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**Golgi staining of neurons: Oxidation-state dependent spread of chemical reaction matches with a testable property of the connectome**

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**Abstract**

In 1873, Camillo Golgi reported reticular nature of the nervous system by a newly developed staining method. In 1888, Ramón y Cajal modified this protocol and obtained staining restricted to individual neurons, which agreed with the cell theory. Close examination shows that Golgi used a single oxidizing agent to treat brain tissue prior to the staining chemical reaction and Cajal used an additional oxidizing agent during the same step. This indicates that the oxidation state of the tissue has a crucial role in determining the extent of spread of Golgi chemical reaction between neurons. Since lateral spread of the Golgi stain indicates extra-synaptic spread of the reaction, it leads to questions, “Are there oxidation state-dependent functional gates operating between the membranes of neurons?” “Does an examination of brain functions reveal any feasible support for their presence?” Since learning-generated changes are expected to reverse following short duration of working memory, gradual increases in blood oxygenation level-dependent (BOLD) signals of functional MRI (fMRI) in a corresponding time frame of seconds following learning prompts to hypothesize a certain functional role for oxygen. This paper explores testable role of oxidation states in the observed similarities between the limitation of spread of Golgi staining and working memory reversal.

**Introduction**

It is necessary to have a structure-function model of nervous system organization that can provide interconnected explanations of the relationships between the brain, mind, and behavior (Lichtman et al., 2014; Swanson and Lichtman, 2016). Since current approaches to study higher brain functions primarily focus on examining behavior, it is necessary to undertake new steps to find a mechanistic explanation for the most important and unique function of the nervous system for the generation of first-person internal sensations. Hence, it is necessary to first undertake theoretical methods to identify a testable mechanism operating in a synaptically-connected circuitry that has the potential to generate first-person internal sensations together with an option to exhibit behavioral motor actions corresponding to those inner sensations. This approach may lead to finding certain hidden system features or missing nano-level connections in the system. This also highlights the importance of re-examining findings from various structural studies undertaken in the past to obtain valuable clues, which will aid in designing experiments to verify structural properties responsible for the anticipated functions.

Argument that understanding higher level neuronal functions necessitates exploration beyond the limits of the neuron doctrine (Bullock et al., 2005) highlights the necessity to thoroughly re-examine structural details of the system towards understanding the generation of first-person inner sensations of various higher brain functions. The remaining roadblock has been remaining to design an approach to study how the brain generates inner sensations. A method to circumvent this was applied by an inductive reasoning approach using constraints from a large number of findings from the system in multiple levels. The main argument used was that if it becomes possible to arrive at a solution that can explain all the findings from the system at different levels in an interconnected manner, then that mechanism is likely correct. In this approach, unmatched findings from different levels are expected to provide most valuable constraints towards solving the system (Vadakkan, 2019). This resulted in the derivation of an inter-postsynaptic functional LINK (IPL) as the key operational structural mechanism of the system (Vadakkan, 2007; 2013; 2016a). This provides an opportunity to re-examine and re-interpret results from past experiments conducted to understand the structural details of the system to identify possible hints regarding the formation of IPLs.

By 1839, Theodor Schwann and Matthias Jakob Schleiden made major contributions to the cell theory (Sharp, 1921) that described cells as the basic structural units in all the organisms. However, there were huge difficulties for staining and observing cells in the brain. Otto Deiters provided a comprehensive description of a nerve cell before his death in 1863. Later, his work was edited and published by Max Schultze (Schultze, 1865). Deiters postulated that dendrites of neurons fuse to form a continuous network. In 1871, Joseph von Gerlach put forward the reticular theory that explained brain cells as a reticulum of cells. In 1873, Camillo Golgi invented Golgi staining method (Golgi, 1873; 1878; 1898; 1906) by first treating the brain tissue with potassium dichromate (K2Cr2O7) followed by a reaction with silver nitrate (AgNO3). This chemical reaction generates a black stain limited to the cell membrane (Koyama, 2013) that spreads between several cells in the brain to generate a continuous network (Golgi, 1906). Golgi’s findings agreed with the reticular theory.

Santiago Ramón y Cajal improved the Golgi staining by using a method that he termed "double impregnation” by adding osmium tetroxide (OsO4) to potassium dichromate solution used by Golgi (De Carlos and Borrell, 2007). Cajal found thatchemical reaction is mainly restricted to individual neurons in the presence of OsO4. This led Cajal to report that the nerve cells are not reticular in nature; but are discrete individual cells (Cajal, 1888; 1894; 1906; 1909) in agreement with the cell theory, which became the basis of neuron doctrine. In the 1950s, electron microscopical (EM) studies showed features that match with arguments in the neuron doctrine. Dye injected into the cytoplasm of individual neurons remained within those neurons, which also supported Cajal’s interpretations. Furthermore, it is possible to grow individual neurons from primary neuronal cultures.

What is the chemical basis for the restriction of Golgi stain to individual neurons in Cajal’s modification, in contrast to the original Golgi staining method? Different interpretations were made to explain this (Millhouse, 1981; Spacek, 1992). Even though these interpretations suggest that the inter-neuronal spread of chemical reaction likely occur under certain conditions through a route other than inter-cytoplasmic route, nature, and properties of the path of spread have been remaining elusive. In the context of the lack of a mechanistic explanation for a) controversial arguments made by Golgi regarding Cajal’s findings (Golgi, 1906), b) continuing dissatisfaction for the explanations for interpretations of Golgi staining results (Sotelo, 2011; Mazzarello, 2018), and c) the fact that we have not yet understood how the nervous system operates to generate first-person properties, it is necessary to fully explore the chemical basis of Golgi staining reaction. Since Golgi reaction preferentially takes place in the dendritic area and not the axonal region, it is necessary to examine the potential nature of chemical reaction between dendrites of neurons that allows the spread of staining reaction between them. It is also necessary to examine whether elements responsible for spread of chemical reaction have the potential to involve in the establishment of any electrical connection between neurons *in vivo* under physiological conditions.

Since Cajal, different modifications of Golgi staining were made (Braitenberg et al., 1967; Smit and Colon, 1969; Ito and Atencio, 1976; Blight, 1978; Somogyi and Smith, 1979; Landas and Phillips, 1982; Ebbesson and Cheek, 1988; Angulo et al., 1994; Angulo et al. 1996; Gibb and Kolb, 1998; Zhfang et al., 2003; Friedland et al., 2006; Ranjan and Mallick 2010; Vints et al., 2019). Each one of them was carried out with an aim to better visualize structural details of dendrites, such as dendritic spines. These modifications use either potassium permanganate (KMnO4) or potassium chromate (K2CrO4) as a second oxidizing agent in addition to K2Cr2O7. This again confirms that the addition of a second oxidizing agent is necessary to restrict the Golgi stain to individual neurons.

What can we learn from Golgi’s findings and Cajal’s modification that restricts staining to individual neurons? K2Cr2O7 has been regarded as an agent that hardens brain tissue, before adding AgNO3, which facilitates sectioning of brain tissue. Both Golgi and Cajal did not refer K2Cr2O7 as an oxidizing agent. However, from the history of analytical chemistry (Szabadváry, 1966) one can infer that prevailing knowledge at the time of Golgi and Cajal was that both K2Cr2O7 and OsO4 are oxidizing agents. This may have led Cajal to qualify his modification a "double impregnation” method. Since K2Cr2O7 and OsO4 are oxidizing agents, it is necessary to understand how increased oxidation state reduces the spread of chemical reaction between neurons in an oxidation state-dependent manner. Since a) no inter-cytoplasmic communication is anticipated between neurons, b) Golgi stain spreads along the membranes (Pannese, 1999), and c) there is no trans-synaptic spread of Golgi staining reaction, it is most likely that the dye spreads through an inter-neuronal inter-membrane route under conditions of decreased oxidation state. This prompts one to ask, “What are the likely inter-neuronal structural interactions that will allow the inter-membrane spread of chemical reaction between neurons in the same neuronal order?” (**Fig.1**) “Is it possible to infer that oxidation state changes regulate a gate through which chemical reaction spreads?” “Does the oxidation state determine the formation of these structural interactions between neurons, which may provide some valuable hints regarding normal operational mechanism of the nervous system?” It is also possible to ask, “What property of the chemical reaction under less oxidizing conditions of the brain tissue allows the spread of staining reaction between neurons that permits the cells to look like a continuous single network by the original Golgi staining method?”

  

***Figure 1****. The spread of Golgi chemical reaction among neurons of the same neuronal order depends on the oxidation state of the tissue.* ***A****) Under original Golgi staining conditions, where only one oxidizing agent was used to prepare brain tissue, spread of chemical reaction occurs between large numbers of neurons producing a reticulated pattern of neurons. Here, only two neurons Neuron 1 and Neuron N along with their spines and an intervening group of spines that belong to different neurons (not shown) in between them are shown to have Golgi staining. An inter-neuronal inter-spine interaction is expected to occur during learning.* ***B****) Under increased oxidation state obtained using more than one oxidizing agent to prepare the brain tissue, spread of Golgi chemical reaction is restricted to individual neurons. This is expected to occur while performing Cajal’s modification of Golgi staining reaction. A fitting explanation for both the effect of oxidation state on reversing IPLs during brain functions and a parallel effect of oxidation state in limiting Golgi staining reaction within individual neurons can be experimentally verified.*

**Probable chemistry: Role of oxidizing agents in the spread of Golgi staining**

Under moderate oxidation states, Golgi stain reaction spreads between neurons even within a single neuronal order. In the presence of a second oxidizing agent, only 1 to 5% of neurons per sample were stained, providing visualization of individual neurons and their dendritic arbor without interference from neighboring neuronal processes in all planes. It is necessary to examine probable chemical changes that allow for differences in the pattern of spread of staining reactions under the above two conditions. K2CrO4 reacts with AgNO3 to form black colored silver chromate that clusters around the cell membranes (Pannese, 1999). Hence, it was named black reaction. Even though one view is that the staining reaction progresses from the cut ends of the neuronal processes, the exact mechanism is not yet known (Pannese, 1999). Introduction of an additional oxidizing agent restricted the black reaction to individual neurons. Even though it is viewed that hydration layer that forms a thin physical and electrical separation between neuronal cell membranes gets lost during fixation of brain tissue by aldehydes, under less oxidizing conditions spread of chemical reaction occurs between neurons through surface contact (Pannese, 1999). This leads to the question, “When membranes are in direct contact with each other, what type of an oxidation state-dependent mechanism allows the spread of Golgi chemical reaction between them?”

**Constraints from Golgi staining and other nervous system findings**

For a system whose operational mechanism is yet to be confirmed, it is necessary to simultaneously examine all the unusual findings that it exhibits in different levels with an aim to find a solution that can inter-connect all the seemingly unrelated findings like solving a large puzzle. Once it becomes possible to arrive at a theoretical solution, it may allow putting forward testable predictions that can be subjected to verifications. The following table shows some of the findings related to both Golgi staining and nervous system functions along with constraints offered by them (**Table 1**).

|  |  |
| --- | --- |
|  **Findings** |  **Constraints** |
| Under moderate oxidizing conditions, the spread of Golgi stain is initially limited to a group of neurons, including those that are present within the same neuronal order (Golgi, 1906). When the oxidation state is further increased, Golgi staining limits to individual neurons  | The oxidation state of the brain tissue determines the extent of spread of Golgi chemical reaction between neurons. Hence, an oxidation state-dependent spread of Golgi staining reaction is present |
| Golgi stain spreads along the cell membranes (Pannese, 1999). Under less oxidizing conditions, Golgi reaction can propagate from one neuron to laterally located neurons within the same neuronal order | There is an oxidation state dependent spread of Golgi staining reaction that indicates the occurrence of membrane-to-membrane spread of chemical reaction between neurons |
| Modified Golgi reaction preferably stains dendritic side of neurons | A gradient of oxidation states is present/occurs along the neuronal processes from the spines (postsynaptic terminals) to the axonal (presynaptic) terminals |
| In the absence of oxygen, brain death occurs within minutes. Lack of oxidative phosphorylation preventing ATP synthesis is one cause. Occurrence of the rapid irreversible death of brain cells faster than cells of other organs needs a compelling reason. | Lack of oxygen decreases tissue oxidation state rapidly. One testable constraint is that it causes irreversible fusion between neuronal processes that belong to different neurons at the locations of their normal interactions that result in ephaptic connections. This leads to mixing of cytoplasmic contents, which in turn causes rapid irreversible death of neurons |
| Blood oxygenation level dependent (BOLD) signals in fMRI appear following a higher brain function or artificial neuronal stimulation at the same location and reach peak values only after a delay of up to 4 seconds (Monti et al., 2010; Murayama et al., 2010)  | Normal synaptic delay is only 1 to 2 milliseconds. Both associative learning and memory retrieval can take place in milliseconds. Hence, it is necessary to explain whether delayed release of oxygen performs a function  |
| Dye injected into the cytoplasm of a neuron remains within that space | Cytoplasm is restricted within a space bounded by cell membranes  |
| Humans do not have the ability to synthesize vitamin C, which is a reducing agent | Certain functions get optimized in the absence of synthesis of this reducing agent  |

***Table 1.*** *Changes in the oxidation and oxygenation states of brain tissue cause changes in the pattern of Golgi staining and BOLD signals of fMRI respectively. Constraints offered by these findings can be used to examine whether they are interconnected in any possible manner. This may assist in searching for the mechanism of operation of the nervous system by asking the questions, “Does the path through which spread of Golgi chemical reaction between neurons get inhibited by increasing the oxidation state of the tissue in Golgi staining reaction?”, “Is this the same as a path operated during higher brain functions where increased oxidation states expected by oxygen release (as evidenced by BOLD signals) occur immediately following a higher brain function?” BOLD: Blood oxygenation level dependent. fMRI: Functional magnetic resonance imaging.*

Questions arising from the above constraints include, “Does membrane-to-membrane spread of Golgi chemical reaction indicate presence of an oxidation-state dependent channel between neuronal membranes?” “Is it possible that such a channel gets formed between membranes during brain functions such as associative learning?” (**Table 2**) “Can such a channel provide explanations for both generation of first-person inner sensations (for example, that of memory and perception) and concurrent motor actions?” Blood oxygenation level-dependent (BOLD) signal changes in fMRI following the release of oxygen that peaks nearly 3 to 4 seconds after performing a brain function. “Can oxygen released during BOLD signals inhibit this channel following learning and explain forgetting?” “Can operation of such a channel allow summated potentials to cross the threshold to fire specific neurons to generate motor actions reminiscent of the arrival of an associatively learned second item?”



***Table 2.*** *A comparison table showing testable effect of oxidation state of the tissue in maintaining or reversing inter-neuronal connections, which can determine both propagation of depolarization in vivo and spread of Golgi stain between neurons in a fixed brain tissue through a similar route. Note black staining of neurons in Golgi staining.*

**Emerging questions and testable answers**

1. **Is there any possibility for a derived mechanism of nervous system functions to operate by interaction between spines belonging to different neurons?**

It is hoped that once we understand how the brain works, we should be able to explain the details of connectome that provides all its functions. At that stage, we are also expected to explain how Golgi stain spreads between neurons across membranes, which is controlled by the oxidation state of brain tissue. In this context, it is reasonable to examine the semblance hypothesis that has derived a mechanism of inter-neuronal membrane interaction to explain generation of first-person inner sensations (Vadakkan, 2007; 2013; 2019). This hypothesis also fulfills the expectations of a mechanism that describes memories as cue-induced, cue-specific hallucinations (inner sensation of a specific item or event in its absence (Minsky, 1980).

Extracellular matrix (ECM) prevents ephaptic spread of depolarization between neuronal processes. Examination of electron microscopic images of the cortex taken after dehydrating the tissue by fixation reveals that inter-membrane ECM is negligible in most locations in the cortex. This increases the possibility for the formation and reversal of a gate between neuronal membranes that may control propagation of depolarization to achieve some brain functions. But displacing the hydration layer between spines is expected to be a highly energy-requiring process similar to displacing the hydration layer between artificial membranes (Cohen and Melikyan, 2004; Martens and McMahon, 2008). Based on the semblance hypothesis, inter-postsynaptic functional LINKs (IPLs) are expected to form functional gates between spines of different neurons during associative learning (Vadakkan, 2013). If there is a biological feature that can easily overcome the energy barrier imposed by the hydration layer, then abutted nature of spines at many locations where dendritic arbor overlaps can provide a beneficial property to the nervous system. After the formation of IPLs, the arrival of one of the stimuli at the time of memory retrieval is expected to reactivate the IPL and induce units of internal sensations at the inter-LINKed spine where the second stimulus had arrived at the time of learning. In the context of the continued quantal release of neurotransmitter molecules at the synapses that depolarize the spine heads and the occasional arrival of action potentials leading to a volley of neurotransmitter release eliciting postsynaptic potentials, any activation of an inter-LINKed spine by depolarization arriving from a lateral direction (other than synaptic transmission from its presynaptic terminal) is expected to induce *a hallucination (semblance) that this inter-LINKed spine is receiving a stimulus from the environment through its presynaptic terminal. In fact, postsynaptic potentials are not generated on the inter-LINKed spine from an action potential arriving at its presynaptic terminal at that moment. Hence, this is called semblance*. This is viewed as a system property of systems where synaptic transmission and propagation of potentials across the IPL contribute vector components to generate oscillating extracellular potentials.

Since brain functions occur only within a narrow range of frequency of oscillating extracellular potentials as evident from electroencephalogram (EEG) studies (Palva and Palva, 2007), the IPL mechanism matches with the expectations of an operational mechanism of the nervous system (Vadakkan, 2019). It also matches with the expectations of K-lines proposed to explain a learning-change that can allow retrieval of memories as cue-induced hallucinations (Minsky, 1980). In the presence of dopamine released during motivational states or associative learning of specific deleterious and beneficial associations (Wise, 2004; O'Carroll et al, 2006) effect of dopamine in expanding dendritic spines (Yagishita et al., 2014) is expected to lead to persistence of IPLs for a long period of time, enabling a mechanism for long-term memory.

IPL mechanism is expected to operate by a spectrum of changes ranging from exclusion of the hydration layer between spines that belong to different neurons to the generation of reversible partial and complete hemifusions between them (Vadakkan, 2013; 2016a; 2019) (**Fig.2A**). Out of the above spectrum of structural changes, IPL formation by exclusion of hydration layer is expected to be the most frequently occurring change that can explain rapid associative learning when an animal moves through the environment and its reversal is responsible for a short duration of retention of learning mechanism, explaining working memory of that learned event.



  

***Figure 2.*** *Inter-neuronal inter-spine structural proximity that can allow interaction between them.* ***A****) Since the mean inter-spine distance is more than the mean spine diameter, abutted spines belonging to two different neurons can interact with each other.A negligible hydration layer of extracellular matrix between these spines that functions as an insulating medium in resting conditions. Even though high energy is expected to overcome this hydration barrier, this can be bypassed through a specific molecular organization during different brain functions such as associative learning to form an inter-postsynaptic functional LINK (IPL. Removal of hydration layer during fixation followed by oxidizing conditions is expected to promote spread of Golgi chemical reaction between spines of different neurons.* ***B****) Neuron 1 and Neuron N**, whose spines are not directly abutted to each other, are expected to get inter-LINKed with each other through several dendritic spines (shown in multiple colors) of other neurons (not shown) under physiological conditions of continued associative learning events. In parallel with this, abutted nature of these inter-neuronal spines favor the spread of Golgi chemical reaction between many neurons during original Golgi staining in the presence of only one oxidizing agent. Inter-neuronal inter-spine interaction is a testable mechanism.* ***C****)**Neuron 1 and Neuron 2, whose spines are directly abutted to each other, are expected to get electrically inter-LINKed with each other through an interaction between their spines during associative learning. This is a reversible, stabilizable, and reactivatable LINK as shown in* ***A****.*

Presence of negligible ECM between neuronal processes in the EM images of dendrites of CA1 neurons of hippocampus (Harris and Stevens, 1989; Harris and Kater, 1994) provides the hint that it may provide a suitable mechanism for both generating a reversible and yet stabilizable learning changes. Dendrites on the dendritic arbors of neighboring CA1 pyramidal cells that overlap each other in 2-D views of Golgi-stained sections (Sorra and Harris, 1993) provides an inference that several spines belonging to different neurons are abutted to each other with negligible ECM in a 3-D field. It is likely that IPLs formed between abutted spines of a large number of neurons can form islets of inter-LINKed spines (**Fig.2B**). A method that identifies locations of synapses (Buhmann et al., 2021) can be utilized to examine the distribution of abutted spines.

When spines of a dendrite are examined by injecting a dye into the soma of its neuron, it is not possible to appreciate their abutted nature with the spines of other neurons. Since the mean inter-spine distance is more than the mean spine diameter (Konur et al., 2003), it is possible to reexamine EM sections (Harris and Kater, 1994) to appreciate the abutted nature of spines that belong to different neurons for the potential formation of IPLs. According to the semblance hypothesis, inter-neuronal inter-spine interaction is necessary to explain classical conditioned learning (and in very rare cases inter-spine interaction between spines of different dendrites of the same neuron, which is expected to generate only units of internal sensation without eliciting any separate motor outputs) (Vadakkan, 2019). Dedicated studies are necessary to confirm the identity of the neurons to which those spines belong. Images from advanced EM studies (Burette et al., 2012) show less than four membrane layers in several locations between abutted cell membranes that match with expected findings at locations of inter-membrane interactions. However, dedicated imaging studies are necessary to a) verify which abutted membranes belong to dendritic spines, and b) confirm whether those spines belong to different neurons (and in very rare cases belong to different dendrites of the same neuron (Vadakkan, 2019)).

1. **Is it possible to correlate fMRI findings in terms of oxidation state-dependent changes during learning?**

Functional magnetic resonance imaging (fRMI) studies show that it takes nearly 3 to 5 seconds for the released oxygen to gradually peak following both a) brain functions, and b) artificial elicitation of neuronal firing at the same location (Monti et al., 2010; Murayama et al., 2010). This is a huge delay compared to only milliseconds needed for both associative learning and memory retrieval. When an animal moves through an environment, it can retrieve memories of majority of newly associated items or events for a short period of time, which can explain the short duration of working memory. Two findings a) learning-changes are short-lasting as evidenced by short duration of working memory, and b) time needed for the BOLD signals to peak matches with the time needed for the disappearance of short-lasting working memory have correlations. In this context, a third finding that Golgi staining reaction between neurons is restricted to single neurons under increased oxidation state prompts to ask, “Does release of oxygen reverse learning-changes through an oxidation state dependent mechanism?”

BOLD signal following learning indicates release of oxygen in the brain areas that may have certain function. If increase in oxidation state can reverse large number of newly formed IPLs, the involved dendritic spines can be used for new sets of associative learning events. In conditions such as motivation-induced dopamine release (Wise, 2004) that can promote spine expansion (Yagishita et al., 2014), strong inter-spine interactions are expected to occur leading to long-term persistence of newly formed IPLs. In conditions that lead to sudden termination of oxygen supply, existing stabilized IPLs will continue to persist. In severe reducing conditions, an extreme end of the spectrum of changes that can initiate fusion between spines involved in newly formed IPLs is expected to occur (Vadakkan, 2016b). This can lead to mixing of cytoplasmic contents of different neurons. Since mRNA profiles of adjacent neurons of even the same neuronal type are different (Kamme et al., 2003; Cembrowski et al., 2016; Tasic et al., 2018) IPL fusion can lead to acute cytotoxic damage of the involved neuronal cells. This can explain signal changes in diffusion weighted imaging (DWI) sequences of MRI following anoxic injury (Achard et al., 2011; Hirsch et al., 2020).

Regulation of oxygen release during brain functions can be seen in the following example. In motion extrapolation experiments, fMRI studies show predictable moving objects to cause decrease in BOLD signals (Schellekens et al., 2016). Using the observation that neurons that fire at regular intervals, it was argued that a predictable stimulus is expected to produce weaker prediction error than unpredicted stimulus (Rao and Ballard, 1999). This needs a mechanistic explanation. Based on IPL mechanism, decrease in oxygen release as evidenced by reduced BOLD signals can facilitate maintenance of newly formed IPLs whose reactivations can be explained to contribute to both internal sensation of anticipation and firing of postsynaptic neurons (Vadakkan, 2013; 2016a).

1. **What is the likely nature of inter-spine interactions?**

DiI dye is a fluorophore-conjugated modified lipid molecule that can replace fatty acids of lipid membranes allowing detection of neuronal processes. Intracellular injection of DiI dye shows neurons as individual cells (Cheng et al., 2014). However, in a few cases dye that function by replacing fatty acids of lipid membranes spreads to adjacent neuronal cells after a few hours (von Bartheld et al., 1990). Since any inter-cytoplasmic connections will lead to mixing of cytoplasmic content, it can lead to homeostatic mechanisms to overcome such connections through removal of involved spines. Failure of the latter can lead to neuronal death (Vadakkan, 2016b). This indicates that normal inter-spine membrane interactions are limited to prevent fusion between membranes. This is the basis for making an inference from the findings of negligible thickness of ECM between neuronal processes in EM studies (Harris and Kater, 1994; Ventura and Harris, 1999; Burette et al., 2012) that IPL structural changes can range only up to inter-membrane hemifusion between two neuronal cells.

1. **Is there any molecular support for the arguments?**

It is possible to inquire whether oxidation state can influence different stages of membrane fusion. The spectrum of IPL changes consists of various stages of membrane fusion except the last stage. One of the candidate mechanisms that facilitates membrane fusion is soluble NSF (N-ethylmaleimide sensitive fusion protein) attachment protein receptor (SNARE) molecule (Sauvola and Littleton, 2021).It is known that unique SNARE proteins are present in the dendritic spines (Jurado et al., 2013). SNARE proteins are associated with the formation of long-lived hemifusion intermediates of membrane fusion (Oelkers et al., 2016). Furthermore, depending on the distance between the membranes, SNARE proteins have ability to hemifuse the outer layers of abutted membranes (Li et al., 2007). Experimental findings that oxidation destabilizes SNARE complex formation (Dabell et al., 2014) and prevents membrane fusion through a SNARE-mediated mechanism (Alpadil et al., 2012) lead to the inference that any condition that increases the oxidation state can result in IPL reversal. Furthermore, molecular dynamic simulations of SNARE complex suggest that oxidation leads to dysfunctional SNARE complex (Bock et al., 2010). These findings match with the inference about the role of oxidation state in the formation and reversal of IPLs. In a fixed brain tissue, proteins are denatured. However, certain fixed inter-membrane interactions in their reduced state may facilitate spread of Golgi stain between neuronal membranes.

1. **Are there any similarities between oxygenation state-dependent reversal of IPLs responsible for working memory and oxidation state-dependent changes in Golgi staining?**

In the context of initial slow release of oxygen that peaks only after 3 to 5 seconds following brain functions, as observed by BOLD signals, one is prompted to ask, “Does oxygen reverse associative learning changes using oxidation properties of oxygen?” In the stratum radiatum layer of the hippocampal CA1 area, pedocytes surround nearly more than 50% of the total number of synapses where they are found in nearly 50% of the perisynaptic space (Ventura and Harris, 1999). Blood brain barrier is formed between the membranes of capillary endothelial cells and astrocytic pedocytes. Since an IPL is formed between membranes of a minimum of two spines (having their synapses on them), release of oxygen is expected to occur towards majority of IPLs. Restriction of Golgi staining chemical reaction to individual neurons under higher oxidation states and oxygenation state-dependent reversal of IPLs responsible for working memory need further explanations. It is necessary to a) prove that oxidation state-dependent reversal of IPLs *in vitro* is responsible for short duration of working memory, and b) find out the chemistry behind restriction of Golgi staining to individual neurons under increased oxidation states.

Even though the effect of oxidation state of tissue in restricting Golgi staining reaction to individual neurons and the effect of oxygenation state in reversing IPLs are expected to occur at the interphase between spine membranes of different neurons, there are major differences between their occurrences. Reversal of IPLs by oxygen is expected to occur at the molecular level due to withdrawal of projected areas of certain molecules from the inter-spine space leading to re-introduction of hydration layer between spine membranes. In Golgi reaction, oxidation change prevents Golgi chemical reaction from spreading between spine membranes of neurons that have already lost hydration layer between them. Hence, it is possible to hypothesize that abutted membrane surfaces of spines are rich in molecules having a wide range of redox potentials and respond according to the presence or absence of oxygen or oxidizing agents.

1. **Is there any advantage for the lack of ability to synthesize vitamin C in mammals?**

Vitamin C (ascorbic acid) is a reducing agent. The last step in the pathway of vitamin C synthesis is oxidation of l-gulonolactone to l-ascorbic acid by l-gulonolactone oxidase. This enzyme is deficient in primates including man, guinea pigs, passeriform birds, bats, and teleost ﬁsh due to mutations of its gene (Linster and Van Schaftingen, 2007). Vitamin C is synthesized by many vertebrates. Its synthesis in sea lamprey (Moreau and Dabrowski, 1998a) indicates that synthesis of vitamin C was present in the early ﬁsh (nearly 590 to 500 million years ago) before the emergence of terrestrial vertebrates (Moreau and Dabrowski, 1998b). The gene in the synthetic pathway was subsequently lost in several species (Birney et al., 1976). Why did this gene get lost? What evolutionary selection might have occurred there? One possibility is the sudden appearance of plants bearing fruits rich in vitamin C. An alternative explanation is that optimized modifications of nervous system operations needed less reducing conditions, which necessitated stoppage of vitamin C synthesis. This provided survival advantage to those animals.

Studies show that brain is one of the organs having the highest vitamin C content and neurons have transporters for vitamin C (Harrison and May, 2009). Furthermore, redox imbalance associated with vitamin C transport takes place in Huntington’s disease (Covarrubias-Pinto et al., 2021), a disease characterized by excessive spine loss (Nithianantharajah and Hannan, 2013). These findings prompt for searching a role of lack of vitamin C in the oxidation state dependent operation of IPLs. One possibility is that IPLs started functioning more optimally to provide survival advantage in the absence of synthesis of vitamin C. For example, vitamin C is an essential factor in the degradation of l-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine. Since dopamine has an important role in motivation-associated learning (Wise, 2004), it is possible to infer that absence of synthesis of vitamin C is contributing to the maintenance of optimum dopamine levels. Thus, lack of synthesis of vitamin C might have started providing an optimum internal milieu where continuous generation of learning-changes takes place followed by their reversal using oxygen explaining short duration of working memory. In other words, creation of a less reducing background state might have allowed reversal of IPLs by the release of oxygen following learning as evidenced by the occurrence of BOLD signals. Such an operational mechanism is suitable for learning very large number of associations from the environment and store only those associations that are either beneficial or deleterious for survival.

**Discussion**

Golgi’s staining method and its modification by Cajal by using an additional oxidizing agent prior to the reaction with AgNO3 informs that background oxidation state determines the extent of spread of Golgi chemical reaction. This raises following questions for which answers are needed.

1. **How to verify the nature of inter-membrane interactions if any**

IPL act like a switch that is turned on by displacement of hydration barrier between abutted spine membranes and turned off by the latter’s re-introduction. Studies using artificial membranes (Leikin et al., 1987) and structural studies of sodium channels (Lenaeus et al., 2017) allow inferring that IPLs span an area between 2to 10 nm2. The substantially small area did not allow their accidental discovery during microstructural studies of neurons. Since IPLs can function fully by propagation of depolarization between the inter-LINKed spines even when their area is only a few square nanometers,and since most newly formed IPLs reverse back, focused search is necessary to detect their presence. Presence of IPLs can be verified by a) dedicated electrophysiological studies of depolarization spread between dendrites whose spines are abutted to each other, and b) structural studies using different colored dyes such as DiI that can be incorporated into the membranes of different neurons. To study how SNARE protein (see subsection f) can overcome high energy barrier between membranes, dedicated experiments (Oelkers et al., 2016) can be undertaken.

1. **What is the functional role for a gradient of redox states between dendrites and axonal terminals of a neuron?**

Modified Golgi protocol stains mainly the dendrites of neurons. Under less oxidizing states there is decreasing staining pattern staring from axonal hillock region towards the presynaptic terminal terminals. This is evident from the reporting that staining of axons is absent after a short distance from the axonal hillock and results in the non-visibility of presynaptic terminals (Morecraft et al., 2014). It is possible to infer that there is a gradient of redox states of molecules between dendritic and axonal terminals, which may have an important testable functional role.

1. **Can oxidizing agents rescue the brain from anoxic injury?**

It was previously explained that under favorable conditions such as presence of fusion proteins, abnormalities in lipid membrane composition and conditions that cause excessive stimulation, there is a theoretical possibility for a spectrum of IPL structures that can progress towards IPL fusion (Vadakkan, 2016b). In reducing states caused by acute onset of anoxia, large number of non-specific IPLs is expected to form. Many of them are expected to lead to fusion between spine membranes at the locations of IPLs and cause mixing of cytoplasmic contents of neurons. Since mRNA profiles of adjacent neurons of even the same type are different (Kamme et al., 2003; Cembrowski et al., 2016; Tasic et al., 2018), it can lead to cellular changes that can cause death of neuronal cells. These changes can explain increased diffusion weighted imaging (DWI) signals indicative of cytotoxic damage occurring in anoxic brain injury (Gutierrez et al., 2010). In these contexts, it is possible to prevent damage due to acute anoxic conditions by exposing brain tissue to oxidizing agents. Even though oxygen inhalation is provided to patients admitted with acute anoxic conditions, it may become possible to reverse IPLs rapidly by intravenous administration of oxidizing agents. Some of the currently approved therapeutic agents can be used for this purpose. For example, it is possible to intravenously administer methylene blue used in the treatment of methemoglobinemia where methemoglobin level is greater than 30% or when there are symptoms despite oxygen therapy (Skold et al., 2011; British National Formulary, 2015). In addition, it will be possible to avoid all therapeutic agents that have reducing properties.

**Conclusion**

One general view is that theories guide one to decide what could be observed (Mazzarello, 2010; Daston and Galison, 2021). At the time of Golgi, there were two theories - reticular theory and cell theory. Golgi made his interpretations based on his findings, which are open for re-examination. Since Golgi was the person who initially developed the technique of staining, one can imagine that he would have done very large number of staining protocols even without oxidizing agents. Prevailing knowledge at the time of Golgi from the fields of a) chemistry (Szabadváry, 1966) that both K2Cr2O7 and OsO4 are oxidizing agents, and b) photography that AgNO3 can be reduced to black metallic silver might have provided valuable insight to Golgi to carry out large number of trial-and-error steps to arrive at a successful staining protocol. It was only when Golgi started using an oxidizing agent that the number of cells that stained decreased significantly to allow uninterrupted views of neurons.

At this juncture one might ask, “Even though Golgi witnessed the effect of adding a second oxidizing agent by Cajal that limits staining to individual neuronal cells, why did Golgi remain firm about his view?” Golgi’s findings might have informed him that nerve cells are interconnected (or intimately interlaced) to form a diffuse web that provided a route for propagation of nerve impulses (Golgi, 1891). He might have decided to stay with his view due to the deep conviction that exposing brain tissue to a second oxidizing agent does not eliminate the formation of a route of chemical reaction between membranes under less oxidizing conditions. Golgi’s view allows one to make a reasonable assumption that inter-neuronal interactions occurring under certain specific oxidation states are structural features of the nervous system related to its functions, which can be subjected to verifications. Furthermore, one possible explanation for Golgi staining of distantly located neurons of the same neuronal order having overlapped dendritic trees (Golgi, 1885; figure in Mazzarello, 2007) is the lateral spread of Golgi chemical reaction through the IPLs.

Cell theory might have led to several questions in Cajal’s mind. Cajal might have thought that if the first oxidizing agent K2Cr2O7 has allowed observation of comparatively a smaller number of cells by some unknown mechanism, then addition of a second oxidizing agent may restrict spread of chemical reaction between individual neurons. This would have allowed Cajal to obtain his results. In summary, close examination of the findings made by Golgi and Cajal shows that there is a gradient of effects produced by exposing the brain tissue to increasing oxidation states. Present work stemmed from the necessity for an explanation for the spread of silver chromate along the cell membranes and a route for this spread. It is reasonable to infer that the effect of oxygenation states to reverse IPLs between neurons under physiological conditions is different from the effect of oxidation states on preventing the spread of Golgi staining reaction between the neurons in terms molecular changes and exact locations of changes. However, since both are expected to occur between laterally located neurons, inter-neuronal inter-spine interaction and a gradient of redox states between dendritic and axonal terminal are common themes.

Present work re-examined previous findings by Golgi and Cajal for testing whether any relationship exist between them and a derived mechanism of nervous system functions that can explain generation of first-person inner sensations, namely the semblance hypothesis. It is noticed that neither Golgi nor Cajal referred K2Cr2O7 or OsO4 as oxidizing agents and were not discussing the role of tissue oxidation states in the extent of spread of chemical reaction. However, since both Golgi and Cajal referred to the chemical reaction as “reduced AgNO3 method”, one can assume that they knew about the nature of chemical reaction behind their staining methods. It is possible to construct a reason behind Golgi’s firm conviction about the reticular nature of neurons. Dedicated experiments to confirm the formation of oxidation state dependent channels between laterally located neurons can settle this controversy.

Oxygen is primarily used for oxidative phosphorylation in the mitochondria for the synthesis of ATP from reducing equivalents formed from different pathways. Oxidation state-dependent changes explained in this work is a new testable function of oxygen. Since areas of brain such as hippocampus, cerebellum and cerebral cortex are very sensitive to hypoxia (Margeta and Perry, 2021), and since these locations are implicated in different brain functions, it highlights the importance of undertaking further studies about the functional role of oxygen. Given the current advances in chemistry, it is now possible to verify a) how Golgi chemical reaction can spread between neurons, particularly those that are present within the same neuronal order, b) inter-membrane route through which such spread occurs, c) whether such oxidation state dependent inter-membrane interaction is possible *in vivo*,and d) whether such interactions are related to any testable mechanism for the generation of internal sensation within the system. Potential methods that can be implemented to limit acute anoxic injury to the brain using currently approved therapeutic agents will be an additional motivation to verify testable inferences made by this work. It is hoped that verifying presence of oxidation state-dependent inter-neuronal connections provide information about a connectivity variable that guides operations of the brain (Bullock, 2003) and will reveal properties of the connectome.

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United States patent number 9477924 pertains to an electronic circuit model of the inter-postsynaptic functional LINK.

**References:**

1. Achard S, Kremer S, Schenck M, Renard F, Ong-Nicolas C, Namer JI, Mutschler V, Schneider F, Delon-Martin C (2011) Global Functional Disconnections in Post-anoxic Coma Patient. *Neuroradiol J.* 24(2):311–315.
2. Alpadi K, Kulkarni A, Comte V, Reinhardt M, Schmidt A, Namjoshi S, Mayer A, Peters C(2012)Sequential analysis of trans-SNARE formation in intracellular membrane fusion. *PLoS Biol.* 10(1):e1001243.
3. Angulo A, Fernández E, Merchán JA, Molina M (1996) A reliable method for Golgi staining of retina and brain slices. *J Neurosci Methods* 66(1):55–59.
4. Angulo, A, Merchan JA, Molina M (1994) Golgi-Colonnier method: correlation of the degree of chromium reduction and pH change with quality of staining. *J Histochem Cytochem*. 42(3):393–403.
5. Birney EC, Jenness R, Ayaz KM (1976) Inability of bats to synthesise L-ascorbic acid. *Nature* 260:626–628.
6. Blight AR (1978) Golgi-staining of "primary" and "secondary" motoneurons in the developing spinal cord of an amphibian. *J Comp Neurol.* 180(4):679–689.
7. Bock LV, Hutchings B, Grubmüller H, Woodbury DJ (2010) Chemomechanical regulation of SNARE proteins studied with molecular dynamics simulations. *Biophys J.* 99(4):1221–1230.
8. Braitenberg V, Guglielmotti V, Sada E (1967) Correlation of crystal growth with the staining of axons by the Golgi procedure. *Stain Technol.* 42(6):277–283.
9. British national formulary: BNF 69 (69 ed.) British Medical Association (2015). [ISBN](https://en.wikipedia.org/wiki/ISBN_%28identifier%29)9780857111562. p.34.
10. Buhmann J, Sheridan A, Malin-Mayor C, Schlegel P, Gerhard S, Kazimiers T, Krause R, Nguyen TM, Heinrich L, Lee WA, Wilson R, Saalfeld S, Jefferis GSXE, Bock DD, Turaga SC, Cook M, Funke J (2021) Automatic detection of synaptic partners in a whole-brain Drosophila electron microscopy data set. *Nat Methods*. 18(7):771–774.
11. Bullock TH (2003) Have brain dynamics evolved? Should we look for unique dynamics in the sapient species? *Neural Comput.* 15(9):2013–2027.
12. Bullock TH, Bennett MV, Johnston D, Josephson R, Marder E, Fields RD (2005) Neuroscience. The neuron doctrine, redux. *Science* 310(5749):791–793.
13. Burette AC, Lesperance T, Crum J, Martone M, Volkmann N, Ellisman MH, Weinberg RJ (2012) Electron tomographic analysis of synaptic ultrastructure. *J Comp Neurol.* 520 (12): 2697–2711.
14. Cajal R (1888) Estructura de los centros nerviosos de las aves (Structure of the nerve centers of birds). *Revista trimestral de histología normal y patológica (Quarterly Journal of Normal and Pathologic Histology).* May 1:1–37.
15. Cajal R (1906) Santiago Ramón y Cajal – Biographical. Nobel Prizes and Laureates. http://www.nobelprize.org/nobel\_prizes/medicine/laureates/1906/cajal- bio.html
16. Cajal R (1909) Histologie du systeme nerveux de l'homme et des vertebres (Histology of the human nervous system and vertebrae) Vols. I and II.
17. Cajal SR (1894) Les nouvelles idées sur la structure du système nerveux chez l'homme et chez les vertébrés (New ideas on the structure of the nervous system in [humans](https://embryo.asu.edu/search?text=humans) and in vertebrates). Paris: C. Reinwald and Cie. https://archive.org/stream/lesnouvellesid00ram#page/n7/mode/2up
18. Cembrowski MS, Bachman JL, Wang L, Sugino K, Shields BC, Spruston N (2016) Spatial Gene-Expression Gradients Underlie Prominent Heterogeneity of CA1 Pyramidal Neurons. *Neuron* 89:351–368.
19. Cheng C, Trzcinski O, Doering LC (2014) Fluorescent labeling of dendritic spines in cell cultures with the carbocyanine dye "DiI". *Front Neuroanat.* 8:30.
20. Cohen FS, Melikyan GB (2004) The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement. *J Membr Biol.* 199:1–14.
21. Covarrubias-Pinto A, Parra AV, Mayorga-Weber G, Papic E, Vicencio I, Ehrenfeld P, Rivera FJ, Castro MA (2021) Impaired intracellular trafficking of sodium-dependent vitamin C transporter 2 contributes to the redox imbalance in Huntington's disease. *J Neurosci Res.* 99(1):223–235.
22. Dabell AM, Reynolds R, Gabrielsen DA, Cardinal JR, Woodbury DJ (2014) In vitro palmitoylation and oxidation of the snare protein SNAP-25. *Biophys J*. 106 (2):311a.
23. Daston L, Galison P (2021) Objectivity. Zone publishers. ISBN: 9781942130611.
24. De Carlos JA, Borrell J (2007) A historical reflection of the contributions of Cajal and Golgi to the foundations of neuroscience. *Brain Res Rev.* 55(1):8–16.
25. Ebbesson SO, Cheek M (1988) The use of cryostat microtomy in a simplified Golgi method for staining vertebrate neurons. *Neurosci Lett.* 88(2):135–138.
26. Friedland DR, Los JG, Ryugo DK (2006) A modified Golgi staining protocol for use in the human brain stem and cerebellum. *J Neurosci Methods* 150(1):90–95.
27. Gibb R Kolb B (1998) A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J Neurosci Methods* 79(1):1–4.
28. Golgi C (1873) Sulla structtura della sostan za grigia dela ervello (On the structure of the gray matter of the brain). *Gazzetta medica Italiana. Lombardia* 33:244–246.
29. Golgi C (1878) Intorno alla distribuzione e terminazione dei nervi nei tendini dell'uomo e di altri vertebrati (Around the distribution and termination of the nerves in humans and other vertebrates' tendons). *Gazzetta medica italiana* 38:221–224.
30. Golgi C (1885) Sulla fina anatomia degli organi centrali del sistema nervoso. Tip. S. Calderini e Figlio, Reggio Emilia.
31. Golgi C (1898) Interno alla struttura delle cellule nervose (Inside the structure of nerve cells). *Bollettino della società medico-chirurgica di pavia* 13:3–16.
32. Golgi C (1906) Nobel letcure. Camilo Golgi – Biographical. Nobel prizes and laureates. http://www.nobelprize.org/nobel\_prizes/medicine/laureates/1906/golgi- bio.html
33. Gutierrez LG, Rovira A, Portela LA, Leite Cda C, Lucato LT (2010) CT and MR in non-neonatal hypoxic-ischemic encephalopathy: radiological findings with pathophysiological correlations. *Neuroradiology* 52(11):949–976.
34. Harris KM, Kater SB (1994) Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci.* 17:341–371.
35. Harris KM, Stevens JK (1989) Dendritic spines of CA1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. *J Neurosci.* 9(8):2982–2997.
36. Harrison FE, May JM (2009) Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic Biol Med.* 46(6):719–730.
37. Hirsch KG, Fischbein N, Mlynash M, Kemp S, Bammer R, Eyngorn I, Tong J, Moseley M, Venkatasubramanian C, Caulfield AF, Albers G (2020) Prognostic value of diffusion-weighted MRI for post-cardiac arrest coma. *Neurology* 94(16):e1684–e1692.
38. Ito H, Atencio F (1976) Staining methods for an electron microscopic analysis of Golgi impregnated nervous tissue and a demonstration of the synaptic distribution upon pulvinar neurons. *J Neurocytol.* 5(3):297–317.
39. Jurado S, Goswami D, Zhang Y, Molina AJ, Südhof TC, Malenka RC (2013) LTP requires a unique postsynaptic SNARE fusion machinery. *Neuron* 77(3): 542–558.
40. Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M (2003) Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. *J Neurosci.* 23(9):3607–3615.
41. Konur S, Rabinowitz D, Fenstermaker VL, Yuste R (2003) Systematic regulation of spine sizes and densities in pyramidal neurons. *J Neurobiol.* 56(2):95–112.
42. Koyama Y (2013) The unending fascination with the Golgi method. *OA Anatomy* 1(3):24.
43. Landas S, Phillips MI (1982) Staining of human and rat brain Vibratome sections by a new Golgi method. *J Neurosci Methods* 5(1–2):147–151.
44. Leikin SL, Kozlov MM, Chernomordik LV, Markin VS, Chizmadzhev YA (1987). Membrane fusion, overcoming of the hydration barrier and local restructuring. *J Theor Biol.* 129:411–425.
45. Lenaeus MJ, Gamal El-Din TM, Ing C, Ramanadane K, Pomès R, Zheng N, Catterall WA (2017) Structures of closed and open states of a voltage-gated sodium channel. *Proc Natl Acad Sci U S A.* 114(15):E3051–E3060.
46. Li F, Pincet F, Perez E, Eng WS, Melia TJ, Rothman JE, Tareste D (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat Struct Mol Biol.* 14(10):890–896.
47. Lichtman JW, Pfister H, Shavit N (2014) The big data challenges of connectomics. *Nat Neurosci.* 17(11):1448–1454.
48. Linster CL, Van Schaftingen E (2007) Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J.* 274(1):1–22.
49. Margeta M, Perry A (2021) Book Chapter – The central nervous system. In, Robbins & Cotran Pathologic Basis of Disease, Chapter 28, p 1241–1304.
50. Martens S, McMahon HT (2008) Mechanisms of membrane fusion: Disparate players and common principles. *Nat Rev Mol Cell Biol*. 9(7):543–556.
51. Mazzarello P (2007) Net without nodes and vice versa, the paradoxical Golgi-Cajal story: a reconciliation? *Brain Res Bull.* 71(4):344–346.
52. Mazzarello P (2010) Golgi: a biography of the founder of modern neuroscience. Oxford University Press, U S A.
53. Mazzarello P (2018) From images to physiology: A strange paradox at the origin of modern neuroscience. *Prog Brain Res.* 243:233–256.
54. Millhouse OE (1981) The Golgi methods. En: Heimer L, Robards MJ, editors. Neuroanatomical tract-tracing methods. New York: Plenum Press. 311–343.
55. Minsky M (1980) K-lines: a theory of memory. *Cog Sci.* 4:117–133.
56. Monti MM, Vanhaudenhuyse A, Coleman MR, Boly M, Pickard JD, Tshibanda L, Owen AM, Laureys S (2010) Willful modulation of brain activity in disorders of consciousness. *N Engl J Med*.362(7):579–589.
57. Moreau R, Dabrowski K (1998a) Body pool and synthesis of ascorbic acid in adult sea lamprey (Petromyzon marinus): an agnathan ﬁsh with gulonolactone oxidase activity. *Proc Natl Acad Sci U S A.* 95:10279–10282.
58. Moreau R, Dabrowski K (1998b) Fish acquired ascorbic acid synthesis prior to terrestrial vertebrate emergence. *Free Radic Biol Med*. 25:989–990.
59. Morecraft RJ, Ugolini G, Lanciego JL, Wouterlood FG, Pandya DN (2014) Chapter 17. Classic and contemporary neural tract-tracing techniques. From Quantitative Measurement to *In vivo* Neuroanatomy. In Diffusion MRI (Second Edition), Editors: Heidi Johansen-Berg and Timothy E.J. Behrens. Pages 359–399.
60. Murayama Y, Biebetamann F, Meinecke FC, Muller KR, Augath M, Oeltermann A, Logothetis NK (2010) Relationship between neural and hemodynamic signals during spontaneous activity studied with temporal kernel CCA. *Magn Reson Imaging* 28(8):1095–1103.
61. Nithianantharajah J, Hannan AJ (2013) Dysregulation of synaptic proteins, dendritic spine abnormalities and pathological plasticity of synapses as experience-dependent mediators of cognitive and psychiatric symptoms in Huntington's disease. *Neuroscience* 251:66–74.
62. O'Carroll CM, Martin SJ, Sandin J, Frenguelli B, Morris RG (2006) Dopaminergic modulation of the persistence of one-trial hippocampus-dependent memory. Learn Mem. 13:760–769.
63. Oelkers M, Witt H, Halder P, Jahn R, Janshoff A (2016) SNARE-mediated membrane fusion trajectories derived from force-clamp experiments. *Proc Natl Acad Sci U S A.* 113(46):13051–13056.
64. Palva S, Palva JM (2007) New vistas for alpha-frequency band oscillations. Trends Neurosci. 30:150–158.
65. Pannese E (1999) The Golgi Stain: invention, diffusion and impact on neurosciences. *J Hist Neurosci.* 8(2):132–140.
66. Ranjan A, Mallick BN (2010) A modified method for consistent and reliable Golgi-cox staining in significantly reduced time. *Front Neurol.* 1:157.
67. Rao RP, Ballard DH (1999) Predictive coding in the visual cortex: a functional interpretation of some extra-classical receptive-field effects. *Nat Neurosci.* 2(1):79–87.
68. Ritchie JM, Rogart RB (1977) Density of sodium channels in mammalian myelinated nerve fibers and nature of the axonal membrane under the myelin sheath. *Proc Natl Acad Sci U S A.* 74(1):211–215.
69. Sauvola CW, Littleton JT (2021) SNARE regulatory proteins in synaptic vesicle fusion and recycling. *Front Mol Neurosci.* 14:733138.
70. Schellekens W, van Wezel RJ, Petridou N, Ramsey NF, Raemaekers M (2016) Predictive coding for motion stimuli in human early visual cortex. *Brain Struct Funct*. 221:879–890.
71. Schultze M (Ed.) (1865) Untersuchungen über Gehirn und Rückenmark des Menschen und der Säugethiere (Studies on the brain and spinal cord of humans and mammals) Vieweg, Braunschweig.
72. Sharp LW (1921) Introduction to cytology. New York: McGraw Hill Book Company Inc. https://archive.org/details/introductiontocy032473mbp/page/n217/mode/2up
73. Skold A, Cosco DL, Klein R (2011) Methemoglobinemia: pathogenesis, diagnosis, and management. *South Med J.* 104(11):757–761.
74. Smit GJ, Colon EJ (1969) Quantitative analysis of the cerebral cortex. I. A selectivity of the Golgi-Cox staining technique. *Brain Res*.13(3):485–510.
75. Somogyi P, Smith AD (1979) Projection of neostriatal spiny neurons to the substantia nigra. Application of a combined Golgi-staining and horseradish peroxidase transport procedure at both light and electron microscopic levels. *Brain Res.*178(1):3–15.
76. Sorra KE, Harris KM (1993) Occurrence and three-dimensional structure of multiple synapses between individual radiatum axons and their target pyramidal cells in hippocampal area CA1. *J Neurosci.* 13(9):3736–3748.
77. Sotelo C (2011). Camillo Golgi and Santiago Ramon y Cajal: the anatomical organization of the cortex of the cerebellum. Can the neuron doctrine still support our actual knowledge on the cerebellar structural arrangement? *Brain Res Rev.* 66(1-2):16–34.
78. Spacek J (1992) Dynamics of Golgi impregnation in neurons. *Microsc Res Tech.* 23(4):264–274.
79. Swanson LW, Lichtman JW (2016) From Cajal to connectome and beyond. *Annu Rev Neurosci.* 39:197–216.
80. Szabadváry F (1966) History of Analytical Chemistry. Oxford: Pergamon Press, ISBN: 978-0-08-010980-0
81. Tasic B, Yao Z, Graybuck LT, Smith KA, Nguyen TN, Bertagnolli D, Goldy J, Garren E, Economo MN, Viswanathan S, Penn O, Bakken T, Menon V, Miller J, Fong O, Hirokawa KE, Lathia K, Rimorin C, Tieu M, Larsen R, Casper T, Barkan E, Kroll M, Parry S, Shapovalova NV, Hirschstein D, Pendergraft J, Sullivan HA, Kim TK, Szafer A, Dee N, Groblewski P, Wickersham I, Cetin A, Harris JA, Levi BP, Sunkin SM, Madisen L, Daigle TL, Looger L, Bernard A, Phillips J, Lein E, Hawrylycz M, Svoboda K, Jones AR, Koch C, Zeng H (2018) Shared and distinct transcriptomic cell types across neocortical areas. *Nature* 563:72–78.
82. Vadakkan KI (2007) Semblance of activity at the shared post-synapses and extracellular matrices - A structure function hypothesis of memory (iUniverse Publishers).
83. Vadakkan KI (2013) A supplementary circuit rule-set for the neuronal wiring. *Front Hum Neurosci.* 7:170.
84. Vadakkan KI (2016a) The functional role of all postsynaptic potentials examined from a first-person frame of reference. *Rev Neurosci.* 27:159–184.
85. Vadakkan KI (2016b) Neurodegenerative disorders share common features of "loss of function" states of a proposed mechanism of nervous system functions. *Biomed Pharmacother.* 83:412–430.
86. Vadakkan KI (2019) From cells to sensations: A window to the physics of mind. *Phys Life Rev.* 31:44–78.
87. Vadakkan KI (2020) A Derived mechanism of nervous system functions explains aging-related neurodegeneration as a gradual loss of an evolutionary adaptation. *Curr Aging Sci.* 13(2):136–152.
88. Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. *J Neurosci.* 19(16):6897–6906.
89. Vints K, Vandael D, Baatsen P, Pavie B, Vernaillen F, Corthout N, Rybakin V, Munck S, Gounko NV (2019) Modernization of Golgi staining techniques for high-resolution, 3-dimensional imaging of individual neurons. *Sci Rep.* 9(1):130.
90. von Bartheld CS, Cunningham DE, Rubel EW (1990) Neuronal tracing with DiI: decalcification, cryosectioning, and photoconversion for light and electron microscopic analysis. *J Histochem Cytochem.* 38(5):725–733.
91. Wise RA (2004) Dopamine, learning and motivation. *Nat Rev Neurosci.* 5:483–494.
92. Yagishita S, Hayashi-Takagi A, Ellis-Davies GC, Urakubo H, Ishii S, Kasai H (2014) A critical time window for dopamine actions on the structural plasticity of dendritic spines*. Science* 345:1616–1620.
93. Zhang H, Weng SJ, Hutsler JJ (2003) Does microwaving enhance the Golgi methods? A quantitative analysis of disparate staining patterns in the cerebral cortex. *J Neurosci Methods* 124(2):145–155.