Investigating pre-clinical dopamine physiology in Parkinson Disease

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This article describes the basic anatomy and physiology of dopamine (DA) neurons in the midbrain region, the substantia nigra pars compacta (SNc). The progressive death of DA neurons in SNc results in Parkinson Disease (PD). The etiology of PD is currently unknown, but post-mortem evidences from human PD brain tissue shows two key features - “selective” loss of DA neurons in the SNc and the presence of protein aggregates called “Lewy bodies”. The neuronal loss in the SNc results in the classical behavioural symptoms seen in PD, such as bradykinesia, rigidity and resting tremor. These symptoms are presented when there is already a substantial (about 50%) loss of SNc DA neurons in the brain, making the disease incurable. We are therefore investigating the physiology of DA neurons “before” the onset of neurodegeneration. We aim to measure the electrical activity of DA neurons and the neurotransmitter release in real time, in living mouse models of PD at sub-second resolution. Any change in physiological parameters will identify a novel detection criterion for identifying the potential risk for PD.

Cellular components of the nervous system:
The basic cellular organization of neurons comprises a cell body called the soma, an extension of the cell body known as the axon and the branching neurites known as dendrites (Figure 1). Dendrites are the primary targets for the reception of neurotransmitters transmitted from axons of other neurons through a specialized region called “synapse”. The electrical signal carried throughout the neurons are called an action potential, which is a self-regenerating wave of electrical discharge (flow of ions) that propagates from the site of initiation to the dendrites. The discharge of an action potential results in the release of neurotransmitter at the synapse. Neurons are classified based on the type of neurotransmitter they release e.g., neurons that release dopamine neurotransmitter are called dopaminergic neurons; neurons that release glutamate neurotransmitter are called glutamatergic neurons. The diversity of neurons arising due to the difference in the neurotransmitter type, anatomical localization and the neuronal connectivity etc. underlies the capacity of the brain to form complicated networks that enables us to perform complex activities and sophisticated behaviours.

The communication of neurons with each other is called synaptic transmission. In detail, a conventional synapse is a junction between two dendritic/axon terminals that is separated by approximately 20-40 nm. An example of a synaptic connection is illustrated in Figure 2.
To drive the release of the neurotransmitter at the synapse, the electrical signal (action potential) must be propagated to the dendritic terminal. The transmitting neuron (pre-synaptic neuron) synthesizes the neurotransmitter and stores them inside packages called synaptic vesicles. When the neurons are electrically excited, meaning when there is an action potential generated due to the movement of ions across the neuronal membranes (in and out of neurons into the extracellular space), it mobilizes the synaptic vesicles to fuse with the membrane surface, so that the neurotransmitters are released into the synaptic cleft. Following the release, the neurotransmitter binds to the receptive neurons (post-synaptic neurons) by receptors that trigger the cellular response (Mroziewicz and Tyndale, 2010). These basic neuronal functions require metabolic support (i.e., glucose and ATP), which is supported by the regional cerebral vascular perfusion.

**Figure 2: The synapse.** Neurotransmitters, such as dopamine and acetylcholine, are chemicals that carry signals from neuron to neuron across gaps called synapses. A pre-synaptic neurons fires an action potential, resulting in the release of the neurotransmitter into the synaptic cleft. Once in the synapse, the neurotransmitter binds to the specific receptor expressed on the post-synaptic neurons to execute downstream cellular responses. The excess neurotransmitters are degraded by the degrading enzymes or taken up by the pre-synaptic neurons through a transporter for recycling the neurotransmitter. (Image source: RF. Tyndale, 2010, Addict Sci Clin Pract.)

**Why Dopamine?**
Dopamine is a neurotransmitter that is synthesized and released from dopamine producing neurons, called dopaminergic (DA) neurons. There are about a million DA neurons in the human brain and about 20,000 DA neurons in the rodent brain, constituting less than 1% of the total brain neurons. DA neurons are involved in crucial functions such as reward-based learning and memory, decision-making, habit formation, motor control and cognition.
These neurons are located in a in a “midbrain” region within two nuclei\(^1\) - the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) (figure 7).

\(^1\) Nucleus/nuclei(p) = cluster of cell bodies (soma and dendrites) of neurons in the different regions of the brain
DA neurons are highly heterogeneous in neurochemical features and anatomical connectivity. DA is synthesized from the amino acid tyrosine by a series of reactions where the action of the enzyme tyrosine hydroxylase (TH) acts as the rate-limiting step. DA neurons have huge axonal branches and form about 360,000 of striatal DA synapses per cell, with an axonal length of about 500 mm (Pissadaki and Bolam, 2013). An example of DA neuronal morphology is illustrated in Figure 3. This huge axon of DA neurons demands an extensive energy supply for its function. The neurons are interspersed in the form of a complex interconnected network in the brain. The neurons in the SNc project to a forebrain region called the striatum\(^2\), and this pathway is termed the nigrostriatal pathway (Figure 4).

DA neurons are spontaneously firing neurons that exhibit either tonic or phasic (burst) firing patterns, both of which are believed to result in the release of DA at the synapses in the striatum. In the striatum, the axon terminals of the DA neurons synapse onto the non-DA neurons, mainly comprising of medium spiny neurons (MSNs) that express either D1 or D2 dopamine receptors and cholinergic interneurons (CHIs) (Lim et al., 2014; Sulzer et al., 2016; Tritsch and Sabatini, 2012).

The electrical activity of the DA neurons in the midbrain drives DA release, thereby triggering postsynaptic DA signalling that enables downstream pathways involved in movement, cognition, decision-making etc. SNc DA neuronal signalling constitutes a portion of a complex circuit, called the basal ganglia circuit, that is critical for the motor control. Dysfunction in the basal ganglia nuclei results in movement disorders such as PD (Leisman et al., 2014). Strikingly, in PD only the DA neurons in the SNc are lost. Another nucleus containing DA neurons called the ventral tegmental area (VTA) that is located only 50 \(\mu\)m medial towards SNc in the midbrain are relatively spared/protected from

\(^2\) Striatum = a region of the subcortical (forebrain) basal ganglia components. This nomenclature is followed in rodents. In humans, the striatum comprises of two nucleus (regions) - referred as caudate nucleus and putamen.
neurodegeneration in PD. This feature is called “selective vulnerability” as the neurodegeneration is selective to SNc and not to VTA. VTA DA neurons therefore act as a control or used to study neuroprotective mechanisms in PD (Brichta and Greengard, 2014; Roeper, 2013).

**Parkinson Disease:**
PD is a chronic, age-dependent, progressive neurodegenerative movement disorder. It is characterized by particular motor symptoms - bradykinesia (difficulty in initiating movement), rigidity (stiff or inflexible muscles) and resting tremor (involuntary trembling of the hands and limbs). In addition there are non-motor and psychological symptoms associated with PD such as constipation, loss of smell, sleep disorders etc (Kalia and Lang, 2015). A pioneering study from Hornykiewicz and colleagues (Goedert et al., 2013) showed that the loss of dopamine (DA) in the brain region called striatum, is the primary cause for the motor symptoms seen in PD. Findings from pathological studies and imaging suggest that by the time neurological symptoms develop, there is already an extensive degeneration (about 50%) of SNc DA neurons and large depletion of striatal dopamine release (Figure:5) (Chesselet and Richter, 2011; Dunnett and Bjorklund, 1999; Kordower et al., 2013). Another key pathological feature seen in DA SNc neurons is the presence of intracytoplasmic protein aggregates called “Lewy bodies” that are predominantly aggregates of proteins including α-synuclein, and ubiquitin (Junn et al., 2003).

![Figure 5: Rate of neuronal loss in the SNc in PD.](image)

**Figure 5:** Rate of neuronal loss in the SNc in PD. There is a 40-50% of dopamine neuron loss in substantia nigra (SNc) by the time the motor symptoms present. Note the rapid loss of neurons as the disease progresses from the time of symptom detection (solid curve). (Image source: review from A. Björklund, 1999, Nature)

![Figure 6: Clinical pathology of PD in human brain.](image)

**Figure 6:** Clinical pathology of PD in human brain. Cross-section of human brain in control (a) and PD (b) condition. Note the complete absence of DA neurons (black outline in a&b) in PD brain. The bottom panel shows the high resolution image of DA neurons in the SNc in control and in PD (c,d) respectively. High-resolution image of a single DA SNc neuron containing Lewy bodies (e). (Image source: http://neuropathology-web.org/chapter9/chapter9dPD.html)

Figure 6 shows the cross-section of human brain in PD (a) and normal (b) conditions. As above, the DA neurons in the SNc (outlined) are completely lost in this PD subject (a). The surviving DA neurons in PD contain Lewy bodies that constitute a primary detection criterion. Note that by the time a subject is

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3 Neurodegeneration = death (degeneration) of neurons
4 Motor = movement related
5 Dopamine = a neurotransmitter
diagnosed, there is already a high loss of SNc neurons and it is too late to effectively implement therapeutics (Somayaji, 2016). Therefore, our aim is to understand the physiology of DA neurons prior to symptom onset to provide alert for potential neurodegeneration in the future and so that preventative therapy can be effective.

**Advantages of using a mouse model to understand human disease**
The common mouse (*Mus muscularis*) has about 90% genome and neural network similarity with that of humans, making it a suitable models to understand the etiology and pathophysiology of the human diseases (figure 7) (Stergachis et al., 2014). In addition to its small size and easy maintenance, the ease with which genetic manipulations are executed, makes it one of the highly manipulatable animal models to understand the brain function. A good mouse model provides a valuable tool to generate models of human disorders, elucidate the mechanisms of pathophysiology and for preclinical testing of potential therapeutic drugs. However, one must be critical wary that mouse models never reproduce the full phenotype of any human disorder and an appropriate animal model requires investigation and careful literature review.

![Figure 7](image)

*Figure 7:* Schematic illustration of the human (left) and the mouse (right) brain showing the nigrostriatal projection. A mouse model is widely used as a surrogate for the human brain to investigate the nigrostriatal pathways and its pathophysiology in PD.

**Neurochemical, Anatomical and Electrophysiological Identification of DA neurons in the midbrain:**
For my previous study, we used a genetically modified mouse line that contains a mutation in the gene, known to increase the risk for PD in humans – the α-synuclein (*SNCA*) (Subramaniam et al., 2014; Venda et al., 2010). This mouse model does not present any classical PD features such as neurodegeneration or Lewy body aggregates, but shows mild motor symptoms and DA release abnormalities in the striatum (Kurz et al., 2010). I therefore chose this model to study the physiology of the vulnerable DA neurons in response to a stressor, in their pre-symptomatic phase where there are no motor symptoms. We chose to study the physiology of neurons *in vivo* so that the entire neuronal network is preserved and this feature mimics the reality.

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6 Etiology = the cause, set of causes, or manner of causation of a disease or condition  
7 Pathophysiology = The disordered physiological processes associated with disease or injury  
8 Phenotype = the set of observable characteristics of an individual resulting from the interaction of its genome with the environment  
9 *In vivo* = effects tested in a living organism
As the experiments are performed when the animal is alive, extreme care is taken and rigorous procedures followed so that the mice do not feel the pain and the experiments are performed according to ethical standards. The mouse is anesthetized with isoflurane, a commonly used inhalation anaesthetic. One anaesthetized, the mouse is head-fixed onto a stereotaxic apparatus. This apparatus is connected to isoflurane anaesthesia that is inhaled and exhaled, so that the mouse is asleep throughout the neuro-surgical and recording procedure. One the mouse is fixed on the set-up and adequately anaesthetized, the skull is exposed to find the coordinates to approach midbrain, where the DA neurons are located. A small hole is drilled through the skull on the exact location that enables the recording electrode to navigate deep into the region of interest. The recording electrode is a tiny glass pipette (1 µm diameter) that contains conductive solutions to measure the voltage changes within the brain, which occurs as a result of ion flow across the neuronal membrane. The DA neurons are then identified in vivo by their unique electrophysiological properties such as – firing frequency between 2-8 Hz, broad action potential duration and shape of their action potential (Ungless and Grace, 2012). DA neurons are electrically active, meaning they produce spontaneous and continuous electrical activity. There are basically two types of electrical discharge patterns of DA neuronal activity – tonic firing (2-5Hz) in which DA neurons discharges the electrical activity (also called firing activity) at regular intervals, and phasic firing, meaning the electrical discharge occurs as packages of bursts (Subramaniam et al., 2014; Ungless and Grace, 2012). The tonic firing pattern maintains a basal level of DA in the brain while the phasic activity results in a massive release of DA that occurs in response to events such as reward and pleasure. Once the neurons are identified electrically, they are recorded for 10 minutes, following which the recorded neuron is selectively labelled in vivo in order to identify the neurons post-hoc by immunocytochemistry. Immunocytochemistry is a technique that is used to visualize a protein in the neuron using fluorescent antibodies. This method allows us to obtain the following information – 1. To identify and record the electrical activity of neurons; 2. To precisely identify the position of the neuron that is recorded in the brain in vivo.

Results from my Ph.D. research performed on a mutant α-synuclein overexpressing (A53T-SNCA) mouse model showed that, in the pre-symptomatic phase, the DA neurons have a higher in vivo firing frequencies compared to control animals. This phenotype was very selective, meaning the neighbouring DA population in the VTA does not exhibit this change in the firing properties. Figure 8 shows that the SNc neurons project to dorsal striatum and the VTA neurons project to the ventral striatum, respectively. Despite their close proximity of these DA neurons within the midbrain, these two DA nuclei modulate

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10 Stereotaxic = A method in neurosurgery and neurological research for locating points within the brain using an external, three-dimensional frame of reference usually based on the reference atlas
11 Recording electrode = a conductor device using which the flow of ions could be measured
12 Electrophysiology = Electrophysiology is the science and branch of physiology that pertains to the flow of ions (ion current) in biological tissues and, in particular, to the electrical recording techniques that enable the measurement of this flow
13 Post-hoc = examining the data after the experiment has concluded
different functions and are differentially vulnerable in PD. VTA DA neurons are involved in modulating learning and memory, cognition and decision making, whereas, SNc DA neurons execute movement via basal ganglia pathway.

Figure 9 shows the 10 sec original extracellular in vivo single-unit recording trace from SNc DA neurons in middle-aged control (A) and A53T-SNCA (B) mice (scale bar: 0.2 mV, 1s). The bottom panel shows corresponding 30s raster plots (scale bar: 1s). The SNc DA neurons in the middle-aged A53T-SNCA mice shows a higher in vivo discharge frequency.

As illustrated in figure 10, the extracted timestamps for the 600s spike-train were plotted into 10ms bins to yield interspike interval histogram (ISIH). ISIH gives a qualitative measure for the regularity and firing pattern of the spike-train. Irregular neuron has a broad ISIH (figure 10A), whereas, a pacemaker has very narrow ISIH (figure 9B). The insert shows the averaged action potential (AP) waveform in high resolution. The bottom panel in figure 10 shows a detailed neurochemical identity of the recorded SNc DA neuron. The success of the juxtacellular labeling was confirmed histologically when the DA neuron showed an immunocytochemical signal for neurobiotin (Nb, red). The DA neurons were always positive for tyrosine hydroxylase (TH, green), a rate-limiting enzyme in the DA biosynthesis that confirms that these are DA neurons. With these two neurochemical identification approaches, the precise anatomical position of the recorded neuron was mapped (bottom right). The immunocytochemical staining moreover provides details regarding the morphology of the SNc DA neuron (the illustrated confocal image shows bipolar SNc DA neuron). This study showed for the first time that DA neurons exhibit increased excitability before the onset of neurodegeneration in A53T-SNCA mouse model (Subramaniam et al., 2014). One of the follow-up questions that remains to be tested (aim1 of the ongoing project, see below) is – does the increase in the firing frequency also result in increased DA release at the axon terminals located in the striatum. Since α-synuclein is a pre-synaptic protein and has a critical role in regulating the fusion of DA vesicles with the membrane, the direct correlation of increased firing frequency with increased release might not be possible. This result will lead to a very novel understanding and interpretation of the local microcircuitry and the role of α-synuclein in the basal ganglia pathway.
Figure 10: ISIH and anatomical localization of SNc DA neurons. ISIH and averaged single action potential waveform (scale bar: 0.2mV, 1ms) of middle-aged control (A) and A53T-SNCA mice (B) respectively. Bottom, confocal images of juxtacellularly labelled neuron (Nb, red), TH (green) (scale=10µm), a marker for DA neurons. The bottom right panel shows the exact localization of the recorded and labelled neuron within the SN. The drawing of the coronal midbrain section was taken from a mouse brain atlas [Franklin and Paxinos, 2007]. Adapted from the Ph.D thesis of Mahalakshmi Subramaniam, Goethe University, 2014.

Figure 11A showed a significantly higher spontaneous in vivo firing frequency of SNc DA neurons in the middle-aged A53T-SNCA mice, compared to respective controls. The mean CV and SFB, showed no differences within middle-aged control and A53T-SNCA SNc DA neurons (figure 11 B,C).

Figure 11: Higher in vivo firing frequency in middle-aged A53T-SNCA mice. (A) Significant increase in the in vivo spontaneous discharge frequency in middle-aged A53T-SNCA mice. No changes in the CV (B) and SFB (C) of SN DA neurons within middle-aged A53T-SNCA mice and control. The line in the box plot represents the median, filled diamond symbol represents the mean, the whiskers are the 25th and 75th percentile respectively and the boundary of the box represents the inter-quartile range. Mann-Whitney U, *** = p<0.0005 ; ns = not significant. Adapted from the Ph.D thesis of Mahalakshmi Subramaniam, Goethe University, 2014.
As a follow-up of the described data, I will be investing the DA physiology at the axon terminals in the upcoming projects. In addition, I will be investigating the role of DA in the process of decision making during appetite-control. To execute these experimental methods, new techniques should be established and integrated into the existing methods available in the laboratory. The critical issue is the integration of \textit{in vivo} electrophysiological recordings with \textit{in vivo} cyclic voltammetry. This new technology will enable us to measure the electrical activity in real time, together with the release of DA at the axon terminals. This is a time consuming and a challenging task as the data acquisition process is completely different and is performed at two different locations in the living mouse brain. Since the method of data acquisition is very delicate and at a very fast time scale (millisecond resolution), extreme care should be taken to evaluate the set-up and in analysing and interpretation of the correct electrical signals. There is a higher chance for acquiring false positive electrical signals and incorrect output for these experiments. Therefore establishing the robust set-up remains to be the critical troubleshooting step in executing the following experiments -

**Aim 1:** In the current project, we aim to investigate the DA release properties \textit{in vivo}, in SNCA mouse model.  
**Hypothesis:** Increased electrical activity of DA neurons neurons in the SNc result in increased release of the neurotransmitter at the axon terminals.  
**Experimental procedure:** Electrical activity of DA neurons \textit{in vivo} will be measured using extracellular recordings on a stereotaxic set-up. The DA release can be measured using a technique called fast scanning cyclic voltammetry (FSCV). If the DA neurons exhibit increased firing rates, this should translate to increased DA release in the striatum, which could be detected by FSCV.  
**Significance of the proposed research:** If the hypothesis holds true, the increased DA release could be a biomarker for early detection for a PD risk.

**Aim 2:** How does the sensory information from food of similar value and smell integrate into the brain circuits thereby enabling decision-making?  
To address these questions, I will employ two techniques simultaneously that will enable detection of released DA from the terminals as well as the electrical activity of neurons at the cell body, in a living mouse. I will be looking at the DA release as well as the electrical activity of DA neurons while the animal is consuming food that it likes (e.g., sweet solution) or while the animal is encountering a familiar smell. This will provide a first means to directly correlate DA neuronal activity with DA release and identify the various sub-groups of neurons that respond differently to the same sensory stimulus.  
The significance of the research projects is that this type of experiments are being performed for the first time and the data output will help understand the basal ganglia and DA circuitry in real-time signal transduction and during hedonic decision making process. The data will provide a platform for testing and understanding novel therapeutic targets and their outcomes. Especially for PD, if there were a pre-symptomatic phenotype that could be detected much ahead of the motor symptom onset, this would enhance the understanding of PD etiology and the quality of life of at-risk PD subjects, significantly.
References: