenital and gastro-intestinal dysfunction as well as sleep-wake disorders, …). The present report focus on motor symptoms (e.g. for review: Parkinson, 1817; Gowers, 1886; Dickson, 1999; Parkinson, 2002; Dauer & Przedborski, 2003; Björklund & Dunnett, 2007; Kempster et al., 2007; Jankovic, 2008; Wolters & Baumann, 2014).

Epidemiology

PD is the second most common neurodegenerative disease after Alzheimer’s disease (AD). PD is a worldwide disease affecting 0.3 % of the population with an increasing rate of 1 % up to 5 % over age 65 to 85, respectively. PD prevalence is higher in men than in women with an average of onset age at 60 years old. PD is often considered as an elderly disease but it is possible to develop PD under 40 years old (uncommon and often originating from genetic parkinsonism). Most of the PD cases are idiopathic (about 10% of genetic origin) and have an average duration of 15 years. The economic cost of PD (direct and indirect burdens) is estimated at 14 billion dollars in United States of America in 2010 (Kowal et al., 2013; Johnson et al., 2013, Wolters & Baumann, 2014; Rodriguez et al., 2015).

Neurodegenerative diseases

Neurodegenerative disorders (e.g. PD, AD, HD, multiple system atrophy (MSA), …) are characterized by an abnormal accumulation of proteins, which form aggregates in specific regions of the central nervous system as well as in the peripheral nervous system. The two famous known proteins are tau (and amyloid-beta) and alpha-synuclein in AD and PD, respectively. Post-mortem analysis of patients suffering of neurodegenerative disease shows an atrophy of the brain (expect for PD patients). This reflects well the high-toxicity of theses aggregations. However, the exact aetiology of PD remains elusive. Two main hypothesis are described: (1) an environmental cause (pesticide containing neurotoxin (eg.: rotenone); and (2) presence of endogenous toxin (such as reactive oxygen species).

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2 He describes the disease as “shaking palsy” or “Paralysis Agitans” : “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being un- injured.” (Parkinson J., 1817).
Both hypotheses are consistent with the neuropathogenesis and pathophysiology of PD (e.g. for review: Dickson, 1999; Dauer & Przedborski, 2003; Wolters & Baumann, 2014).

**Neuropathogenesis and pathophysiology**

Two pathologies occur in PD: (1) the degeneration of the midbrain DA neurons in SNc\(^3\); and (2) the alpha-synuclein protein aggregation (alpha-synucleinopathy) forming cytoplasmic inclusions called Lewy Body (LB) and neuritic deposition (Lewy neurites (LN)) ([**Figure 6**](#)). Both together are hallmarks for PD. Indeed LB pathology is not specific to PD; LB are also found in dementia with Lewy Body (DLB).

![Substantia nigra pars compacta in a normal human subject](#) ![Dopamine neurons with two alpha-synuclein-positive Lewy bodies](#)

**Figure 6**: (A) Substantia nigra pars compacta in a normal human subject (C), in whom the neuromelanin of dopamine neurons is visible. (P) Parkinson’s disease patient in whom, a loss of dopamine neurons into the substantia nigra pars compacta appears (Mackenzie, 2001). (B) Dopamine neurons with two alpha-synuclein-positive Lewy bodies. Scale = 8 µm (Spillantini et al., 1997).

The SNc cells loss appears to be more pronounced in the dorsolateral part of the putamen, the main projection of SNc. Interestingly, the mesolimbic system (projection from VTA) is much less affected in PD. Consequently, the caudate nucleus (main VTA projection) has much less DA depletion. In order to explain the pathogenesis of SNc degeneration, three clues from animal-model studies emerged: (1) the SNc cell death topography (ventrolateral and caudal in PD patients are affected, whereas the dorsomedial part is affected in normal aging), (2) the proportion of striatal terminal loss seems higher than nigral cell bodies loss. This could be a result of a “dying back” process suggesting that the terminal is the first target by cellular death, and (3) DA transporters (DAT) mainly ensure the striatal DA uptake, whereas in prefrontal cortex (one of the VTA projection) this is not the case (this could help to the understanding of the DA VTA neurons resistance). However, the exact cause of SNc cell death remains unclear. Since many years, a lot of hypotheses have been proposed. The oxidative stress has been shown in PD patients with a reduction of glutathione (major brain antioxidant) and an increase of

\(^{3}\) It is important to note that non-DA neurons also degenerate in PD such as cholinergic nucleus basalis of Meynert, norepinephrine neurons of the locus ceruleus, serotonin neurons of the raphe nucleus,... However, these neurons are affected later in the disease.
iron accumulation. The role of inflammation has been quoted with an increase of cytokine expression (because microglia is activated) and inflammatory-associated factors present in PD patients. Glutamate-mediated excitotoxicity as well as apoptosis process (evidence of cytochrome C) belong to other hypotheses. To finish, a mitochondria dysfunction (inhibition of complex I of respiratory chain) could explain cells loss. Unfortunately, all these hypotheses failed to resolve the primary cause because either they are not present in all PD patients and/or clinical treatments (such as inhibitor of glial activation, antagonist of NMDA receptor, anti-apoptotic agents and antioxidant) failed to significantly improve symptoms in PD patients.

Nevertheless, the proteolytic stress can elucidate the PD aetiology, at least for genetic parkinsonism. In normal cells, a misfolded and/or unwanted protein is ubiquitinated and degraded by the proteasome; amino acids can be further recycled. When too many misfolded proteins are present, the cellular mechanism is stressed (proteolytic stress). Consequently proteasome cannot degrade all of the proteins and could results by an abnormal accumulation of protein and aggregation formation. The aggregation may interfere with intracellular trafficking in neurons and cause cell death. However, protein that could be important for cell survival can be sequestered in protein inclusion. Alpha-synuclein, coded by the SNCA gene, is highly expressed in pre-synaptic terminal where it interacts with synaptic vesicle and cellular membrane. In PD, alpha-synuclein may be involved in the core proteolytic stress process (Figure 7).

![Figure 7: Suggested mechanisms of neurodegeneration. Accumulation of misfolded proteins is probably a key in neurodegeneration process. In genetic parkinsonism, genetic mutation occurs (Parkin, UCH-L1) leading to proteolytic stress. Dysfunction within mitochondria may lead to oxidative stress and formation of misfolded protein. It is unclear if Lewy bodies or misfolded proteins lead to neurodegeneration. ATP depletion may play also a role (Dauer & Przedborski, 2003).](image-url)
Alpha-synucleinopathy with Lewy pathology evolves by predictable stages (Braak stages). The stage 1 starts in the olfactory bulb and the dorsal motor vagus nucleus. The propagation continues (stages 2/4) through the brain stem and affects different regions (e.g. raphe nuclei). The stage 5/6 is defined by reaching the neocortex. The clinical manifestation appears during the stage 5/6 when the loss of nigrostriatal dopaminergic cells passes a threshold (Figure 8). It is argued that the first affected region belong to autonomic regions. In the stage 1/2 (beginning around 50 years old), olfactory and autonomic dysfunctions appear, followed by a REM sleep disorder and depression in stage 3 (no cell death yet). In the stage 4 (around 60 years old), cardinal motors symptoms appear (cf. below) (because of SNc cell death) and a diagnosis can be established. Stage 5 (cognitive decline) and stage 6 (dementia) are both at the end of the disease, 15 years after stage 4 (e.g. for review: Polymeropoulos, 1997; Spillantini et al., 1997; Clayton & George, 1998; Takahashi & Wakabayashi, 2001; Muchowski, 2002; Braak et al., 2003; Olanow, 2007; Büeler, 2009; Dickson et al., 2009; Schulz & Falkenburger, 2009; Doty, 2012; Hirsch et al., 2012; Sulzer & Surmeier, 2013; Wolters & Baumann, 2014).

**Figure 8:** Braak stages showing the initiation of Parkinson's disease and the progression of alpha-synucleinopathy with Lewy pathology. During the stage 4, cardinal motor symptoms appear and a diagnosis can be established. Parkinson's disease continues leading to stage 6 during which dementia appears (Doty, 2012).
Motor manifestations, clinical diagnosis and treatments

Parkinson’s disease is diagnosed when the cardinal motor symptoms appear: bradykinesia (slowness of movement), postural instability, resting tremor and rigidity (Figure 9). Cardinal symptoms can be explained by the degradation of SNc (cf. Figure 5B) but not by Lewy pathologies, which remain stable over time in PD. The degradation of SNc begins approximately 5 years before the onset of cardinal symptoms, which occur when 50 to 60% of DA neurons of the SNc have been lost. Moreover, an asymmetry of symptoms and a robust response to levodopa treatment (see below) are considered to be indispensable to make a PD diagnosis and exclude PD-related disorders. An optional assessment to diagnose PD is neuroimaging. Indeed, it is possible to evaluate DA depletion with positron emission tomography (PET) (by “staining” DA neurons with a precursor of DA radio ligand like [18F] Fluorodopa) and single-photon emission tomography (SPECT) (by “staining” DAT with radio ligand such as [123I] FP-CIT) (Figure 10). The main advantage of neuroimaging is the possibility to diagnose early stage of PD before the cardinal motor symptoms onset.

Figure 9: The aspect of patient is very characteristic. The head and the trunk are bent forward. Arms are fixed indicating rigidity. Resting tremor is usually unilateral. Voluntary movements are performed slowly (bradykinesia). Gait is performed with little quick steps with postural instability (Gowers, 1886).

4 It is important to note that parkinsonism symptoms may be caused by: (1) diverse idiopathic neurodegenerative disease (such PD, MSA, progressive supranuclear palsy (PSP)...), (2) genetic disorders, (3) cerebrovascular incidents and (4) drugs and toxins. However, about 80% of the case is due to idiopathic PD.
Cardinal symptoms of PD can be attenuated using conventional oral pharmacologic treatment, levodopa. Levodopa can cross the blood-brain barrier (BBB) and then is transported to nigrostriatal terminations where it will be converted into DA. In order to prevent the conversion to DA in the periphery, an inhibitor to decarboxylase (Carbidopa) has to be taken. Levodopa is the most efficient motor treatment for cardinal symptoms and it improves quality of life. However, complications have been associated with levodopa treatment ("on-off" motor fluctuation and dyskinesia). The main problem is that "off" motor fluctuation can be reversed by increasing the dosage of levodopa. In contrary, to reduce dyskinesia, a decrease of levodopa dosage is necessary. Therefore, an optimization of medication (regarding each patient) and/or addition of other medication (e.g. inhibitors of diverse enzyme in DA synthesis pathway), which could lead to side effects, are imperative. Another symptomatic treatment is the deep brain stimulation (DBS) consisting of high-frequency stimulation (generated though an implantable generator subcutaneously) by implanted microelectrodes in specific nuclei in BG (commonly STN and GPI), in order to restore the balance between the indirect and direct pathways. In combination with medication, STN DBS can improve up to 50-60% the cardinal symptoms. However, STN DBS can lead to adverse effects (such as cognitive dysfunction, depression, even suicide) because of the smaller size of STN (less segregation of limbic, associative and motor loops). In addition, young patient (in early stage of PD) is more suitable to receive in DBS. These two classic treatments remain symptomatic and do not stop the propagation of PD (eg. for review: Maier, 1983; Fabbrini et al., 1987, Hughes et al., 1992; Uitti et al., 1993; Booij et al., 1999; Hughes, 2002; Rascol et al., 2002; Braak et al., 2003; Eshuis et al., 2008; Greffard et al., 2010, Bronstein et al., 2011; Merola et al., 2011; Stoessl et al., 2011; Wolters & Baumann, 2014).
Cell therapies for Parkinson’s disease

Since the 70s, many laboratories explore DA cells replacement as a therapeutic strategy in animal model of PD, based on stem cells transplantations. The major approach is to implant DA cells derived from: (1) human fetal brain (neural stem cells (NSCs)), (2) embryonic tissues (pluripotent embryonic stem cells (ESCs)), (3) fibroblast (induced-pluripotent stem cells (iPSCs)), and (4) bone marrow (mesenchymal stem cells (MSCs)) (Figure 11).

*Figure 11: Possible source of stem cells: (1) human fetal brain (neural stem cells (NSCs)), (2) embryonic tissues (pluripotent embryonic stem cells (ESCs)), (3) fibroblast (induced-pluripotent stem cells (iPSCs)), and (4) bone marrow (mesenchymal stem cells (MSCs)) (Politis & Lindvall, 2012).*

**Neural stem cells**

NSCs are derived from the ventral mesencephalon (VM) of human aborted fetal brain. This strategy represents the main sole approach used in clinic (one clinical trial with MSCs have been tested). The first open-trial was in 1987. By now, more than 400 patients receive VM-derived DA neurons transplantations. In general, bradykinesia and postural instability are recovered and $^{18}$F-Dopa PET scan shows an increased uptake within the DA system. However, double-blind trials are very disappointing. Indeed, some patient develops dyskinesia (graft-induced dyskinesia (GID)) and beneficial effects of the graft were lost around six months post-transplantation (after stopping immunosuppressive treatments). Recently, Kefalopoulou et al., (2014) showed a long-term survival (15 and 18 years) of VM-derived DA neurons transplantations (in striatum) in two patients. Both exhibit a significant improvement of motor functions without DA medication. This study gives the proof-of-principle that VM-derived DA neurons transplantation can be a successful outcome. However, postmortem studies of patients (not those described before) show LB in the graft, suggesting that PD progression does not stop by affecting the transplanted cells. In addition, ethical controversies, immune rejection and lack of fetal donors remain a major problem (Björklund & Stenevi, 1979; Morizane & Brundin, 2007; Politis & Lindvall, 2012; Björklund & Kordower, 2013; Kefalopoulou et al., 2014).
Embryonic stem cells

ESCs represent another approach in cell therapy. Pluripotent cells are taken from the inner cell mass of human blastocyst and then differentiate in DA neurons. Human ESCs-derived DA neurons are highly proliferative with an unacceptable risk of tumor formations (incapability to transfer such protocol in human). However, recent study (Grealish et al., 2014) shows that transplantation of human ESCs-derived DA neurons can lead to functional recovery in a rat model of PD. Moreover, they have a long-term survival and restore DA transmission. So, their outcomes are similar to VM-derived DA neurons (phenotype, morphology, number of survival neurons,…) and they can project over long distance and target specific brain regions (from the nigral transplant to striatum for example). Nevertheless, this study does not show whether the graft expresses neurotrophic factors, which could provide additional benefit and slow down the PD progression (Lindvall & Kokaia, 2009; de Munter & Wolters, 2013; de Munter et al., 2013; Barker, 2014; Grealish et al., 2014).

Induced-pluripotent stem cells

DA neurons can be obtained by a recent technique consisting of reprogramming somatic cells. For example, fibroblasts can be reprogrammed into iPSCs, by adding virus expressing Yamanaka genes (c-myc, sox2, oct4 and klf4). Then, iPSCs can be differentiated into DA neurons to further be transplanted in specific brain regions. The main advantage of such technique is the attenuation of immune rejection (autologous source; the host is its own donor) and elimination of ethical controversies. Nevertheless, there is a high risk of tumor (teratoma) formation. Furthermore, because of the autologous source, DA neurons derived from iPSCs can carry the susceptibility to develop PD. Despite those limitations, a recent study in non-human primate model of PD shows that iPSC-derived DA neurons (from fibroblasts) exhibit the capacity to provide a long-term (up to two years) functional recovery, survive and reinnervate the host brain. As for ESCs-derived DA neurons, iPSCS-derived DA neurons do not express neurotrophic factors (Morizane & Brundin, 2007; Deleidi et al., 2011; Politis & Lindvall, 2012; Hallett et al., 2015).

Mesenchymal stem cells

In mice models of PD, MSCs can differentiate into tyrosine hydroxylase (TH)-positive neurons (if cells are pretreated with neurotrophic GDNF) and improve motor performances. One clinical trial (Venkataramana et al., 2010) has conducted in seven PD patients using the MSCs from bone marrow. These cells were isolated but no DA differentiation has been made. Motor improvements were weak and no PET investigation was done in order to evaluate the graft fate. This approach needs further assessments (Dezawa et al., 2004; Venkataramana et al., 2010; Politis, 2011; Politis & Lindvall, 2012).
Limitations

For now, only the human VM-derived DA neurons approach shows real positive outcomes, mainly due to early clinical trials. However, better symptomatic treatments are available such as DBS. Moreover, major limitations such as lack of fetal donor, ethical controversies and immune rejections encouraged turning towards ESCs and iPSCs assessments in clinical trials. However, both are too proliferative and induce tumorigenesis. Consequently, a good manufacturing practice (GMP) protocol is required (for example: cells have to contain no marker of pluripotency,…).

Nevertheless, therapies based on stem cells seem to be symptomatic treatments (by replacement of DA neurons) as well as DBS and levodopa. Whereas, one of the major challenges to treat PD is to stop its propagation, PD may be counteracted by increasing neurotrophic factors such as GDNF (glial-derived neurotrophic factor) and BDNF (brain-derived neurotrophic factor). Both promote the survival of many types of neurons (including DA neurons). PD patients exhibit a reduction of both GDNF and BDNF. So, neuroprotection effect (administration of GDNF/BDNF) can ensure the survival rate of neurons in PD patients and can be provided by development of drugs and/or cell-based and gene-therapies. However, it remains challenging to promote such therapies in PD patient (Björklund & Stenevi, 1979; de Mogi et al., 1999; de Mogi et al., 2001; Politis & Lindvall, 2012; Björklund & Kordower, 2013; de Munter & Wolters, 2013; de Munter et al., 2013; Barker, 2014, Wolters & Baumann, 2014).

Autologous neural cell ecosystems (ANCE) transplantation

A recent technique called autologous neural cells ecosystem (ANCE) transplantation has emerged and abolishes the limitations of stem cells therapies. The ANCE transplantation consists of performing a cortical biopsy in the dorsolateral prefrontal cortex (dIPFC). Tissue is then cultivated, in GMP conditions according to previous reports (Brunet et al., 2002, Brunet et al., 2003; Brunet et al., 2005; Brunet et al., 2009, Kaeser et al., 2011, Bloch et al., 2014), to be subsequently labeled with fluorescent membrane protein (PKH67) and finally implanted in brain regions of interest (Figure 12). According to Bloch et al. (2011), progenitor cells are present in the whole primate adult cortex. They express proteins such as glial fibrillary protein (GFAP) and doublecortin (DCX) and are supposed to have a role in brain plasticity and development. In the liquid cell culture, progenitor cells (obtained from the cortical biopsy) divide by asymmetric division and generate quiescent neuroblasts (DCX and nestin (proliferating and migrating marker) positive-cells). The division carries on until obtention of a “nodule” (aggregation of cells). A nodule contains few progenitor cells (five to ten) surrounded by quiescent neuroblasts (300 to 1000). Progenitor cells need to be close to astrocytes in order to promote cell survival. For this reason, the term “ecosystem” was chosen. ANCE transplantation has already shown promising results in non-human primate model of motor cortex lesion (Kaeser et al., 2011) and PD (Bloch et al., 2014). Both showed an improvement of functional recovery. Moreover, cells have the ability to migrate through to corpus callosum (not seen in stem cells therapy) and have a high survival rate (up to 60%). In VM-derived DA neurons therapy, grafts suffer from 90-95% of cell death (although
if functional recovery is observed). Interestingly, in asymptomatic PD monkeys, implanted cells are not TH-positive, suggesting that they do not replace DA cell loss but seems to have a neuroprotective effect (GDNF and/or BDNF positive-cells). A dIPFC biopsy induces negligible side effects, as compared to existing therapies. Therefore, ANCE transplantation might be a future promising technique. Nevertheless, only few studies assessed this therapeutic strategy and further investigation are needed (Brunet et al., 2002; Brunet et al., 2003; Brunet et al, 2005; Brunet et al., 2009; Bloch et al., 2011; Kaeser et al., 2011; Bloch et al., 2014).

![Figure 12: Procedure of autologous neural cell ecosystems transplantation.](image)

1. Cortical biopsy
2. Cells culture
3. Cells labeling
4. Cells implantation

**Non-human primates model of Parkinson’s disease**

Animal models are needed in order to investigate cell therapies, for example. A neurotoxin, called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was used to mimic PD symptoms in primates.

MPTP was discovered accidentally in humans in the 80’s (drug addicts having used a poor quality substance containing MPTP; these young subjects developed PD symptoms). MPTP can cross easily the blood-brain barrier (BBB) because of its lipophilic characteristic. Glial cells contain the enzyme monoamine oxidase-B (MAO-B), which converts the MPTP in MPP+ (1-methyl-4-phenylpyridin-1-ium), which turns out to be toxic. MPP+ can freely exit its glial environment and enters in DA) neurons via the DAT system. Once in DA neurons, MPP+ can move to different cell compartments, including
mitochondria, in which it penetrates by diffusion. Once in the inner membrane of mitochondria, MPP+ inhibits the cellular respiration through the complex I inhibition leading to a reduction of ATP, in combination with the generation of oxygen free radicals (reactive oxygen species), which leads to an oxidative stress, ending with cell death. (Figure 13) (Davis et al., 1979; Langston et al., 1983; Langston et al., 1984; Ramsay and Singer, 1986; Cleeter et al., 1992; Inazu et al., 2002; Smeyne and Jackson-Lewis, 2005; Emborg, 2006).

Figure 13: MPTP neurotoxin mechanisms. MPTP can easily cross the blood-brain barrier to be subsequently converted into MPP+ in glial cell. MPP+ penetrates into DA neurons through the DAT system. Then it can be stored in vesicle (not shown) and/or inhibit respiratory chain in mitochondria leading to ATP decrease. Another possibility is the creation of reactive oxygen species. Both pathways lead to cell death. BBB = blood-brain barrier; DA = dopamine neuron; DAT = dopamine transporter; MAO-B = monoamine oxidase-B, MPP+ = 1-methyl-4-phenylpyridin-1-ium, MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. (NB: scale of different components is not respected for a better understanding).

Interestingly, DA neurons located in the ventral tegmental area (VTA) are much less affected by MPTP. This property is likely due to a protective transcriptional response to MPTP of these DA cells. Given that, MPTP neurotoxin is widely used in order to generate PD-like symptoms in non-human primates and rodents. Indeed, severe loss of SNc DA neurons (which is a hallmark of PD) is observed after an acute MPTP administration (Jenner et al., 1983; Elsworth et al., 1987).

There are many ways to use MPTP in various non-human primate models (old-world as well as new-world primates). The famous MPTP model that has been developed is the unilateral model after intracarotidian MPTP administration (Benazzouz et al., 1992). Moreover, systemic administration of MPTP (intramuscular, subcutaneous, intraperitoneal and intravenous administration) is widely used (e.g. Rose et al., 1993; Bezard et al., 1997; Guehl et al., 2003; Boulet et al., 2008). With these characteristics, MPTP is largely used to mimic Parkinson’s disease (symptoms/pathophysiology) in non-human primates (which are considered as the optimal MPTP models) (Porras et al., 2012; Potts et al., 2013).
Nevertheless, some limitations of MPTP models have been reported. First, it is difficult to reproduce the progressive degeneration of the DA neurons as observed in PD. Investigators decided to administer chronic low dose of MPTP rather than an acute treatment. However, such a protocol requires a treatment for 17 to 21 months (Schneider and Kovelowski, 1990; Hantraye et al., 1993). Second, the response in MPTP treatment varies substantially among individuals. The symptoms severity has a high inter-individual variability even with the same dose and the same MPTP protocol treatment (Elsworth et al., 2000). The last limitation of MPTP model is the existence of a spontaneous recovery that is not seen in idiopathic PD patients (Eidelberg et al., 1986; Taylor et al., 1997). According to Mounayar et al. (2007), with an acute protocol of MPTP (two series of daily injections), monkeys fail to reproduce spontaneous recovery and exhibit stable motor symptoms. In contrast, monkeys receiving a progressive intoxication protocol (five injections spaced by four or five days) showed recovery. Interestingly, both group of monkey exhibit a dramatic DA neurons death in SNc.

The project

The aim of the present research project was to test the therapeutic potential of autologous neural cell ecosystems (ANCE) transplantation (Figure 12) in four non-human primates subjected to Parkinson’s disease-symptoms. Results obtained from different analyses were compared between pre- versus post-lesion and post-transplantation phases on each monkey. The time course of the experiment is shown in Figure 14. All four animals (Mk-LY, Mk-LO, Mk-MY, and Mk-MI) were subjected to a systemic MPTP lesion and the autologous ANCE transplantation. This report presents the behavioral assessment as well as the in-vivo brain image follow-up from the pre-lesion phase until 4.5 months in the post-transplantation phase (April 2015).

Figure 14: Time course of the experiment. In green the pre-lesion phase during which monkeys were supposed to be at a plateau of motor performance. In red the post-lesion phase during which the hypothetic deficit can be evaluated. In blue, the post-transplantation phase during which the effect of transplantation can be assessed. Different time points show discrete events (brain imaging, biopsy and Mk-LY sacrifice). Grey color represents the behavioral follow-up (during the MPTP lesion, it was impossible to approach monkey because of the MPTP toxicity and after the transplantation, 2 weeks of rest were necessary because of the impact of the surgery).
II. Materials and methods

Subjects
Four female adult macaques (Macaca fascicularis) weighing between 3 and 5 kg and ranging from 6 to 10 years old at the beginning of the pre-lesion phase, were involved in this study. They were all trained at least three years before the pre-lesion phase. All four animals were kept in the animal house facility of the University of Fribourg in an enriched room of 45m³. Animals can interact with each other and were free to move. They had free-access to water and they were not-food deprived. The entire experiment was in accordance with the law on animal protection and accepted by the Federal and local veterinary authorities.

Locomotors and activity assessment
Before the daily behavioral tasks, monkeys were individually placed in a cage during 40 minutes. The first 10 minutes were aimed to habituate monkeys to the environment. During the next 30 minutes, locomotor and other activities (free behaving) were assessed using the VigiePrimate® image analyzer system (View Point, Lyon, France). Video cameras were connected to an analyzing system (PC computer). During these 30 minutes, the system calculated in real time the quantity of movements, obtained by change of the different 256 pixels grey level. Different thresholds defined freezing, middle and burst activities (middle activity threshold at 500 and burst activity threshold at 2000 pixels change per seconds (Figure 15). Time spent in the three different level of activity were then imported in Microsoft Excel 2010 files, to be subsequently analyzed in SigmaPlot/SigmaStat 13.0. Statistical analyses (t-test/Mann-Whitney test) compared each three different phases with another phase (pre-lesion, post-lesion and/or post-transplantation). Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05).

Figure 15: (A) Video camera filming the activity of the monkey during 30 minutes. Red pixels show pixel in movement. (B) Raw activity curves calculated by PC computer, based on the three different thresholds.
After the 40 minutes activity session, the monkey was placed in a custom Plexiglas® primate chair adapted to the monkey morphology (Figure 16) (Schmidlin et al., 2011). The monkey body weight was monitored (experiment had to stop if the body weight dropped by 10% or more; this was not the case in the present study). Finally, the monkey was transported to the experimental room, in order to perform specific motor tasks, as explained below.

Figure 16: To perform behavioral tasks, the monkey was trained to sit in a custom Plexiglas® primate chair adapted to the monkey morphology. Two frontal doors can be opened or closed in order to choose with which hand the monkey has to perform the task.

Modified Brinkman board task

The four animals were daily trained at least three years before the beginning of the experiment. In the « modified Brinkman board task » (Figure 17) (Brinkman & Kuypers, 1973; Rouiller et al., 1998), monkeys had to retrieve banana food pellets from 25 vertical and 25 horizontal slots (15mm long, 8 mm wide and 6mm deep) using the opposition of thumb and index finger (precision grip). In pre-lesion phase, monkeys performed daily the task; firstly bimanually. If the task was correctly executed, monkey received an extra reward (raisins, almond,…). Then, monkey repeated the task unimanually, received an extra reward. Finally, the subject did the task unimanually with the other hand (the hand used first was alternated session after session) and obtained additional food (monkey’s pellets, fruits, vegetables,...) directly after the end of the experiment. Sessions were recorded with three different video cameras (Sony®; DCR-SX33): one video camera at the top of the board and two video cameras placed laterally from the board. During the post-lesion and the post-transplantation phases, monkeys performed the task three times per week. Every two days, a “reach and grasp drawer task” was done (in the three phases) directly after the « modified Brinkman board task » (the present manuscript does not report the results for the “reach and grasp drawer task”).
Figure 17: The « modified Brinkman board task », in which the monkey has to retrieve banana food pellets, from 25 horizontal and 25 vertical slots. Dimension of the slots: 15 mm long, 8 mm wide and 6 mm deep (see Schmidlin et al., 2011).

Video sequences (in MPEG format) of the « modified Brinkman board task » were analyzed frame by frame (0.02 seconds separated each frame) using the software Dartfish 7. The analyzed parameters allowed to measure quantitatively different motor aspects: (1) dexterity scores: (i) score in first 30 seconds (number of pellets correctly retrieved in 30 seconds), and (ii) the total score (number of pellets correctly taken during the entire task); and (2) the contact time (CT) corresponding to the time interval (in seconds) from the finger contact with the pellet to the successful pellet retrieval from the slot. For each session, the contact time was measured in the first five explored vertical slots and the first five explored horizontal slots. Moreover, the 50 slots were numerated following their location (from left to right and from up to down) (Figure 18A). Using Matlab R2014b, the temporal sequences of prehention was represented by color code for each session allowing a representation of strategies used to perform the task (Figure 18B).

Figure 18: (A) Geographical position of the 25 horizontal (in grey) and 25 vertical slots (in yellow). (B) Example of a picking sequence. Blue color represents the first visited slots and red the last visited slots.
Motor scores and contact times were then imported in Microsoft Excel 2010 files, to be subsequently analyzed in SigmaPlot/SigmaStat 13.0. Statistical analyses (t-test/Mann-Whitney test) compared each three different phases with another phase (pre-lesion, post-lesion and/or post-transplantation). Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05).

**Positron emission tomography (PET) scans**

Positron emission tomography (PET) scans with $^{18}$Fluoro-Dopa ($^{18}$F-dopa) (Radiopharmazie, Klinik für Nuklearmedizin, Zürich, Switzerland) were conducted for each animal during each experimental phase. Monkeys received one hour before the $^{18}$F-dopa injection, 50 mg per os of Carbidopa (Pharmacie internationale Golaz, Lausanne, Switzerland) in order to have a better acquisition. Then, they were sedated with a intramuscular injection of a cocktail containing ketamine (Ketasol-100®; Graeub; 10 mg/kg) and benzodiazepine (midazolam) (Dormicum®; Roche; 0.1 mg/kg) in order to transport them (around 15 minutes) to the Hospital of Fribourg (HFR). On HFR site, the monkeys received an additional dose of ketamine if necessary. They were then anesthetized with an intravenous cocktail (3.75 mg/kg/h) of 1% propofol (Fresenius Kabi®), ringer-lactate (Hartmann ISOTON®) and ketamine (syringe preparation: 20 ml of propofol, 20 ml of ringer-lactate and 1.25 ml ketamine). Then, the monkeys were placed in pronation position in a custom made stereotaxic plastic apparatus (ear bars were initially covered with local anesthetics (Lidohex®, Bichsel AG)). Oxygen tube was fixed in front of the monkey nose with a flux of 3 l/min (Figure 19A). Five latex gloves filled with hot water were positioned all around the subjects and they were cover with bubble wrap in order to prevent temperature decrease. The level of anesthesia was kept at optimal level with a perfusion rate of the anesthetic cocktail of 0.1 ml/min/kg. Electrocardiogramm (ECG) was monitored and reported all five minutes during the entire acquisition. After the acquisition, monkeys were transported back to University, placed under a hot lamp until the complete awakening, fed, hydrated and finally put back with the group. PET scans with $^{18}$F-Fluodeoxyglucose ($^{18}$F-FDG) were done in all four animals in each phase. This report does not present the results for these analyses.

![A.](image1) ![B.](image2)

**Figure 19:** (A) Head position in the stereotaxic frame with the oxygen tube in front of monkey nose. (B) Monkey in pronation position in the scanner during the acquisition, lasting about 90 minutes.
Monkeys, in pronation position, were introduced in to the scanner (Philips Ingenuity TF) (Figure 19B). First, a computer tomography (CT) acquisition was done. Then, an injection of $^{18}$F-dopa (about 120 MBq) was performed intravenously. Directly after the injection, the dynamic $^{18}$F-dopa acquisition begun with four first frames of 30 seconds, followed by three frames of 60 seconds, two frames of 120 seconds, then 7 frames of 180 seconds and finished with 12 frames of 300 seconds (for a total of 28 frames and 90 minutes of acquisitions). 128 slices composed one frame.

Analysis of $^{18}$F-dopa scans was done with the software PMOD (v3.605). First, native images were flipped, converted from DICOM format to NIfTI format. With help of the CT scan, native images were aligned (CoRegistration) with T1-weighted MRI images, in which several regions of interest (ROIs) were defined. Images from the 10 last frames were summed and used to adjust precisely the ROIs. The adjusted ROIs were then transferred in dynamic images. The software calculated the amount of radioactivity per ROIs (Kbq/cc) in all frames. The amount of radioactivity of each ROI per frame was calculated and averaged for caudate nucleus, putamen and occipital area, differentiating the left and right hemisphere (except for the occipital area). The influx rate constant ($K_i$) was calculated using the Patlak-analysis (occipital area served as reference for non-specific uptake of $^{18}$F-dopa). The final striatal $K_i$ was obtained by averaging the average of left and right putamen with the average of left and right caudate nucleus.

**Magnetic resonance imaging (MRI)**

Magnetic resonance imaging (MRI) (GE Medical system®; Discovery MR750; 3 Tesla) was carried out for each animal during the pre-lesion phase and after the cortical biopsies (post-lesion phase). The animal care was the same as described for the PET scans (without the administration of Carbidopa). On HFR site, monkeys were under anesthesia with an intramuscular injection of a mixture of ketamine (4 mg/kg) and medetomidine (Dorbene®; Graeub; 0.04 mg/kg). (The anesthetic protocol described above for PET scan was also used in Mk-LY; Mk-LO and Mk-MY in post-lesion phase). Then, they were placed in a pronation position (with hot water gloves, bubble wrap and oxygen tube (3 l/min)). The head was placed on a knee antenna (C-GE-HDx TR Knee PA®, G-CoilType=8). As for PET scan, ECG was monitored and reported. After acquisitions, monkeys, under Dorbene® protocol, were injected with atipamezole (Alzane®; Graeub; 0.2 mg/kg) for a faster awakening and brought back to University of Fribourg. Finally, they were under a hot lamp until the complete awakening, fed, hydrated and finally put back within the group of monkeys. The parameters of the acquisition were: (1) acquisition matrix: 256x256; (2) 0.7 mm isocubic thick; (3) echo time (TE) = 3.1; (4) repetition time (TR) = 7.3; (5) 3DT1 and (6) 3D sagittal.

**1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesion**

During the MPTP protocol lesion (Figure 20A, Table 1), animals were exceptionally housed separately in cage. They received daily a ration of food (monkey’s pellets, fruits and vegetables) and water. For intramuscular injections of MPTP (Sigma; 0.5 mg/kg), the animals were transferred into a smaller cage, in which a custom Plexigas® plate can mechanically constrain the monkey against the cage leading to an easier access to muscle (this method was chosen to avoid a sedation that could be
more deleterious for the animals). Then, the animals were re-transferred to their home cage. After two series of daily injections, the protocol continued with two single injections. After 10 days of break (during which cortical biopsies took place, see below), we prolonged injections (4 to 5 injections per animal) with twice less MPTP amount (0.25 mg/kg), depending on the symptoms of animals. Motor symptoms were evaluated using the Schneider rating scale (Schneider et al., 1994). All the MPTP procedures were conducted under a strict and safety protocol for experimenters (Przedborski et al., 2001) (Figure 20B).

**Figure 20:** (A) Time course of the MPTP lesion protocol. Red arrows represent single injection and green arrows represent breaks. (B) Monkey room under negative pressure. The two experimenters carried protection wear (mask with filters, coat, double pair of gloves, etc.).

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<td>6.25</td>
<td>6.25</td>
<td>7.75</td>
</tr>
</tbody>
</table>

**Table 1:** Table showing the dose of single injection for each monkey. During the third break, the cortical biopsy took place.
**Cortical biopsies**

Before the surgery, the animals were tranquilized as described above but received an additional intramuscular dose of methadone (Methadon®, Streuli; 0.2 mg/kg). Then, they were treated with analgesic Carprofen (Rymadil®, Pfizer; 4 mg/kg; subcutaneously), atropine (atropine; 0.05 mg/kg; intramuscularly) in order to reduce bronchial secretions, antibiotics (Synulox®, Pfizer; 8.75 mg/kg; subcutaneously) and dexamethasone (Dexadreson®, Intervet; 0.3 ml/kg; diluted 1:1 in saline; intramuscularly). Monkeys were then anesthetized with an intravenous cocktail of propofol 1% and ringer-lactate (1 volume of propofol for 2 volumes of ringer) and placed in pronation position in a stereotaxic frame. Before the skin incision, a local analgesic was injected subcutaneously (Rapidocain®, 10 mg/ml; Sintetica). During the craniotomy, monkeys received intravenously an opioid analgesia (Fentanyl®, Sintetica; 6 µg/kg/h; diluted 1:1 in saline). Craniotomy (boned square of about 8x8 mm) was done on the left hemisphere (except for Mk-MY) (Figure 21A). Using a surgical blade, the dura-matter was excised and a piece of cortical tissue (from 7 to 14 mm³) was removed from the dorsolateral prefrontal cortex (dlPFC) (Figure 21B). The cortical tissue was placed in sterile cold medium (see below for the cells cultures). The bone was put back in place and anchored with histological glue (histoacryl®, B. Braun). The wound was sutured and monkey was place under hot lamp until the complete awakening. All the surgery was practiced under sterile conditions. ECG, oxygen saturation and rectal temperature were monitored and reported during the procedure. After surgery, monkeys received per os antibiotics (Clavubatin®, Graeub; 12.5 mg/kg) and anti-inflammatory (Rimadyl®, Pfizer; 10 mg) twice a day during 5 days. After the convalescence, MPTP protocol lesion carried on. A pilot biopsy (following the same protocol) was performed, in all four animals, five months before the pre-lesion phase in order to evaluate the possible impacts of such biopsy in motor tasks execution (see Appendix 1) and to test the good manufactured practices (GMP) protocols.

Figure 21: (A) Craniotomy on the left side of the skull. (B) Cortical tissue (red circle) taken from the left dorsolateral prefrontal cortex (dlPFC).
Cells cultures and preparation for transplantation

The cells cultures protocol was the same as described in previous studies (Brunet et al., 2005; Brunet et al., 2009; Kaeser et al., 2011; Bloch et al., 2014). Cortical biopsies were put in steril cold medium (during few hours) until transport to the cells production center (CPC, CHUV, Lausanne). Tissues were dissected with a razor blade in order to collect enriched fractions of gray matter. Primary cultures were obtained by mincing and mechanically triturating (comings and goings with a fire-polished glass pipette of decreasing diameters) the tissue. Cells were re-suspended at 50000 cells/ml in RPMI-1640 medium (1x) without L-Glutamine (31870-025, Invitrogen AG, Basel, Switzerland) supplemented with NaHCO3 44mM, 20% fetal bovine serum (FBS), and an antibiotic/antimycotic cocktail (A7292 Sigma) directly into 25 ml-glass erlenmeyer at 37°C in a water-saturated atmosphere containing 6.5% CO2/93.5 % air under horizontal agitation at 70 rotations/min. In a 24 wells plate, a cell suspension was plated on glass coverslips under the same incubator conditions. Fifteen days later, the serum concentration was diminished to 10%. When the cells became confluent (after 20 days) on the coverslips (Figure 22), they were maintained without serum until the transplantation (35 days later).

Figure 22: Cells aggregate (nodule) composed of few progenitor cells surrounded by quiescent neuroblasts. Nodule was surrounded by astrocytes forming long filament. Scale bar = 200 µm.

The day before the transplantation, cell aggregates from one flask were pooled by centrifugation at 800 rotations/min. For the re-suspension, the supernatant was recuperated. Aggregates were re-suspended in 500 µl diluent C with 5 µM fluorescent viable dyes PKH67 (MINI67, Sigma) for three minutes. 500 µl of fetal calf serum (FCS) was added for one minute and aggregates were washed three times with RPMI medium. After the last wash, a recovered medium was centrifuged at 4000 rotations per minute (to eliminate cellular wastes) and added in order to re-suspend aggregates. Tubes containing cellular stained aggregates were completely filled for transportation at room temperature from Lausanne to Fribourg. For transplantation, aggregates were formed and supernatant was partially removed until a volume of 100 µl in which aggregates were re-suspended at a cell concentration of 3000/µl.
Cells transplantation

For each animal, two implantations sites in the putamen and one in caudate nucleus per hemisphere (six total implantation sites per animal) were determined based on T1-weighted MRI scan (with OsiriX (v.4.1.2)) of the post-lesion phase (see Appendix 2), and then compared with the Paxinos atlas of the macaque brain (Paxinos et al., 1999). Transplantations surgery followed the same surgical procedures as described for biopsy. The craniotomy was performed bilaterally in order to have access to stereotaxic coordinates of implantation sites. Implantation sites were reached vertically with a Hamilton microsyringe (Figure 23B). Once the site reached, a total volume of 10 µl culture medium (corresponding to approximately 300’000 cells) was automatically injected (2 µl/min during 5 minutes for each site) using a nano-injector (Stoelting, Wood Dale, IL, USA) (Figure 23A). After the six sites implantations, the bone flap was put back in place, the muscles and skin were sutured as described for the biopsy surgery. After 2 weeks of convalescence, the behavioral assessments restarted.

Figure 23: (A) Stereotaxic frame with the Hamilton microsyringe on the right (N.B: during the surgery, the microsyringe was placed perpendicularly to monkey’s head). (B) Implantation of ANCE with microsyringe in the striatum on the left hemisphere.
III. Results

General comments

The results are presented by comparing pre-versus post-lesion and/or post-transplantation phases on each monkey. Qualitative images for the $^{18}$F-dopa PET scans were normalized and extracted from the software (PMOD) allowing a direct visual comparison of the striatum (Figure 24). Final striatal uptakes ($K_i$) were reported in Table 2 in order to compare them quantitatively for each experimental phase.

Results of the manual dexterity performance (score in 30 seconds and total score) as well as temporal sequence of prehention in the “modified Brinkman board task” (Figure 26, 30) were presented following the time course of the experiment in order to visualize the dynamics of manual dexterity performance and strategy (temporal sequence to visit the 50 individual slots). The time (x-axis) is represented as “sessions from lesion” because in the pre-lesion phase one session per week during 6 months was analyzed, whereas during the post-lesion and post-transplantation phases, all sessions were analyzed.

All statistical analyses and graphs (manual dexterity performances, contact times and spontaneous activities) were performed with SigmaPlot/SigmaStat (13.0). Graphs were represented with box and whisker plots and visually improved with CorelDRAW (Home & Student X7): in white the pre-lesion phase, in black the post-lesion phase and in grey the post-transplantation phase. Statistical analyses (t-test/Mann-Whitney Rank Sum Test) were used to compare the three different phases among each other. Statistically significant differences were considered when the P-value was smaller than 0.05 ($P \leq 0.05$). Statistically significant differences are indicated with: * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$. "n.s" meaning statistically non-significant. All P-values of Figure 25, Figure 27, Figure 28 and Figure 29 are shown in Appendix 4. In the box and whisker plots, the horizontal line in the box represents the median value. The 75% and 25% percentiles are indicated by the top and bottom lines of the box, respectively.

MPTP lesion protocol and cardinal symptom evaluations

During the MPTP lesion protocol, motor symptoms were quantitatively assessed with the Schneider rating scale (Schneider et al., 1995). In Mk-LY and Mk-LO, motor symptoms (especially tremor, bradykinesia and lower limb movement impairments) were moderately and intermittently present after the two series of daily injections. Two weeks after the MPTP treatment, both exhibited a drop of motor symptoms (sometimes slight tremor were observed) (Appendix 3, Figure 35A,B). In Mk-MY, cardinal symptoms (especially tremor and bradykinesia) appeared progressively at the end of the MPTP lesion protocol and were stable after the complete MPTP lesion protocol (Appendix 3, Figure 35C). In Mk-MI, virtually no motor signs of PD were observed during the entire MPTP lesion protocol. Few days after the end of the protocol, a dramatic increase of the motor symptoms emerged and persisted without spontaneous recovery (Appendix 3, Figure 35D).
**18F-dopa PET scans**

The final striatal uptake (Kₐ) was calculated by averaging the uptake in caudate nucleus and putamen on both hemispheres. During the pre-lesion phase, all four animals showed comparable striatal uptake (Kₐ) (**Figure 24, Table 2**). After the MPTP lesion, dramatic decrease of striatal uptake (up to 80%) was observed in all four animals except for Mk-LY (17%). Consistent with a systemic lesion, the striatum (putamen and caudate nuclei) was affected bilaterally (**Figure 24, all anatomic planes**). Mk-LO lost 84.2%, Mk-MI lost 82.8 % and Mk-MY lost 81.5% of 18F-dopa uptake within the striatum (**Table 2**). At the present step, only the post-transplantation 18F-dopa PET scan in Mk-LY was conducted (will be done later for the three other monkeys). In Mk-LY, the 18F-dopa uptake returned to the level as in pre-lesion phase (**Figure 24, Table 2A**) with an uptake of 100.5%, as compared to the pre-lesion phase. In Mk-LO during the post-lesion phase, “defluorinization” phenomenon of the 18F-dopa molecule took place and the radioactive molecules deposited in the skull and glands.
Figure 24: Normalized images of $^{18}$F-dopa PET scans in all the four animals. Red color represents high $^{18}$F-dopa uptake, whereas the blue color represents low $^{18}$F-dopa uptake. The three anatomical planes were shown. For all animals (except in Mk-LY) a dramatic loss of striatal $^{18}$F-dopa uptake was observed (loss > 80%). Mk-LY showed a moderate loss of striatal uptake. The post-transplantation PET scan was conducted only in Mk-LY at that step. The $^{18}$F-dopa uptake in Mk-LY returned to a level as in the pre-lesion phase. See also Table 2.
### Table 2

Table showing the striatal ¹⁸F-dopa uptakes (Kᵢ) obtained with the Patlak-analysis. The final striatal Kᵢ were calculated by averaging the averages of the left caudate nucleus and left putamen by the averages of the right caudate nucleus and the right putamen. Final striatal Kᵢ were considered as 100% of activity. (A) The post-lesion final striatal Kᵢ in Mk-LY represented 83% of the pre-lesion Kᵢ. The post-transplantation Kᵢ represented 100.5% of the pre-lesion Kᵢ. (B) In Mk-LO, the post-lesion final striatal Kᵢ represented 15.8% of the pre-lesion final striatal Kᵢ. (C) In Mk-MY, the post-lesion final striatal Kᵢ represented 18.5% of the pre-lesion final striatal Kᵢ. (D) In Mk-MI, the post-lesion final striatal Kᵢ represented 17.2% of the pre-lesion final striatal Kᵢ.

<table>
<thead>
<tr>
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<th>Pre-lesion</th>
<th>Post-lesion</th>
<th>Post-transplantation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td><strong>A.</strong> Mk-LY</td>
<td></td>
<td></td>
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<tr>
<td>¹⁸F-dopa uptake (Kᵢ)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Kᵢ in caudate nucleus</td>
<td>0.006</td>
<td>0.00646</td>
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<td>0.007355</td>
<td>0.006105</td>
<td>0.007394</td>
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<tr>
<td></td>
<td>100%</td>
<td>83.00%</td>
<td>100.5%</td>
</tr>
<tr>
<td><strong>B.</strong> Mk-LO</td>
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<td></td>
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<td></td>
</tr>
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<td>0.0012</td>
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<tr>
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<td>0.0012453</td>
<td>?</td>
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<tr>
<td></td>
<td>100%</td>
<td>15.80%</td>
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<tr>
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<tr>
<td></td>
<td>100%</td>
<td>18.50%</td>
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</tr>
<tr>
<td><strong>D.</strong> Mk-MI</td>
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<td>¹⁸F-dopa uptake (Kᵢ)</td>
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<tr>
<td></td>
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<td>17.20%</td>
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</table>
Spontaneous activity time (VigiePrimate®)

All four monkeys spent a higher time in freezing activity after the MPTP lesion (P≤0.001). Consequently, the mild and burst activities decreased (P≤0.001). An augmentation in time spent in freezing activity of (1) 158% in Mk-LY, (2) 283% in Mk-LO, (3) 343% in Mk-MY and (4) 195% in Mk-MI were observed, as compared to the pre-lesion phase (Figure 25A,B,C,D). After the transplantation, only Mk-LY showed a spontaneous activity time comparable to the post-lesion phase (p>0.05; n.s.), in all three levels of activity (Figure 25A). Contrary to Mk-LY, in the other three monkeys, a statistically significant reduction of time spent in freezing activity was observed after transplantation as compared to the post-lesion phase (Figure 25B,C,D). Consistent with those decreases, the three monkeys exhibited increases of mild and burst activities. As compared to the reference (pre-lesion phase), the time spent in freezing activity in the post-transplantation phase represented 248% (reduction of 35% with respect to the post-lesion phase), 332% (reduction of 11% with respect to the post-lesion phase) and 190% (reduction of 5% with respect to the post-lesion phase), in Mk-LO, Mk-MY and Mk-MI, respectively. All those reductions with respect to the post-lesion phase in freezing activity were statistically significant (P≤0.01) (Figure 25B,C,D).
Figure 25: Box and whisker plots showing the spontaneous activity time during 30 minutes (1800 seconds). For each monkey the time spent in the three activity levels are separated. For each activity level, time spent was compared between pre- versus post-lesion and post-transplantation phases. The average of all data points in the pre-lesion phase was considered as 100% of time spent in freezing activity (reference value). (A,B,C,D) All four monkeys showed an increased time spent in freezing activity after the MPTP lesion. After the transplantation, a statistically significant reduction of time spent in freezing activity was observed in all four monkeys, except in Mk-LY. Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05). Statistically differences are indicated with: * for P≤0.05, ** for P≤0.01, *** for P≤0.001. ‘n.s’ meaning statistically non-significant. pre = pre-lesion phase; post 1 = post-lesion phase; post 2 = post-transplantation phase.
**Modified Brinkman board task**

*Manual dexterity performance*

For the score in 30 seconds, the description of the sum of vertical and horizontal slots is discussed (total score). For both hands, Mk-LY exhibited a lot of variability before the lesion ([Figure 26A,B]). No significant change between the pre-lesion and the post-lesion phases were observed for both hands. However, Mk-LY showed an improvement of the score in 30 seconds for the left hand post-transplantation, as compared to pre-lesion ([Figure 27A]). No plateau of manual dexterity performance was defined as the monkey remained stable during the entire experiment and showed a strong variability across daily sessions. Consequently, all data were included in [Figure 27A].

In the pre-lesion phase, Mk-MY reached a plateau of manual dexterity performance. After the MPTP lesion a drop of the grasping score was observed for both hands, followed by a progressive spontaneous recovery reaching a plateau (plateau was arbitrarily defined when the slope of the linear regression was smallest than 0.2), after 39 and 35 days for the left and the right hands, respectively ([Figure 26C,D]). The scores at plateau of the post-lesion phase represented 77% (calculated with the average of all data at the plateau) (for the left hand) and 72% (for the right hand) of the pre-lesion score of reference ([Figure 27B]). After the transplantation, a progressive recovery carried on until reaching a plateau after 40 and 49 days for the left and the right hands, respectively ([Figure 26C,D]). The scores at the plateau in the post-transplantation phase represented 92% (for the left hand) and 93% (for the right hand) of the pre-lesion plateau. For the left hand, an increase in manual dexterity performance of 15% was observed after the transplantation as compared to the post-lesion plateau, whereas for the right hand, an improvement of 21% of manual dexterity was observed. The difference between the post-lesion and post-transplantation plateau was statistically significant for both hands (P≤0.001) ([Figure 27B]).

The other two subjects (Mk-LO and Mk-MI) were excluded from the score in 30 seconds analysis. Indeed, Mk-LO did not perform the task adequately and Mk-MI could not empty entirely the board after the MPTP lesion. Consequently, for both animals, the motor performance was evaluated with the total score (number of pellets successively retrieved during the entire task). In addition, in Mk-MI, the left index and the third digit were injured (incapability to perform the precision grip). Then, only the right hand was considered.

Mk-LO showed a lot of variability during the pre-lesion phase. This variability even increased during the post-lesion phase and post-transplantation phase. A significant decrease of total score was observed during the post-lesion phase for both hands ([Figure 27C]). However, this decrease was present mainly at the end of the post-lesion phase for both hands ([Figure 26E,F]). During the post-transplantation phase, an increase of total score was observed followed by a decrease for both hands ([Figure 26E,F]). Mk-LO exhibited non-significant change in total score between the post-lesion and post-transplantation phases for both hands ([Figure 27C]). No plateau of manual dexterity performance was defined because Mk-LO showed a lot of variability during the entire experiment. Consequently, in [Figure 27C], all scores were included in the box and whisker plots.
Mk-MI (right hand only) was at a plateau of motor performance during the pre-lesion phase. After the MPTP lesion, there was a dramatic decrease (99% of loss) of total score. After 48 days post-transplantation, Mk-MI was able to perform the task again and reached a plateau after 62 days (Figure 26G). The post-transplantation plateau represented 63% of the pre-lesion plateau. An increase of 62% of motor performance was observed as compared to post-lesion plateau (Figure 27G). Plateau were arbitrarily defined as described for Mk-MY. Moreover, a large variability of total score was observed after the transplantation.
Figure 26: Time course in daily sessions of the manual dexterity performance (score in 30 seconds and total score). Scores are given by the number of pellets successively retrieved by the monkeys during 30 seconds or during the entire task. Vertical red lines (session 0) represent the MPTP lesion (lasting for 6 weeks) and vertical blue lines represent the time point in which the ANCE transplantation took place. Dashed lines (C,D,G) indicates the time when a plateau was reached (see text for criterion to define the plateau).
Figure 27: Box and whisker plots showing the manual dexterity score at the plateau of performance in Mk-MY (B) and Mk-MI (D). In Mk-LY (A) and Mk-LO (C) box and whisker plots include all the data for each phase (see text for criterion of the plateau exclusion). (B,D) The average of all data in the pre-lesion phase was considered as 100% of manual dexterity score. Percentages represent the pre- or post-transplantation phases performance with respect to the pre-lesion phase. Percentages above the horizontal bars display the recuperation of manual dexterity performance after the transplantation. Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05). Statistically significant differences are indicated with: * for P≤0.05, ** for P≤0.01, *** for P≤0.001. “n.s” meaning statistically non-significant. pre = pre-lesion phase; post 1 = post-lesion phase; post 2 = post-transplantation phase.
Contact time

The contact time (CT) represents a fine manual dexterity measurement (manipulation of the pellet between the thumb and the index finger) and can be resumed by the time spent into the slot to successfully retrieve the pellet. In other words, the CT is the time interval between first contact between the pellet and the first finger entering the slot (usually the index finger) and the time point at which the pellet is successfully retrieved from the slot. Data were collected from all the daily sessions in each phase (see materials and methods chapter “modified Brinkman board task”). The CTs were presented separately for the vertical (Figure 28) and horizontal (Figure 29) slots. The post-transplantation phase (grey box-plot) was separated into months (post 2a = January; post 2b = February; etc.). Y-axes are not all at the same scale in order to have a better graphical representation of data.

Mk-LY exhibited an increase (statistically significant: P≤0.001) of the CT for the vertical slots for the left hand after the lesion. During the post-transplantation phase (in April), non-significant change was observed as compared to the pre-lesion phase (Figure 28A). For the right hand, there was no significant changes for the CTs on each phase (Figure 28B). The same observation was valid for the horizontal slots for both hands (Figure 29A,B).

Mk-LO had stable CT for horizontal and vertical slots during the entire experiment for the right hand (Figure 28D, 29D). Nevertheless, an unexpected decrease of the CTs for vertical slots for the left hand was observed (P=0.007) after the MPTP lesion (Figure 28C). This decrease was maintained until April during the post-transplantation phase. The CTs for horizontal slots for the left hand increased during the post-transplantation phase in January with respect to the post-lesion phase. However, the horizontal slots CTs remained stable during the post-transplantation phase in April as compared to the pre-lesion phase (Figure 29C).

Mk-MY exhibited an augmentation of the CTs (vertical as well as horizontal slots) for both hands after the lesion (P≤0.001) (Figure 28E,F; 29E,F). In January, after the transplantation, no significant change was observed in both hands and for both slot orientations with respect to the post-lesion phase. In April, the CTs returned to a level as in pre-lesion phase, except for the left hand in horizontal slots, where a decrease was observed (P=0.038) (Figure 29E).

In Mk-MI, only 8 vertical CTs could be measured and 3 for the horizontal slots during the post-lesion phase. Consequently, statistical analyses were omitted during the post-lesion phase. Post-transplantation, in April, the CTs for vertical and horizontal were higher (P≤0.001) than in pre-lesion phase. However, CT decreases were observed along the months during the post-transplantation phase for vertical slots, whereas for horizontal slots, Mk-MI remained stable from February until April (Figure 28G, 29G).
Figure 28: Box and whisker plots showing the contact time (CT) for the vertical slots. The post-transplantation phase is separated into months (Post 2a; Post 2b; Post2c; Post 2d). Data were collected from all the sessions in each experimental phase. Y-axes are not all at the same scale in order to have a better graphical representation of data (justified by the more pertinent comparison across phases within the same hand, rather than crossed comparisons between hands and monkeys). Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05). Statistically significant differences are indicated with: * for P≤0.05, ** for P≤0.01, *** for P≤0.001. “n.s” meaning statistically non-significant.
Figure 29: Box and whisker plots showing the contact time (CT) for the horizontal slots. The post-transplantation phase is separated into months (Post 2a; Post 2b; Post2c; Post 2d). Data were collected from all the sessions in each experimental phase. Y-axes are not all at the same scale in order to have a better graphical representation of data. (justified by the more pertinent comparison across phases within the same hand, rather than crossed comparisons between hands and monkeys). Statistically significant differences were considered when the P-value was smaller than 0.05 (P≤0.05) Statistically significant differences are indicated with: * for P≤0.05, ** for P≤0.01, *** for P≤0.001. "n.s" meaning statistically non-significant.
**Temporal sequence of pellet retrieval (strategy)**

The picking sequence (along the vertical axis of the board) in the modified Brinkman board task was qualitatively assessed only in Mk-LY and Mk-MY, for the same reasons as previously described for the manual dexterity performance. When looking at the data, the picking sequence was not randomly performed. Indeed, the monkeys retrieved the pellets following a more or less constant strategy pattern (especially in Mk-MY) along the vertical axis (up-down strategy) across the daily sessions.

In Mk-LY, the up-down strategy remained relatively unchanged during the entire experiment. Indeed, for the left hand, the slots visited first (blue circles) were located predominantly in the “middle” of the board along the vertical axis and the slots visited last (red circles) were at variable locations, most often at the bottom or at the top. For the right hand, there was a general preference to scan the board from top to bottom (Figure 30A,B). Furthermore, this trend was enhanced during the post-transplantation phase for the right hand (Figure 30A,B).

In Mk-MY, the picking sequence was affected after the lesion (especially for the right hand). During the pre-lesion phase, Mk-MY retrieved first the pellets located at the middle and the top of the board (blue circles), moving gradually to the bottom part of the board (green and yellow circles) and finally terminated at the extreme bottom part of the board (red circles). This up-down strategy was even more prominent when performing the task with the right hand. After the lesion, the first visited slots were virtually never located at the middle of the board but at the top positions, then the monkey scanned the board from top to bottom. Nevertheless, the initial strategy (as in the pre-lesion phase) was re-exhibited after the transplantation but already begun at the end of the post-lesion phase (Figure 30C,D).
Figure 30: Picking temporal sequence along the top-down (vertical) axis in the modified Brinkman board task. X-axis represents the consecutive behavioral daily sessions from the MPTP lesion. One column corresponds to one daily session. Y-axis displays the geographical location along the vertical axis of the board. Colors indicate the temporal picking sequence (E), ranging from 1 (blue, first pellet retrieved) to 50 (red, last pellet retrieved). The red arrow represents the MPTP lesion and the blue arrow the transplantation.
IV. Discussion

Limitations of interpretation

The interpretation of the present study is limited for two reasons. First, the limited number of animals and the absence of control monkeys (MPTP-treated monkeys not subjected to the ANCE transplantation) remain a challenge to correctly interpret the results. However, the present study focused on the fate of the implanted cells, reason why all four monkeys were ANCE re-implanted. Furthermore, the absence of control monkeys (sham re-implantation) in the present study was decided based on a previous experiment using the same autologous adult progenitor cells transplantation strategy, which showed that, in comparison to control monkeys, ANCE re-implanted monkeys recovered better from the MPTP lesion (Bloch et al., 2014). Capitalizing on this recent demonstration of the benefit of the ANCE transplantation, the present study was designed in order to put emphasis on less often assessed parameters in MPTP monkey models of PD, namely the motor function of distal forelimb muscles, imaging (PET scans) and fate of re-implanted cells. As far as the number of monkeys is concerned, the limitation to four monkeys was dictated by the limited capacity of the animal facility, as well as by the number of available experimenters. In any case, the hypothetic addition of two control monkeys to the four re-implanted monkeys would not have properly addressed this number issue, considering the large inter-individual variability of the MPTP monkey model (Elsworth et al., 2000), as well as the optimal number of treated monkeys as derived from a previous lesion work in our laboratory (Freund et al., 2009). Second, the present manuscript reports preliminary results, limited to 4.5 months post-transplantation. Indeed, beyond the writing of the present manuscript, the project will be pursued during the year 2015 with behavioral follow-up until sacrifice (a total of at least 6 months post-transplantation evaluation) and histological readout. Without the histology, it remains difficult to interpret possible mechanisms of functional recovery. In addition, at that step, only one animal (Mk-LY) was subjected to the post-transplantation $^{18}$F-dopa PET scan. Therefore, $^{18}$F-dopa PET scans were useful only to quantify the MPTP lesion in-vivo. Despite those two limitations, some observations and proof-of-principle interpretations were derived from the data and may be profitable for general knowledge about PD and regenerative process after such a brain dysfunction.

MPTP lesion protocol and motor symptom evaluations

As mentioned above, the response to MPTP treatment varies substantially among individuals (Elsworth et al., 2000). According to Mounayar et al. (2007), an acute MPTP intoxication (two series of daily injections) is sufficient to exhibit stable motor symptoms with no spontaneous recovery. In the present study, an acute MPTP intoxication was chosen following the same protocol (Mounayar et al., 2007). However, it fails to produce stable PD motor symptoms after the two series of daily injections in all four animals. The MPTP protocol had to be pursued a couple of additional days until reaching motor symptoms qualitatively evaluated with the Schneider rating scale (Schneider et al., 1995). In Mk-LY and Mk-LO, motor symptoms were moderate and intermittently present after the two series of
daily injections. Consequently, low dose of MPTP were administrated after the two series of daily
injections. Two weeks after the MPTP treatment, both exhibited some spontaneous recovery regarding
the Schneider rating scale (sometimes slight tremor were observed). In Mk-MY, motor symptoms
especially tremor and bradykinesia) appeared progressively and were stable after the complete
MPTP protocol treatment. Slight spontaneous motor recovery was seen during the post-lesion phase
(see results in “modified Brinkman board task”). In Mk-MI, virtually no motor signs of PD were
observable during the entire MPTP protocol. Few days after the end of the protocol, a dramatic
increase of motor symptoms emerged and persisted without spontaneous recovery. After the MPTP
intoxication protocol, Mk-LY and Mk-LO were considered as “recovered parkinsonian monkeys”, Mk-
MY as “moderated parkinsonian monkey” and Mk-MI as “severe parkinsonian monkey”. These
assessments seem consistent with the motor symptoms. However, they have to be nuanced with
respect to the 18F-dopa PET scans.

Correlation between nigrostriatal lesion and motor symptoms

18F-dopa PET scan allows an evaluation of DA depletion (reflected by a decrease in the activity of the
enzyme dopa decarboxylase) within the striatum in-vivo. Direct highly significant positive correlation
between striatal in vivo 18F-dopa uptake and post-mortem numbers of DA neurons has been shown in
MPTP-treated monkeys: striatal 18F-dopa uptake measurement by PET scan provides a good indicator
of the nigrostriatal state in-vivo (Pate et al., 1993; Strome & Doudet, 2007).

As mentioned before, two “recovered PD monkeys” have been considered regarding the cardinal
symptoms. Nevertheless, a distinction between both is necessary. Indeed, Mk-LY seemed to show a
resistance to MPTP with a loss of 17% of 18F-dopa uptake after the lesion. The failure of the MPTP
lesion to generate nigrostriatal lesion in Mk-LY may be due to a resistance to MPTP. Indeed, mice
housed in enriched environment (as the monkey detention room in this study) are protected against
MPTP. This protection might be mediated by an up-regulation of neurotrophic factor such as BDNF
(Bezard et al., 2003). Moreover, resistance to MPTP was observed in mice when they had the
possibility to interact with each other (as the monkeys in this study) (Goldberg, 2012). However, with
such explanations, all the monkeys should have exhibited resistance to MPTP, whereas this is not the
case in the three others monkeys (up to 80% of 18F-dopa uptake reduction after the lesion). Thus,
MPTP resistance in Mk-LY should be explained by metabolic differences and/or clearance of MPP+
(Johannessen et al., 1985; Capitano & Emborg, 2008; Potts et al., 2013). On the contrary to Mk-LY,
Mk-LO seemed to exhibit almost complete spontaneous recovery although a broad nigrostriatal lesion
(84.2% of decrease in 18F-dopa striatal uptake) was observed. Spontaneous recovery mechanisms
remain uncertain. Nevertheless, a few hypotheses may be proposed. Here three of them will be
discussed.

First, during the MPTP lesion protocol, the down-regulation of tyrosine hydroxylase (TH; enzyme
converting tyrosine into L-dopa (DA precursor)) may be dysfunctional but DA neurons may not die.
Thus, after the MPTP lesion protocol, DA neurons may be “re-activated” (Song & Haber, 2000).
Second, the associative territory of the striatum (less affected by MPTP) could compensate by sprouting fibers to the sensorimotor territory of the striatum (areas more affected by MPTP) (Song & Haber, 2000) and increasing the DA release, from residual DAergic system (Boulet et al., 2008). Both hypotheses can be rejected as Mk-LO showed an \(^{18}\)F-dopa reduction of 84.2%. But both explanations may also explain the weak DA depletion in Mk-LY. The third hypothesis in accordance with Mounayar et al. (2007) and Boulet et al. (2008), suggests a role of the serotonin (5-HT) neurotransmission, which might play a role in motor recovery. This last hypothesis has not been tested yet (with the histological readout for example) as Mk-LO was subjected to the ANCE transplantation. Mk-LO can still be considered as “recovered parkinsonian monkey”, whereas Mk-LY has to be considered as “resistant parkinsonian monkey” or “asymptomatic parkinsonian monkey” (Blesa et al., 2012). Moreover, both monkeys are four years old, younger than Mk-MY and Mk-MI. It has been shown that younger-onset PD patients seem to have more efficient compensatory mechanisms as compared to older-onset patients. Indeed, the disease progresses slower and seems to endure more damage of the nigrostriatal system before the first motor symptoms appearance (la Fuente-Fernandez et al., 2011). Mk-LY and Mk-LO cases illustrate the well-known complexity and variability of the MPTP model in non-human primates.

The two other monkeys (Mk-MY and Mk-MI) exhibited motor symptoms consistent with a broad nigrostriatal lesion (81.5% and 82.8% in Mk-MY and Mk-MI, respectively) as expected with a chronic high dose systemic intoxication with MPTP (Albanese et al., 1990; Emborg, 2006; Porras et al., 2012). Although Mk-MY displayed less motor symptoms as compared to Mk-MI, both animals were optimal to evaluate the potential therapeutic effect of the ANCE transplantation on motor functions. Additionally, Mk-MY was able to perform the “modified Brinkman board task” during the post-lesion phase given the possibility to assess the nigrostriatal function on the fine manual dexterity performance.

**Spontaneous activity time**

In the case of MPTP studies, quantification of the animal movement is indispensable in order to study the motor impairments. Rather than assessing motor symptoms with a rating scale, which depends on the subjective observer scoring according to his experience, an automatic video image analyzer system (VigiePrimate®) provides a more objective and reproducible method to investigate the free motor behavior of the animal (Chassain et al., 2001). In the present study, three levels of activity (freezing, mild and burst activities) were defined in order to quantify the spontaneous activity of monkeys during daily sessions of 30 minutes. All four monkeys exhibited an increase of time spent in freezing activity.

In the asymptomatic parkinsonian monkey (Mk-LY), a freezing activity augmentation of 58% as compared to pre-lesion plateau was observed. This non-consistent result (17% of DA loss in Mk-LY) does likely not reflect bradykinesia and/or akinesia. Indeed, a decreased level of DA about 60% is necessary to generate cardinal symptoms such as bradykinesia. This discrepancy in Mk-LY may be explained by cognitive changes after the MPTP treatment. Indeed, during the pre-lesion phase Mk-LY
showed stereotypic behavior during the activity session. It has been shown that nigrostriatal pathway plays a role in the production of stereotypies (Fog & Pakkenberg, 1971; Ridley, 1994; Graybiel et al., 2000). An intra-striatal injection of DA agonist in rats into the striatum produced stereotypies. It might be possible that 17% DA depletion is sufficient to reduce the stereotypy. Furthermore, recent studies claimed that cognitive declines induced by MPTP (Vezoli et al., 2011) and social behavioral changes in MPTP-treated monkeys (Durand et al., 2015) are developed during the pre-symptomatic motor state. Thus, it might be plausible that cognitive changes emerged in Mk-LY despite a weak DA depletion. Indeed, it has been demonstrated that PD patients (in early stage of the disease) suffering of cognitive deficits, have less striatal $^{18}$F-dopa uptake as compared to healthy subjects ($K_i$ values 10% to 40% smaller than the control subjects) accompanied with stronger uptakes in frontal areas (medial frontal cortex, dorsolateral prefrontal cortex and anterior cingulate) (Brück et al., 2005).

Interestingly, after the ANCE transplantation, Mk-LY exhibited stable time spent in freezing activity as compared to post-lesion phase. However, the $^{18}$F-dopa PET scan showed a total recuperation in DA activity. The reducing stereotypy-behavior could be elicited by a complex striatal activity change. Indeed, responses of neurons in dorsal striatum during amphetamine-induced focused stereotypy (in rats) are changed and provide the evidence that stereotypy is driven by multiple synaptic mechanisms (Rebec et al., 1997). In Mk-LY, these mechanisms could be maintained after the ANCE transplantation. However, Mk-LY showed a lot of variability (see modified Brinkman board task) and this could explain this stable time spent in freezing activity after the ANCE transplantation.

The other three monkeys showed a longer time spent in freezing activity, likely due to bradykinesia and akinesia (consistent with 80% of DA depletion). In Mk-LO, the time spent in freezing activity was enhanced by 183% as compared to pre-lesion baseline. Although MK-LO was considered as “recovered parkinsonian monkey” by the rating scale, the sensitivity of the video analyzer system may ensure a finest way to measure motor deficits (especially bradykinesia) after MPTP lesion (Chassain et al., 2001). The inter-individual MPTP sensitivity can be seen in Mk-MY with a dramatic increase of 243% of the time spent in freezing activity. In Mk-MI, an increase in time spent in freezing activity of 95% was observed. Both monkeys spent more or less the same time in freezing activity after the lesion. However, Mk.MI spent the highest time in freezing activity before the lesion (reference value), as compared to the other three monkeys. A cognitive cause explaining these increases in freezing activity cannot be rejected (they also exhibit stereotypic behavior as Mk-LY). Nevertheless, bradykinesia seems more likely (up to 80% of DA depletion). After the ANCE transplantation, significant recoveries were observed in the three animals. Percentages of recovery were inversely related to their severity of motor symptoms evaluated with the rating scale (and with the modified Brinkman board task). Indeed, Mk-LO showed the better recovery (35%), whereas Mk-MI exhibited the worst recovery (5%). This observation might be logical: the more the lesion affected the animal; the more it takes time to exhibit recovery. This phenomenon was also seen in spontaneous recovery after MPTP treatment (Schneider et al., 1995; Blesa et al., 2012). The functional recovery after the ANCE transplantation could be a result of a beneficial effect of the transplantation (Bloch et al., 2014).
Modified Brinkman board task

The fine manual dexterity performance can be quantitatively assessed with the modified Brinkman board task by measuring two parameters: (1) the score, reflecting the global voluntary movement (from reaching the pellet to bringing it to the mouth); (2) the contact time (CT), reflecting the precise manipulation of the pellet between the thumb and index finger (precision grip) (Schmidlin et al., 2011).

Mk-LY (asymptomatic monkey) and Mk-LO (recovered monkey) showed intra-individual variability in the manual dexterity performance. Their intra-individual variability is surprising as the monkeys were trained for two years before the pre-lesion phase. Indeed, motor learning leads to an optimization of motor performance (score as well as CTs) and a decrease of intra-individual variability (Kaeser et al. 2014; Chatagny et al., 2013). Over-training and possible biases due to inattention and/or lack of motivation could explain this intra-individual variability (Chatagny et al., 2013). Indeed, in the score, it is impossible to discriminate the motor performance from the strategy used (2 or 4 pellets taken together) and/or from the number of errors (pellet dropping). When looking at the CTs both monkeys showed much less intra-variability as the CT measures the time spent into the slot. During the entire experiment Mk-LY showed no change in score (except an increase of the score post-transplantation compared to pre-lesion phase for the left hand, probably explained by intra-variability) as well as for the CTs (despite a significant post-lesion increase for the left hand in vertical slots, also explained by intra-variability because non-significant change was observed in the post-lesion phase and transplantation phase as well as post-transplantation phase and pre-lesion phase). Moreover, the temporal sequence of prehention in Mk-LY showed also intra-individual variability during the entire experiment. In Mk-LO, a decrease of the score post-lesion was seen but remained stable after the transplantation. The decrease is also explained by the intra-individual variability as Mk-LO had stable score just after the lesion and progressively decreased. Moreover, after the transplantation, the score increased, followed by a decrease. This pattern of motor performance reflects well the intra-individual variability of the animal. Indeed, sometimes Mk-LO performed the task adequately (one pellet after another) but sometimes took several pellets and kept them into the palm before bringing them to the mouth (as seen in Kaeser et al., 2014). Mk-LO exhibited a decrease of the CTs throughout the experiment for the left hand (the right hand remained stable) also reflecting the intra-individual variability. The results in the modified Brinkman board task are consistent for both animals according to their general assessments: asymptomatic and recovered parkinsonian monkeys. Indeed, no changes were observed in the execution of voluntary movement in the modified Brinkman board task after the MPTP lesion (also see temporal sequence of prehention in Mk-LY). On the contrary to Mk-LY and Mk-LO, the other two monkeys showed stable motor performance during the pre-lesion phase.

In Mk-MY, drops of 23% (for the left hand) and 28% (for the right hand) of score as compared to pre-lesion were observed. Mk-MI showed a loss of 99% of motor performance after the lesion. The CTs showed less disruption as compared to the score in Mk-MY (Mk-MI retrieved only 8 pellets in the vertical slots and 3 pellets in the horizontal slots, consequently no statistical analyzes were performed). Moreover, the temporal sequence of prehention showed a change in strategy in Mk-MY, probably due to rigidity. Indeed, proximal movements (arm flexion) seem more difficult to perform. In
PD, the impairment of voluntary movement is mainly characterized by bradykinesia (the deficit in the sensorimotor processing results as a generalized slowness of movement) and rigidity (for review: Lukos et al., 2014). However, it remains unclear if the deficit in finger dexterity is due to bradykinesia or to limb-kinetic apraxia (LKA, distinctive disorder affecting manual dexterity and motor performance over and above the CST and/or BG disorder). In PD patients, LKA deficits respond marginally to conventional DA treatment as compared to bradykinesia. Moreover, it has been shown, again in human subjects, that LKA may be related to an intrinsic dysfunction of the primary somatosensory cortex (S1) and/or a disruption of the fronto-parietal circuits involved in grasping and manipulation of objects. Thus, several studies argue that impairments in fine manual dexterity is mainly due to LKA rather than bradykinesia (Leiguarda et al., 2003; Quencer et al., 2007; Gebhardt, 2008; Foki et al., 2015). Nevertheless, the post-lesion results in Mk-MY (and Mk-MI) suggest that the deficit in fine manual dexterity occurred as a result of cardinal symptoms (bradykinesia and rigidity). Moreover, CTs recovered one month after the transplantation. The increase during post-lesion phase was mainly due to tremor once the pellet reached. This observation supports that cardinals symptoms may be involved rather than LKA. The fact to reject LKA as impairment in manual dexterity performance reinforces bradykinesia as a result in the increase of the freezing activity (see above). Indeed, it has been shown that LKA emerged in later stage of PD when cerebral cortex is affected resulting as a cognitive-motor model of praxis (Vanbellingen et al., 2011).

At least 40 days after the transplantation, Mk-MY reached a stable plateau of recovery, which represented 92% (for the left hand) and 93% (for the right hand) of the pre-lesion plateau, whereas in Mk-MI the plateau was reached 62 days after the transplantation, representing 63% of the pre-lesion plateau. For both monkeys, the amelioration of the score is likely due to the transplanted cells rather than spontaneous recovery. Mounayar and colleagues (2007) have shown that the total spontaneous recovery was reached one month after the MPTP lesion protocol. In Mk-MY, the post-lesion plateau was defined one month after the last MPTP injection (spontaneous recovery can be seen during the first month in both the score and the temporal sequence of prehention). In Mk-MI, the post-lesion plateau was not defined because Mk-MI was not able to perform the task few weeks after the last MPTP injection. Moreover, the post-transplantation plateaux were reached at least 40 days after the transplantation (about 70 days after the last MPTP injection). With these time intervals, spontaneous recovery could be rejected to be involved in the recovery. Furthermore, Bloch and colleagues (2014) have shown that ANCE transplantation enhances biological and behavioral damage in MPTP-treated monkeys. A rebound of motor performance may emerge later, leading to a second post-transplantation plateau as seen in the case of motor cortical lesions (Kaeser et al., 2011).

**ANCE transplantation effect and future perspectives**

Overall, the MPTP lesion engendered motor symptoms and degeneration of DA neurons at least in three monkeys. Their motor symptoms have been largely restored after the ANCE transplantation in accordance with Bloch et al. (2014). Interestingly, the post-transplantation PET scan in MK-LY
suggests a restoration of the nigrostriatal system. 100% of recovery of DAergic system by compensatory mechanisms has not been shown yet in MPTP monkeys (Blesa et al., 2012; Vezoli et al., 2014). This suggests that implanted cells “re-boosted” the remaining DAergic system probably by releasing neurotrophic factors (GDNF and/or BDNF) (Brunet et al., 2009; Bloch et al., 2014). However, it seems improbable that the implanted cells could restore at 100% the DAergic system in the three other monkeys. Indeed, Mk-LY suffered of only 17% of DA depletion against up to 80% in the three other monkeys. Nevertheless, an increase remains an open possibility. In asymptomatic monkeys (Brunet et al., 2009), implanted cells did not become TH-positive cells and consequently did not participate to cell replacement. Interestingly, TH-positive cells were enhanced after the ANCE transplantation supporting a neuroprotection effect. GDNF were largely used in order to improve motor symptoms in various animal model of PD (e.g. for review: Kordower et al., 2000; Olanow & Jankovic, 2005; Capitanio & Emborg, 2008). Promising results have even led to clinical trials in phase II but failed probably because the drug bioavailability (delivered with catheter) was limited to a small portion of the human putamen (Salvatore et al., 2006). Recent techniques using adeno-associated virus with GDNF (AAV-GDNF) have shown promising outcomes (e.g. for review: McCown, 2011). However it requires genetic manipulations and could be less stable than ANCE transplantation (same genetic background). Moreover, the implanted cells may ensure several functions such as migration and other type of neurotrophic factors release (Brunet et al., 2009; Kaeser et al., 2011; Bloch et al., 2014). In contrast to other types of cell therapies (see introduction), ANCE transplantation did not show any signs of tumor formation and/or rejection (immunosuppressive treatment is not required) (Brunet et al., 2009, Kaeser et al., 2011; Bloch et al., 2014). In the present manuscript, the fate of implanted cells remains uncertain but show promising and positive outcomes. The post-transplantation PET scans in the three remaining monkeys and the histological readout will confirm whether the implanted cells migrated, survived, delivered neurotrophic factors and enhanced the remaining DAergic system.
V. Conclusion

The present study based on autologous neural cell ecosystems (ANCE) transplantation provides a proof-of-principle evidence that non-human primate models of Parkinson's disease integrate the grafts and show an enhancement of motor functions. Nevertheless, the histological readouts are missing in the present manuscript letting the veritable implanted cells fate in question. In contrast to other cell therapies, ANCE is not subject of ethical controversies, immune rejection, tumor formation and/or high rate of cell death. Furthermore, a major role of neuroprotection has been shown with ANCE in non-human primates model of Parkinson's disease. Thus, ANCE transplantation represents an attractive approach in order to recover from brain dysfunction or brain lesion.

The behavioral follow-up as well as the in vivo investigation with Positron Emission Topography (PET) scans allows the validation of the well-known complexity of non-human primate models of Parkinson's disease. Moreover, fine manual dexterity assessments provide new interrogations in the general knowledge of basal ganglia functions in non-human primates.
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VII. Appendix

1. Impact of dlPFC biopsy in fine motor tasks execution

A pilot biopsy was performed in the dorsolateral prefrontal cortex (dlPFC), in all four animals, five months before the pre-lesion phase in order to evaluate the possible impacts of such biopsy on motor tasks execution. Behavioral assessments were the “modified Brinkman board task” (as explained in the present manuscript) and a “reach and grasp drawer task”. In the “reach and grasp drawer task”, monkeys had to pull open a drawer against increasing resistances (R0, R3, R5). The force required opening the drawer (load force) and the force applied on the knob (grip force) were recorded (Figure 31). One of the animals (Mk-MI) was excluded from the reach and grasp drawer task analysis as the task was not executed adequately. Here results are briefly presented.

**Figure 31**: (A) Representation of the «reach and grasp drawer task» setup with the adjustable resistances in Newtons. (B) Raw data curves are shown for a single trial: 1. The “tic” represents the time point when the monkey touched the knob of the drawer. 2. The “tic” represents the time point when the drawer started to open. 3. The displacement of the drawer. 4. The grip force (the force applied on the knob in between the thumb and the index finger) and 5. The load force (the force applied to open the drawer) against the resistances.

As expected for a small biopsy volume in dlPFC (Figure 32), there was no significant change both in motor performance (number of pellets retrieved) and in motor strategy (temporal sequence of visiting the slots) for the “modified-Brinkman board task”, in line with a previous report (Kaeser et al., 2013) (Figure 33). In contrast, in the “reach and grasp drawer task”, a significant decrease of the maximum grip force was observed post-biopsy in 3 animals. In Mk-MY, the maximal grip force decrease was present for both hands and at all resistances (Figure 34C) whereas, in Mk-LO, the effect on grip force was restricted to the ipsilesional hand (Figure 34E); in addition, a decrease of the load force was largely observed in the ipsilesional hand (Figure 34F). Mk-LY showed a decrease of maximal grip force for each hand, except for one resistance (Figure 34A); in addition, the load force was affected in the contralesional hand.
To sum up, in Mk-LO, the ipsilesional hand was more affected (in load force and grip force) than the contralesional hand (only the load force at R5); the biopsy was located more caudally than in the other two monkeys. In addition, the load force amplitude in Mk-LY was only affected in the contralesional hand and the grip force decrease in both hands (except for one resistance); the biopsy was located more rostrally than in Mk-LL. Mk-MY, in which the biopsy was located more rostrally than in the other two monkeys, showed a decrease only for the grip force at all resistances and for both hands.

**Mk-LY - Volume of dlPFC biopsy = 14 mm$^3$**

![Image A](A.png) ![Image B](B.png) ![Image C](C.png)

**Mk-MY - Volume of dlPFC biopsy = 7 mm$^3$**

![Image D](D.png) ![Image E](E.png) ![Image F](F.png)

**Mk-LO - Volume of dlPFC = 7 mm$^3$**

![Image G](G.png) ![Image H](H.png) ![Image I](I.png)

Figure 33: The color coded graphs show the temporal sequences and the motor performance in the modified-Brinkman board task, respectively. The x-axis displays the sessions from the biopsy (biopsy = session 0). (A,D) Graphs illustrate the right-left temporal sequences. The y-axis shows the right-left positions of the 50 slots. (B,E) An example of a right-left temporal sequence with the corresponding color code in an individual daily session. The blue color represents the first slot visited by the monkey. The red color is the last slot visited by the monkey. (C,F) Graphs show the motor performance (number of pellets taken in 30 seconds = score). N.B.: comparable results were obtained in Mk-MI and Mk-LO (only in motor performance; the temporal sequences could not be measured as the task was not performed adequately).
Figure 34: The graphs show the quantitative assessment in the “reach and grasp drawer task”, separately for the 3 resistances R0, R3 and R5. For each resistance, the left hand (LH) and the right hand (RH) are presented. Box plots are composed of all trials before (pre) and after (post) the cortical biopsy. Statistical analyses (t-test/Mann-Whitney test) compare maximal grip and load forces between pre-biopsy and post-biopsy sessions for each resistance and for each hand. Statistically significant differences are indicated with: * is for p≤0.05, ** for p≤0.01, *** for p≤0.001. «n.s» meaning statistically non-significant. (A,B) The graphs show the maximal grip force and maximal load force for Mk-LY where the right hand is the contralesional hand. (C,D) The graphs show the maximal grip force and maximal load force for Mk-MY where the right hand is the ipsilesional hand. (E,F) The graphs show the maximal grip force and maximal load force for Mk-LO where the right hand is the contralesional hand.
The results suggest a contribution of the dlPFC in the control of the grip force amplitude in monkeys, more precisely in the prediction of the grip force required. Indeed, at each resistance tested, the monkeys performed ten consecutive trials, allowing prediction based on working memory of the grip force to be produced (except for the first trial when the resistance was changed). These data in non-human primates are consistent with a recent report in human subjects, arguing for a role of dlPFC in the prediction of grip force (Wasson et al., 2010). However, variations between the three monkeys may be due to different positions and sizes of the dlPFC biopsies. These various positions suggest a more pronounced contribution of the rostral dlPFC in the grip force prediction. Moreover, indirect interactions between dlPFC and primary motor cortex (M1) (contra- and ipsi-lateral) may vary according to either the rostro-caudal axis and the type of movement (precision grip, proximal movement,...).

References:

2. Stereotaxical coordinates of implantation sites

**Mk-LY**

**Caudate nucleus**
Medio-lateral: 5.5 mm  
Rostro-caudal: 23.3 mm  
Vertical: 14 mm

**Putamen 1**
Medio-lateral: 10.7 mm  
Rostro-caudal: 20.8 mm  
Vertical: 15 mm

**Putamen 2**
Medio-lateral: 12.7 mm  
Rostro-caudal: 16.4 mm  
Vertical: 17 mm

The stereotaxical coordinates were based on the interaural and orbital ridge references. For the right hemisphere, same coordinates were used. Vertical coordinates corresponded to the vertical distance between the dura-mater and the site.
The stereotaxical coordinates were based on the interaural and orbital ridge references. For the right hemisphere, same coordinates were used. Vertical coordinates corresponded to the vertical distance between the dura-mater and the site.
The stereotaxical coordinates were based on the interaural and orbital ridge references. For the right hemisphere, same coordinates were used. Vertical coordinates corresponded to the vertical distance between the dura-mater and the site.
The stereotaxical coordinates were based on the interaural and orbital ridge references. For the right hemisphere, same coordinates were used. Vertical coordinates corresponded to the vertical distance between the dura-mater and the site.
3. Motor symptoms evaluation using the Schneider rating scale

Figure 35: Quantitative evaluation of the motor symptoms during the MPTP lesion protocol (lasting for 6 weeks) in order to adjust the MPTP dosage. All the four animals exhibited motor symptoms (especially tremor, bradykinesia and impairment in lower limb movement) three weeks after the first MPTP injection (A,B); in Mk-LY and Mk-LO, the motor symptoms emerged at the end of the MPTP lesion protocol and progressively decreased two weeks after the last MPTP injection. (C) In Mk-MY, the motor symptoms progressively appeared during the MPTP lesion protocol and were stable through the months after the last MPTP injection. (D) Mk-MI showed virtually no motor symptoms during the MPTP lesion protocol. Few days after the last MPTP injection, motor symptoms exponentially emerged and remained stable for several months.
<table>
<thead>
<tr>
<th>Lower limb movement</th>
<th>Bradykinesia/akinesia</th>
<th>Hyperkinesia</th>
<th>Tremor (note if resting or intention and which body part)</th>
<th>Dystonia (note affected body part)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(noted separately for left and right)</td>
<td>0 – normal</td>
<td>0 – absent</td>
<td>0 – absent</td>
<td>0 – absent</td>
</tr>
<tr>
<td>1 – noticeable decrease in capacity and frequency of limb use</td>
<td>1 – mild decrease in grasping food; occasional dropping of food</td>
<td>1 – mild increase in spontaneous movements</td>
<td>1 – slight and intermittent</td>
<td>1 – mild reduction, but able to extend limb almost completely</td>
</tr>
<tr>
<td>2 – severe decrease in capacity and frequency to use limb</td>
<td>2 – moderate decrease in the ability to grasp/handle; drops food often; may not be able to get food to mouth – may bring mouth to food</td>
<td>2 – moderate /but definite increase in spontaneous movements</td>
<td>2 – moderate and more persistent</td>
<td>2 – moderate reduction, unable to fully extend; exhibits flexor posturing of limb at rest</td>
</tr>
<tr>
<td>3 – no effective limb movement</td>
<td>3 – severe slowing; great difficulty in initiating and maintaining movement</td>
<td>3 – severe, persistent excessive movements</td>
<td>3 – severe, present most of the time</td>
<td>3 – severe, present most of the time</td>
</tr>
</tbody>
</table>

Table 3: Table showing the motor evaluations in MPTP-treated monkeys used in the present study. A score of 0 is considered as normal whereas, a score of 3 represents the worse symptom (Schneider et al., 1995).
4. Statistical analyses: P-Values (P)

**Table 4:** P-Values, which refer to Figure 25. P-Values were obtained with: (1) One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks (KW) when the Normality Test (Shapiro-Wilk) failed) or (2) Student’s test (Mann-Whitney Rank Sum Test (MW) when the Normality Test (Shapiro-Wilk) failed). “n.s.” meaning statistically non-significant (P>0.05). pre = pre-lesion phase; post 1 = post-lesion phase; post 2 = post-transplantation phase.

<table>
<thead>
<tr>
<th></th>
<th>Freezing activity</th>
<th>Mild activity</th>
<th>Burst activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mt-LO</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = 0.001 (KW)</td>
<td>P = &lt;0.001</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = 0.003 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>pre VS post 2</td>
</tr>
<tr>
<td><strong>Mt-MY</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = &lt;0.001 (MW)</td>
<td>P = &lt;0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>P = 0.006 (MW)</td>
<td>P = 0.007 (MW)</td>
<td>pre VS post 2</td>
</tr>
<tr>
<td><strong>Mt-MI</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = &lt;0.001 (MW)</td>
<td>P = &lt;0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>P = 0.001 (MW)</td>
<td>P = 0.001 (MW)</td>
<td>pre VS post 2</td>
</tr>
</tbody>
</table>

Score in 30 seconds in modified-Brinkman board task

<table>
<thead>
<tr>
<th></th>
<th>Left hand</th>
<th>Right hand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mt-LY</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = 0.007 (KW)</td>
<td>n.s (KW)</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = 0.003</td>
<td>n.s (MW)</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>n.s</td>
<td>post 1 VS post 2</td>
</tr>
<tr>
<td><strong>Mt-MY</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = &lt;0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>P = &lt;0.001</td>
<td>pre VS post 2</td>
</tr>
</tbody>
</table>

Score total in modified-Brinkman board task

<table>
<thead>
<tr>
<th></th>
<th>Left hand</th>
<th>Right hand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mt-LO</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = 0.006 (KW)</td>
<td>P = 0.015 (KW)</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = 0.004</td>
<td>P = 0.028</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>n.s</td>
<td>post 1 VS post 2</td>
</tr>
<tr>
<td><strong>Mt-MI</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td>pre VS post 1</td>
<td>P = &lt;0.001 (KW)</td>
<td>P = &lt;0.001</td>
</tr>
<tr>
<td>pre VS post 2</td>
<td>P = &lt;0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td>post 1 VS post 2</td>
<td>P = &lt;0.001</td>
<td>pre VS post 2</td>
</tr>
</tbody>
</table>

**Table 5:** P-Values, which refer to Figure 27. P-Values were obtained with: (1) One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks (KW) when the Normality Test (Shapiro-Wilk) failed) or (2) Student’s test (Mann-Whitney Rank Sum Test (MW) when the Normality Test (Shapiro-Wilk) failed). “n.s.” meaning statistically non-significant (P>0.05). pre = pre-lesion phase; post 1 = post-lesion phase; post 2 = post-transplantation phase.
Table 6: P-Values, which refer to Figure 28. P-Values were obtained with: (1) One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks (KW) when the Normality Test (Shapiro-Wilk) failed) or (2) Student’s test (Mann-Whitney Rank Sum Test (MW) when the Normality Test (Shapiro-Wilk) failed). “n.s.” meaning statistically non-significant (P>0.05). pre = pre-lesion phase; post 1 = post-lesion phase; post 2a = January post-transplantation phase; post 2b = February post-transplantation phase; post 2c = March post-transplantation phase; post 2d = April post-transplantation phase.

<table>
<thead>
<tr>
<th></th>
<th>Left hand</th>
<th>Right hand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mk-LY</strong></td>
<td>One Way Anova</td>
<td>One Way Anova</td>
</tr>
<tr>
<td></td>
<td>P = 0.001 (KW)</td>
<td>n.s. (KW)</td>
</tr>
<tr>
<td></td>
<td>P = 0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2a</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2a</td>
<td>post 1 VS post 2a</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2b</td>
<td>p &lt; 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2c</td>
<td>p &lt; 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2c</td>
<td>p &lt; 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2d</td>
<td>n.s. (MW)</td>
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</table>

**Mk-LO**

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P = 0.001 (KW)</td>
<td>n.s. (KW)</td>
</tr>
<tr>
<td></td>
<td>P = 0.007 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2a</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2a</td>
<td>post 1 VS post 2a</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2b</td>
<td>p = 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2a</td>
<td>p = 0.019 (MW)</td>
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<td>post 2b VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2c</td>
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</tr>
<tr>
<td></td>
<td>post 2a VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2c</td>
<td>p = 0.006 (MW)</td>
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<tr>
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<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 3d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 3d</td>
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</tr>
<tr>
<td></td>
<td>post 2b VS post 3d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2c VS post 3d</td>
<td>n.s. (MW)</td>
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**Mk-MY**

<table>
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<th></th>
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<td>n.s. (KW)</td>
</tr>
<tr>
<td></td>
<td>P = 0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2a</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2a</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2d</td>
<td>P = 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2c VS post 2d</td>
<td>n.s. (MW)</td>
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</table>

**Mk-ML**

<table>
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</thead>
<tbody>
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<td></td>
<td>P = 0.001 (KW)</td>
<td>n.s. (KW)</td>
</tr>
<tr>
<td></td>
<td>P = 0.001 (MW)</td>
<td>pre VS post 1</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2a</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>pre VS post 2b</td>
<td>P = 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 1 VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2a VS post 2b</td>
<td>P = 0.001 (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2b</td>
<td>n.s. (MW)</td>
</tr>
<tr>
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<td>post 1 VS post 2c</td>
<td>n.s. (MW)</td>
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<td>post 2a VS post 2c</td>
<td>n.s. (MW)</td>
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<td>post 2b VS post 2c</td>
<td>n.s. (MW)</td>
</tr>
<tr>
<td></td>
<td>post 2b VS post 2d</td>
<td>P = 0.001 (MW)</td>
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<td>post 2c VS post 2d</td>
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</tr>
<tr>
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<td>post 2c VS post 2d</td>
<td>n.s. (MW)</td>
</tr>
</tbody>
</table>
Table 7: P-Values, which refer to Figure 29. P-Values were obtained with: (1) One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks (KW) when the Normality Test (Shapiro-Wilk failed) or (2) Student’s test (Mann-Whitney Rank Sum Test (MW) when the Normality Test (Shapiro-Wilk failed). “n.s.” meaning statistically non-significant (P>0.05). pre = pre-lesion phase; post 1 = post-lesion phase; post 2a = January post-transplantation phase; post 2b = February post-transplantation phase; post 2c = March post-transplantation phase; post 2d = April post-transplantation phase.


Brinkman J, Kuypers HGJM (1973) Cerebral control of contralateral and ipsilateral arm, hand and finger movements in the split-brain rhesus monkey. Brain 96:653–674


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