Routes of the thalamus through the history of neuroanatomy

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Abstract (170 words)

The most distant roots of neuroanatomy trace back to antiquity, with the first human dissections, but no document which would identify the thalamus as a brain structure has reached us. Claudius Galenus (Galen) gave to the thalamus the name '*thalamus nervorum opticorum'*, but later on, other names were used (e.g., *anchae*, or buttocks-like). In 1543, Andreas Vesalius provided the first quality illustrations of the thalamus. During the 19th century, tissue staining techniques and ablative studies contributed to the breakdown of the thalamus into subregions and nuclei. The next step was taken using radiomarkers to identify connections in the absence of lesions. Anterograde and retrograde tracing methods arose in the late 1960s, supporting extension, revision, or confirmation of previously established knowledge. The use of the first viral tracers introduced a new methodological breakthrough in the mid-1970s. Another important step was supported by advances in neuroimaging of the thalamus in the 21th century. The current review follows the history of the thalamus through these technical revolutions from Antiquity to the present day.

Keywords:

Antiquity; Connectivity tracing; History; Human dissection; Neuroanatomy; Staining; Thalamus

Content

List of abbreviations

AAV : adeno-associated virus
BCE : before common era
BDA : biotinylated dextran amines
BOLD : Blood oxygenation level-dependent
CE : common era
CNS : central nervous system
CA1 : hippocampal region CA1 (*cornu ammonis* 1)
ChAT : choline acetyltransferase
CTB : Cholera toxin subunit B
DA : dopamine (dopaminergic)
DBS : deep brain stimulation
DTI : diffusion tensor imaging
DWI : diffusion weighed MRI
EEG : electroencephalography
EGFP : enhanced green fluorescent protein
FITC : Fluorescein isothiocyanate
GABA : gamma aminobutyric acid
GFP : green fluorescent protein
HRP : horseradish peroxydase
HSV : herpes simplex virus
LIPv : ventral lateral intraparietal area
MD : mediodorsal
MIP : medial intraparietal area
mPFC : medial prefrontal cortex
MRI : magnetic resonance imaging
NPY : neuropeptide Y
PHA-l: Phaseolus vulgaris) lectin
PNS : peripheral nervous system
PRV : pseudorabies virus
PV : parvalbumin
Re : reuniens nucleus
RNA : ribonucleic acid

TH : tyrosine hydroxylase

TK : thymidine kinase

TRITC : Tetramethylrhodamine-isothiocyanate

VLo : ventralis lateralis pars oralis

VMpo : posterior part of the ventral medial (thalamic) nucleus

VPLo : ventralis posterior lateralis pars oralis

VPm : ventral posteromedial

VSV : vesicular stomatitis virus

WGA: wheat germ agglutinin

ZI : zona incerta

"Scientific practice is above all a story-telling practice. (...) Biology as a way of knowing the world is kin to Romantic literature, with its discourse about organic form and function. Biology is the fiction appropriate to objects called organisms; biology fashions the facts "discovered" about organic beings."

Donna Haraway (1985)

(Primate Visions: Gender, Race and Nature in the World of Modern Science)

1. Introduction

Perhaps the oldest known document to mention a link between a region of the central nervous system and a behavioral function is the about 36-century old Edwin Smith papyrus (e.g., Kamp et al., 2012), which could be a copy of an even older document. Edwin Smith was an American antiquity dealer who acquired this papyrus in 1862 and subsequently tried to translate it, but he never published his translation. Smith kept the hieroglyphic script until he passed away in 1906. After his death, his daughter gave the papyrus to the New York Historical Society, and the American Egyptologist James Henry Breasted (1865-1935) published his own translation of it in 1930 (e.g., van Middendorp et al., 2010). Therein, words like 'brain' ('skull offal' is a closer translation; Standring, 2016), 'meninges' or 'spinal cord' are used. The document describes 48 cases of more or less dramatic wounds or trauma, each of them according to a standardized presentation principle: heading, description of symptoms, diagnosis, and treatment (or prognosis pointing to hopelessness). Twenty-seven of these reported cases concern head injuries, of which 22 descriptions are in line with traumatic brain injuries (Kamp et al., 2012). Among them, case 20, and perhaps case 22, might be the first descriptions of aphasia (Minagar et al., 2003). Remarkably, this papyrus is probably the oldest document providing neuropsychological (as one would say today) arguments in favor of localization of functions in the brain. When open and penetrating fractures of the skull were noticed, where the head had been hit obviously correlated with the type of functions lost or altered. No trace, however, of evidence for neuroanatomical knowledge pointing to the brain as an ensemble of structural entities delimited by specific and clearly-identified landmarks.

Regarding brain organization, various proposals and types of (sometimes fanciful) anatomo-functional conceptions arose later on. Some of them relied on empirical evidence. For instance, Christian philosopher Nemesius (350?-420? CE), bishop of Emesa (now Homs in Syria), defended a view according to which the different higher functions of an organism originated in the ventricles. In his view, the senses and imagination had a rostral ventricular location, memory a posterior one, and intellectual thought seated in between the two others. Nemesius grounded his anatomo-functional proposal on clinical support (van der Eijk, 2008). Indeed, he had observed that frontal injuries impaired senses, not the other functions, middle injuries affected thought but preserved sensation and memory, and posterior injuries altered only memory. This ventricular doctrine, however, disregarded the cerebral substance, or brain parenchyma, as the crucial support of an organism's functions, and therefore remained far away from the first relevant foundations of brain mapping.

In such a view, there was no place for cerebral tissue, no place for neuroanatomy other than ventricular, hence no place for the thalamus. However, it is not impossible that these anatomofunctional considerations influenced later localisationnist thoughts about functional organization of brain tissue. The current review, which is partly historical, will not discuss in detail the different conceptions of the anatomical and anatomo-functional organization of the thalamus over the ages. Rather, it will focus on showing how the evolution of conceptions of, and knowledge about anatomy of the brain and of the thalamus depended on the investigation possibilities opened up by ideological, initially, and then technical progress.

2. Greek philosophers and physicians

Describing the brain and identifying the thalamus as a structural subdivision is not conceivable without accepting dissection as a fundamental, unmissable principle of investigation of an organism's and organ's external and internal organization. Human dissection has an old, sometimes chaotic history that could have roots in mummification, an embalming art used in ancient Egypt to prepare the dead for afterlife. For mummification of the body, the brain was removed with hooks introduced into the skull through the nostrils, while the stomach and intestines were dissolved in intra-abdominally injected cedar oil, or were removed together with the lungs and heart after an incision of the flank; these organs were separately placed

into a jar (Turliuc et al., 2015). Turliuc et al. (2015) think that this kind of practice might have led Egyptian priests to overcome their inhibitions with regard to the dissection of human cadavers. At this time, however, the process of dismantling a corpse did not go beyond the field of funeral tradition. Alcmeon of Crotone (exact dates of birth and death are unknown), a Greek philosopher of science and medical theory born in Crotone (now a city in Calabria, Italy) near the end of the 6th century BCE, is considered one of the earliest supporters of anatomical dissections for scientific purposes. Whether he dissected human bodies or only other animals is still under discussion, but his work and insightful analyses led him to consider the brain as the seat of intelligence, as did Hippocrates (460?-377 BCE) and others later on. This opinion stands in clear contradiction with Aristotle's (385-323 BCE) influent cardio-centric hypothesis (e.g., Crivellato and Ribatti, 2007). For Aristotle, the heart, and not the brain, was in fact the seat of intelligence, motion, and sensation, and other organs such as the brain or lungs had a role in cooling the heart and mitigating the passions arising therefrom (for more details, see e.g., Engelhardt, 2018). Surprisingly, this debate between cardio-centrism and encephalo-centrism lasted until the 16th century (Crivellato and Ribatti, 2007).

Alcmeon of Crotone is credited with a first study of the paths of the optic nerve as well as a theory of senses in which he established a functional connection between sensing and the brain: organs of senses (eyes, ears) are connected to the brain by the way of ducts (*porois* in Greek). He also distinguished understanding/thinking and sense perception as different processes.

Dissection, whether of human cadavers or of less evolved animals, and thus anatomical knowledge, probably traces back to even earlier than ancient Greece. The documents which could have attested to it, however, did not reach our time, presumably because they disappeared during the destruction of the library of Alexandria (which extended over about seven centuries) or for other reasons. The same may have occurred with the contributions of Herophilus of Chalcedon (about 325?-255? BCE; Chalcedon is now a district of Istanbul), and Erasistratus of Ceos (310?-250? BCE; Ceos is now Kea, an island in Greece). Both belonged to the prestigious school of Greek medicine in Alexandria. Herophilus and Erasistratus performed human dissections, and presumably even human vivisections (Strkalj and

Chorn, 2008). The first two kings of the Ptolemaic dynasty authorized these dissections and vivisections. It is a matter of fact that most writings and documents of Herophilus were lost or have been destroyed, and the information available today is at least second hand. Nevertheless, it is known that Herophilus identified, described, and named numerous anatomical structures of the nervous system. For instance, he distinguished motor from sensory nerves, described seven pairs of cranial nerves, differentiated the cerebrum (encephalon), cerebellum (parencephalon), and meninges, discriminated the four ventricles, and described the different layers of the eye. His dissection/vivisection subjects were criminals provided by Ptolemy I and II, and one thinks that Herophilus dissected up to about six hundred people over about 40 years of practice (Acar et al., 2005). No trace, however, exists of a reference to a subdivision of the brain that could correspond to the thalamus (Strkalj and Chorn, 2008).

3. Galen

Galen (131-210 CE), or Claudius Galenus, was a philosopher and physician of the Roman Empire. During Galen's lifetime, dissections of the human body were no longer acceptable. Following Aristotle's proposal, who defended the idea of referring to the parts of other animals that appeared comparable to those of humans, Galen focused on bovid brains, and also on brains from North African baboons (Acar et al., 2005), which he described in *De Anatomicis Admnistrationibus*. He was the one who used the word 'thalamus' for the first time. This word derives from the Greek word 'θάλαμος', or 'thalamos' (Gailloud et al., 2003). In fact the name Galen gave to this structure was '*thalamus nervorum opticorum*'. 'Thalamus' means inner chamber or anteroom in a given semantic context and, in another one, bridal bed or bridal chamber (Gailloud et al., 2003; Jones, 1985; Serra et al., 2019). According to Gailloud et al. (2003) and Jones (1985), 'thalamos' may originate in the even older word 'thalam', which designates the first of the three chambers of an Egyptian temple (see Figure 1.2. in Jones, 1985). Interestingly, according to Jones (1985) who cites Morrison and Williams (1968), the rower sitting on the lowest bench of a Greek battle galley (i.e., a trireme) was called a *thalamite*, and the oar-hole corresponding to this place was the *thalamia*, which could be another origin of the word. The rower was called a *thalamian*. Why '*opticorum*' (which can be translated by 'optical') then? *Thalamus opticorum* means optical bed, a designation that may sound logical if one

considers that, at this time, dissections were started from the ventral side of the brain and what was visible in the region encompassing the thalamus was the optic nerve tracing to an oval mass located near ventricles, on which it seemed to be extending like on a bed. It is possible that Galen saw in this organization a resemblance with the aforementioned *thalamia* from which the oar was emerging. Therefore, for Galen and for those who subsequently relayed his teaching for more than fifteen centuries, the thalamus was a structure not dissociable from visual functions. It is not exceptional that brain structures were named according to their resemblance with objects (e.g., pulvinar or habenula; Turliuc et al., 2016), animals (e.g., the hippocampus), or body parts (e.g., mammillary bodies).

It may sound weird that two parenchymal structures like the left and right thalamus received a name designating a room, and thus an empty volume. The explanation for this might be related to both what Galen was actually observing (see below) and his *pneuma* (vital spirit) hypothesis of physiology, which was inspired by the nerve physiology of Herophilus and may have driven his observations. Herophilus, and later Galen, considered that all the pneuma or "breath" in the body came from the outside air having entered the body. The inspired air underwent changes in the lungs, then in the left ventricle of the heart, from where the vital *pneuma* went to the ventricles of the brain by the arteries. It is there, in the brain, that the vital *pneuma* (*pneuma zoticon*) in turn became elaborated and refined into the psychic *pneuma (pneuma psykhicon)*. From there, the psychic *pneuma* was able to flow into sensory organs and muscles, making sensory experiences and body movements possible. Therefore, it would not be surprising that what Galen defined as the thalamus in fact corresponded not to the two oval diencephalic structures made of gray matter that the noun thalamus nowadays refers to, but in fact to a rather middle inner part of the left and right lateral ventricles that border the thalamus dorsally. Hence, Galen's thalamus was a storeroom for the vital spirit. Burdach (see below) was even convinced that Galen had never seen the thalamus, as he stated in 1822 (Serra et al., 2019).

Because they were taught to physicians over centuries, Galen's views and theories remained extremely influential during almost fifteen centuries. Human cadaveric dissection was prohibited almost throughout this long period, in part because of the

growing influence of religious authorities, who advocated the superiority of the soul over the body, and for whom dissection of human cadavers was therefore considered useless and blasphemous (Ghosh, 2015). To this, one must add that the bulk of the population also viewed dissections of human bodies in a negative light, as already had been the case at the time Herophilus and Erasistratus practiced anatomical explorations in Alexandria (Ghosh, 2015).

4. Albertus Magnus

The ventricular conception of brain function, which had roots in the 3rd century BCE (see below), was another obstacle. It was still very relevant in the 13th century, as shown by Albert Magnus' proposals. Albertus Magnus (1193-1280) was a German bishop, theologian, astrologer, and philosopher. He described the ventricular system as the seat of human faculties and distinguished three compartments: anterior for presumably what we name the lateral ventricles, middle for the third ventricle, and posterior for the fourth ventricle. The ventricles contained so-called 'sensory vapor' (Stratton, 1931), a point of view inspired by Erasistratus of Ceos' explanation of the function of the *pneuma psykhicon* which, from the lateral ventricles, controlled structure-function relationships and set the body in motion by getting through the motor nerves to the muscles (Duque-Parra et al., 2017). For Magnus, common sense and basic imagination were located in the anterior ventricle. Imaginative, cogitative and formative powers, and evaluation capacities were located in the middle ventricle. Finally, mnemonic power operated from the posterior ventricle (Figure 1).

This ventricular doctrine was also clearly influenced by Avicenna's (980-1037) conceptions. He considered the following faculties to be arranged along the rostro-caudal axis of the ventricular system according to the following enumeration order: five senses, representation, sensitive imagination, evaluation, retention, and recall. Despite his anatomo-functional elaboration, Albertus Magnus did not seem to pay much attention to the cerebral and cerebellar substance. It was also the case of Gregor Reisch's encyclopedic compilation (1504), in which a drawing shows lines illustrating the convergence to the most anterior ventricle of sensory information arising from the eyes, nose, tongue, and ears (Figure 2). Such views are reminiscent of the views of Nemesius we mentioned in our introduction. They gave little chance that a tissue-made structure of the brain came under focus, be it the thalamus or any

other parenchymal region. It is interesting to note that the ventricular theory was preserved for more than fifteen centuries before Leonardo da Vinci provided the first realistic drawing of the ventricular system. He performed it from a wax cast of an ox brain (Duque-Parra et al., 2017).

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Insert Figures 1 & 2 about here

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5. De Liuzzi

During the 12th century, a period corresponding to the setting up of the first European universities (e.g., Paris in 1150, Bologna 8 years later, and Oxford another 9 years later), the Church ceased to forbid human cadaveric dissections in the domain of anatomical science. The emperor Frederick II even made it mandatory in 1231 (1238 in Sinha, 2015) for all people who were to practice medicine or surgery (Ghosh, 2015). The emperial decision was that one dissection be performed every fifth year (Sinha, 2015). In 1299, however, Pope Boniface VIII issued a Bull that prohibited the boiling and dismembering of human cadavers, a practice that had enabled the dissemination of different pieces of a same corpse for e.g., reasons of prestige (pieces of a same person in different burials), ease of transport (much used by the crusaders), respect of the premortem will to rest in different places, or whatever. This Bull, however, had little effects on dissection of humans motivated by scientific reasons, perhaps more in Italy than elsewhere.

In Bologna, Mondino de Liuzzi (1270-1326) introduced public dissections of executed criminals as a support to anatomical studies and lectures. De Liuzzi broke with the tradition that required the eminent teacher to read a descriptive text – Galen's work was often used for this purpose – while a barber (the sector) performed the gestures of the dissection, and a third person (the ostensor) pointed to the organs named by the lecturer (Standring, 2016). In fact, de Liuzzi performed some dissections on his own, taking on all roles, as in 1315 in Bologna when he publicly dissected what could have been the cadaver of an executed woman. Surprisingly, he let Galen's mistakes (due to transposition or extrapolation from animal anatomy) uncorrected and only made a few additions to Galen's work. These additions encompassed the description

of structures located underneath the lateral ventricles and bordering the third ventricle, which "are of the same substance as the brain", and which he called *'anchae*' (a latin word that, placed in its medical context, could mean 'shaped like buttocks'; see Gailloud et al., 2003). There is no evidence, however, that de Liuzzi's '*anchae*' actually designated the thalamus. Indeed, what he named so could have been parts of the caudate nuclei instead (Swanson, 2015). Whatever may have been, it seems that until 1610, when Jean Riolan (1580?-1657) resumed the expression '*thalamus nervorum opticorum'*, the word thalamus was abandoned in favor of other names, including de Liuzzi's *anchae*, but also *monticulus* (little mountain), *nates* (buttocks), and even *testes* (Serra et al., 2019).

6. Vesalius and Eustachius

With the birth of printing (around 1470), anatomical engravings underwent a real boom and were more than exceptionally the work of artists who acquired unquenchable fame during their lifetime or after their death. Beside Dürer, Donatello, Michelangelo, and Raphael, one can also mention Leonardo da Vinci (Sinha, 2015; Swanson, 2000). He illegally practiced overnight candle-lit dissections of human cadavers in the crypt of Santa Maria Nuova hospital, of which he left incredibly remarkable drawings (e.g., Perloff, 2013). Sinha (2015) mentions that da Vinci made 750 drawings and might have written more than 100 anatomical books. As was already the case at the end of the 13th century, a main problem for these dissections was the shortage of usable corpses, which led to illegal conducts such as grave openings for cadaver stealing. What could have been the first printed anatomical illustration of the brain structure is the representation published by Magnus Hundt (1449-1519) in 1501 in his *Antropologium de Hominis Dignitate, Natura et Proprietatibus, de Elementis, Partibus et Membris Humani Corporis*. Showing the head with the organs of senses and their connections, the illustration emphasized the ventricular cells, thereby attesting to Galen's still very significant influence.

In 1519, Lorenz Fries (1490?-1550) published *Spiegel der Artzney* (literally: medicine mirror) showing six modern-like representations of brain dissections arranged around the drawing of an open human trunk (Swanson, 2000). On these drawings, however, nothing visible that could correspond to the thalamus! In 1543 appeared the *De Humani Corporis Fabrica*, a masterpiece of anatomy by Andreas Vesalius (1514-

1564). Vesalius was reputed for his public dissections of human cadavers, sometimes with 500 observers around him it is said (Scatliff and Johnston, 2014), including artists sketching what was presented and commented by the anatomist. The book encompassed 273 woodcut illustrations of so far unmatched quality and precision, among which 25 were of brains (Scatliff and Johnston, 2014). Incredibly, at the beginning of the 1930s, typograph Willy Wiegland found 227 of Vesalius' original woodblocks from the *Fabrica*, and of a summary thereof published a few days earlier and named *Epitome*. In 1932, American physician Samuel Lambert, who thought that this material had perhaps not disappeared, asked Wiegand to investigate. The blocks were discovered in an attic at the University of Münich. The pictures corresponding to the originally in Basel(Swizerland)-printed woodcuts were republished in 1934 (Lambert, 1934) but, unfortunately, all these blocks were destroyed in 1944 during the bombing of Münich by the allies (Scatliff and Johnston, 2014).

It is in Vesalius' remarkable anatomical monograph that one can find the first illustrations of the human thalamus, one as a posterior view covering the brainstem and the thalami, and one as a horizontal view of a section through a brain coiled in an open skull (Figure 3). The beauty of the design is impressive. While Vesalius' illustrations were printed from woodcuts, Bartolomeo Eustachius (1513?-1574), another founder of anatomy, used copper plate engravings, which, thanks to the better resolution they offered, permitted the inclusion of even more details. The copper-engraving technique had been much developed by German painter and printmaker Albrecht Dürer (14712-1528). In 1552, Eustachius prepared 47 drawings on copper plates, but only 8 of them showing skeletons and muscles were published during his life, in *Opuscula Anatomica*, in 1564. The 39 other plates disappeared and reappeared almost 150 years after Eustachius' death, in 1722 (Adanir and Bahsi, 2019). On one of those plates, Eustachius had represented for the first time the interthalamic commissure (a problematic denomination, as this structure is devoid of crossing axons). Therefore, intrathalamic adhesion is a better name (Gailloud et al., 2003) to designate this thalamic structure, which apparently not all human subjects possess (Borghei et al., 2020; 2021; Damle et al., 2017).

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Insert Figure 3 about here

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7. Willis, Vieussens, Vicq d'Azur

Before Thomas Willis (1621-1675), there was no monograph which focused exclusively on the nervous system. So far, monographs dealing with anatomy covered the whole body, the brain being only part of their topics. *De Cerebri Anatome* (1664) by Willis, and *Nevrographia Universalis* (1884) by Raymond Vieussens (1641?-1715) were the first monographs to deal exclusively with the anatomy of the nervous system (Meyer, 1971). Willis produced remarkable drawings of the thalamus, which he named *thalamus opticus*. He also insisted on the fact that there was no cavitation in this structure, despite being otherwise rather seduced by Galen's *pneuma* hypothesis at a purely functional level. His drawing placed the thalamus underneath the striated bodies, for the first time illustrated at the top of the medulla oblongata (i.e., the brainstem). The outstanding quality of Willis' observations and analyses (e.g., the conception that voluntary movements originate in the cortex, involuntary ones in the cerebellum, that the cortex is the substrate of cognition, etc. Arraez-Albar et al., 2015) most probably owe a lot to the revision of the university statutes at Oxford in 1636. Among other changes, this revision introduced dissection as mandatory in medical studies and allowed, for the purpose of dissection, the anatomy reader to be given the corpse of any person executed in a radius of 21 miles around Oxford. Before this, teaching of anatomy largely relied on Aristotle's, Hippocrate's, and Galen's outdated contributions (Arraez-Albar et al., 2015).

A famous anecdote of that time might be worth a short relating. Anne Greene, a maid, was wrongly accused of having murdered her, in fact, miscarried baby. On December 14, 1650, she was hung and, after her apparent death, taken off the gallows and placed in a coffin that was brought to the private home of Dr William Petty, the reader in anatomy. When Petty and Willis opened the coffin, they were dumbfounded to hear a breath and a noise in Ms Greene's throat. The idea of dissecting her was given up and they both, with the help of colleagues, tried to reanimate her, which they actually managed to do. Over days and weeks, Anne Greene returned to normal life and died 15 years later after having married. From her and her husband's nuptial bed, three children were born in the meantime (e.g., Hughes, 1982).

Beside coining neuroanatomical terms (Molnar, 2004), Willis also strongly relied on careful observations of clinical cases to which he tried to relate his anatomical observations (Finger, 1994); he is well known for correlating symptoms and brain morphology in his patients (Molnar, 2004). Lacking a traditional medical education, he had also much interest in experimental sciences (Arraez-Albar et al., 2015). For more details on Willis, see Molnar (2004). According to Gailloud et al. (2003), Raymond Vieussens, who was the first to introduce the distinction between white and gray matter, placed the thalamus on the back of the caudate nucleus and documented the connection of the optic nerve with the posterior part of the thalamus. He also named the *corpus album subrotundum*, which corresponds to the part of the anteroventral nucleus protruding into the third ventricle. Félix Vicq d'Azur (1748-1794), who was the first neuroanatomist to systematically dissect the human brain in the coronal plan, provided a first accurate description of the mamillo-thalamic tract, along with a precise description of this bundle's course through the thalamus. Vicq d'Azur is also known for advocating the adoption of a single nomenclature to designate the same anatomical parts in all species (Parent, 2007).

8. Burdach, Luys and Forel

In his *Vom Baue und Leben des Gehirns* published in 1822 (see illustration in Figure 4), Karl Friedrich Burdach (1776-1847) introduced the word pulvinar (meaning pillow or cushion) to denominate the largest nucleus of the thalamus. He also identified the *lamina medullaris interna* and *externa*, which he used as delimitations to subdivide the thalamus into superior, internal, and external regions. Thus, the thalamus encompassed four regions if one adds the pulvinar to the aforementioned three. Thereby, Burdach did not only open the possibility of addressing the question of a link between architectonic thalamic subdivisions and possible functional specializations, assuming the two can actually be related to each other, but also paved the way towards a modern nomenclature of the thalamic architecture (Meyer, 1970). Jean Bernard Luys (1828-1897), in his *Recherches sur le Système Nerveux Cérébrospinal: sa Structure, ses Fonctions et ses Maladies* (1865), identified 4 thalamic subregions he called centers because of their functional implications, all related to senses: the anterior center (*centre antérieur*; olfactory), the middle center (*centre moyen*; optic), the median center (*centre médian*; somaesthetic), and the

posterior center (*centre postérieur*; acoustic). According to Jones (1985), Luys saw in the thalamus a kind of relay between spinal cord-associated functions and high level activities of the brain. In his 1881 book, Luys wrote: *"From a physiological standpoint, the optic thalami are intermediary regions interposed between the purely reflex phenomena of the spinal cord and the activities of psychical life…"*. Luys also named 'Luys body' what we now call the subthalamic nucleus (Pearce, 2001). Finally, in 1877, the year he became *Privatdozent* in Münich (Germany), Auguste-Henri Forel (1848-1931) introduced the distinction of four different thalamic regions. He named them anterior, posterior, ventral, and lateral, to which he added a fifth center he called medial (Gailloud et al., 2003). This nomenclature subsequently inspired denominations when it came up that these regions had to be split into smaller subregions based on staining and connectivity studies (see below). The thalamus was the topic of Forel's PhD thesis defended in 1872 (Osiro et al., 2012).

From antiquity to the turn of the 19th century, knowledge about the thalamus progressed very slowly, partly because of technical limitations, partly because of ideological pressures and beliefs. What can be retained from this period, however, is the impressive progress in the artistic quality of the drawings made over the last three centuries. They all related to essentially a macroscopic description of external and much less frequently internal aspects of the thalamus. Details about the partition of the human thalamus and the inner organization of the nuclei were still lacking, but the latest studies in primates enabled to propose a subdivision of the thalamus into a more or less important number of major subregions based on anatomical landmarks. Knowledge about the connectivity of thalamic subdivisions with other brain regions remained entirely to be established, being waiting for highly reliable staining techniques. But things would quickly change, partly because of technical progress regarding tracing methods, partly because more and more animal studies were undertaken, although animals were also a source of confusion, rats, mice, cats and inframammalians having thalamic maps differing, in several aspects, from those of humans (e.g., Butler, 2008). What was still missing, was a precise idea about the functions the thalamus and even more about what its subdivisions were contributing to. Obviously, as advocated by Meynert (1872), considering functional outputs rather than anatomical cues was also one possible criterion to setup a nuclear nomenclature of the thalamus.

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9. Staining-based studies

During the second half of the 19th century and in addition to the popularization of the microscope as a revolutionary tool to explore what so far was invisible to the naked eye, two major technical breakthroughs contributed to further progress in the knowledge of the thalamus, and more generally of brain anatomy. The first one concerned tissue staining techniques, and the second one the use of so-called ablative studies in a large variety of animal models. These two approaches will converge and combine, permitting further gain in neuroanatomical accuracy. It might be worth noting that Joseph Gerlach (1820-1896) introduced the first stain for nervous tissue in 1858. It was carmine, a red powder obtained by grinding the dried body and eggs of the female cochineal (Dactylopius coccus), a South American and Mexican cactus parasite (Swanson, 2000). Gerlach's carmine-based staining was useful to stain cell bodies and therein more specifically their nucleus.

Other methods were developed later on, and to name just two of the determinant ones, let us start with Franz Nissl (1860-1919) in 1884, at a time he was a medical student. Nissl's method was based on alcohol fixation of brain tissue, which was subsequently sectioned and stained with magenta red, methylene blue, and toluidine blue, exactly in this sequence (da Mota Gomes, 2019). Although this method did not permit to visualize the morphology of neurons (the stain binds to nucleic acids and therefore mainly stains the cell body), it proved extremely useful in characterizing the cytoarchitecture peculiarities of different brain structures, including the thalamus. For morphological details, one had to use another method developed by Camillo Golgi (1843-1926) in 1873, the famous black reaction (*la reazione nera*) or silver impregnation method. The reason why this method stains only a small proportion (ca. 3%) of neurons is still unknown (Bentivoglio et al., 2019). The staining principle of Golgi's method required that tissues be hardened in potassium bichromate to be soaked in a silver nitrate solution. This technique enabled the perfect visualization of

some neurons in their entirety (i.e., axon, dendrites, dendritic spines, and soma) on a well-contrasted background, i.e., devoid of staining residues.

When Nissl died in 1919, he was studying the connectivity between the thalamus and the cortex. Thirty years earlier, hence in 1889, he had given a talk about the organization of the thalamic nuclei in the rabbit during the meeting of the *Naturforscherversammlung* (meeting of naturalists) in Heidelberg (Germany). Based on histological arguments, Nissl distinguished 16 nuclei as such: anterior dorsal, anterior ventral, anterior medial, medial middle, posterior medial, anterior lateral, posterior lateral, magnocellularis, medial ventral, lateral ventral, dorsal ventral, posterior, midline, and ventral, dorsal and lateral *Gitterkerne* (lattice nuclei), the latter three corresponding to the reticular nucleus (Andô, 1937). For reasons unbeknown to us, Nissl did not publish the paper corresponding to the talk he gave in Heidelberg before 1913. At this time, his nomenclature encompassed 18 subregions, some of which had already been considered entities in 1889: anterior dorsal, anterior ventral, anterior dorsomedial, anterior ventromedial, middle medial, posterior medial, anterior lateral, posterior lateral, magnocellularis, posterior dorsal, posterior ventral, central, par-ependymal, prebigeminal, the ventral anterior and ventral posterior groups of nuclei, dorsal and ventral *Gitterkerne* (Andô, 1937).

Meanwhile, Münzer and Wiener (1902) distinguished 14 nuclei, Winkler and Potter (1911) an identical number, d'Hollander (1913) 17, and Miura (1933) 23, which he grouped in 5 *ensembles*. Andô (1937) later proposed a breakdown of the thalamus into 6 *ensembles* encompassing 26 nuclei. These discrepant breakdown proposals, which are just a few selected examples, clearly show that observation of the same brain structure using comparable or constantly improving staining methods in the same (or different) species did not lead to a consensual view on the cytoarchitectonic organization of the thalamus. Such divergencies, essential as they are in the evolution of knowledge, reveal that beyond the brain preparations examined, the species studied, the instruments used, the eye of the observer was most probably just as important as technical tools.

Le Gros Clark also conducted staining-based (and ablation-based, see hereafter) studies of the thalamus. In an important review article (Le Gros Clark, 1932) on the

structures and connections of the thalamus, he emphasized how important it is to have a clear nomenclature in order to be able to apply the appropriate experimental methodology for progressing on anatomical knowledge about this structure. And as the thalamus is a complex structure in high order mammals, he pleaded for comparative anatomy, defending the idea that it was necessary to start from the thalamic organization in lower vertebrates in order to follow its phylogenesis up to primates. Indeed, the thalamus evolved from a structure being mainly concerned by sensory impulses from brain stem, spinal cord and hypothalamus in cyclostomes, to a structure on which evolution superimposed an upper level connected with association areas of the cortex, reaching a culmination in primates (for more details, see e.g., Butler, 2008). Le Gros Clark was also the one to propose that, beyond cellular groupings, subregions of the thalamus be delimited by additional consideration of fiber connections, pointing to the fact that differences in cytoarchitecture do not necessarily parallel functional differentiations. This is perhaps more the case for intralaminar nuclei than for others.

10. Ablation-based studies

Beside these histology-based approaches, an ablation-based strategy aiming to characterize the organization of the thalamus according to its connectivity emerged. One of the first scientists to use such a strategy was Bartolomeo Panizza (1785-1867). He damaged the visual pathway in newborn animals (e.g., rabbits, dogs) at some place (e.g., a unilateral ablation of the eye bulb) in order to, later on in adults, post-mortem trace the lesion-induced degeneration indicated by the shrunken regions (Figure 5). Sometimes he also used animals with congenital or accidental lesions of e.g., an eye bulb. This strategy allowed Panizza to realize that following postnatal unilateral enucleation, parts of the thalamus (and of the posterior cerebral cortex) had substantially shrunken. When the thalamic parts he had found to have degenerated were then destroyed in the intact adult brain, the animals showed blindness contralateral to the lesion (Colombo et al., 2002). Observations of this kind provided additional functional support to the implication of thalamic regions in visual functions. They also contributed to extend knowledge about thalamic connectivity.

Other ablative studies were performed to study the connections between the thalamus and cortical regions, the principle being then to damage circumscribed

622 regions of the cortical mantle and observe the resulting retrograde degeneration in
623 the thalamus. When thalamic degeneration was observed it was interpreted as
624 evidence for existing connections between the damaged cortical and the
625 degenerated thalamic zones. It soon emerged that after lesions restricted to
626 particular cortical regions only delimited neuronal clusters of the thalamus underwent
627 degeneration, and the location of these clusters depended on which of the cortical
628 regions had been damaged. Bernhard von Gudden (1824-1886) was one of the first
629 scientists to use this ablation method to describe the organization of the thalamus
630 from a connectivity and possibly functional point of view. As the animals with lesions
631 had to survive post-surgically for a while, sometimes several months, they could be
632 observed and behavioral consequences of their lesions could be evaluated. Von
633 Gudden also invented a microtome, this instrument enabled the preparation of clean
634 human brain sections, 55 µm in thickness after Forel's improvements. Von Gudden is
635 best known for his studies showing a partial decussation of the optic paths, on which
636 he spent about 30 years of his researcher life (Sarikcioglu, 2007). As a renowned
637 psychiatrist, von Gudden was assigned to follow the health of King Ludwig II of
638 Bavaria, who was suffering mental illness diagnosed as paranoia. On June 13th,
639 1886, they both had an after-dinner walk by the Starnberg Lake, in whose waters
640 they were found dead half an hour before midnight. What exactly happened remains
641 mysterious, but it is well possible that King Ludwig II, who was very corpulent, killed
642 von Guden before himself dying of cold water shock.
643
644 Before von Gudden, Luigi Rolando (1773-1831) had performed thalamic lesions in a
645 variety of animals (lambs, pigs, dogs, hamsters, chickens, ducks, turtles), and he
646 noticed that motor incoordination emerged in mammals, blindness in birds, and
647 cognitive decline in reptiles (Serra et al., 2019). On his side, François Magendie
648 (1783-1855), who also used lesion approaches in frogs and birds, found functional
649 impairments that went beyond the visual sphere as they encroached onto motor
650 functions. In general, however, the ablative methods lacked – sometimes seriously –
651 precision. For instance, when used in young animals or after very long survival times,
652 as sometimes in brain-damaged humans, degeneration could extend both
653 retrogradelly and anterogradelly over a few synapses. Therefore, although pointing
654 towards interesting directions, the von Gudden method did not permit an accurate
655 description of the organization and functions of the thalamus. As a result, where a

consensual agreement would have been welcome, the room for controversy and dispute remained large. In particular, Marie Jean Pierre Flourens (1794-1867) was inexhaustible in matters of sharp criticism of the ablative method, which he nevertheless systematized by introducing a principle of rigorous delimitation of the damaged region and a requirement of reproducibility of the observations made (Serra et al., 2019). At this time, however, the thalamus was clearly more than just a question of visual functions. It is noteworthy that, despite this skepticism, until the 1940s, the retrograde cell degeneration method was frequently used (Jones, 1985).

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Insert Figure 5 about here

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11. Combination of staining- and ablation-based studies

A true progress in connectivity tracing occurred when Vittorio Marchi (1851-1908) went to Camillo Golgi's laboratory as a specialization fellow, and there developed a novel staining method that enabled unambiguous visualization of degenerating nervous fibers (Talamonti et al., 2013). Quickly this method became very popular in the field of neuroanatomical studies. It consists in placing tissues in a mixture of osmium tetroxide and potassium chlorate used as an oxidizing agent. So exposed, degenerating myelin becomes black; the material stained is a cholesterol ester or a polysaccharide (Strich, 1968). As with the aforementioned ablation method, a lesion was essential for successful tracing with the Marchi fluid, but this technique now allowed perfect localization of the pathways that underwent Wallerian degeneration triggered by the lesion. A major drawback, however, was that it only reveals degenerating myelin sheaths, and thus was restricted to tracks of myelinated fibers (Figure 5). Furthermore, it was reputed capricious, variable and generating artefacts (Pricket and Stevens, 1939).

Nevertheless, the technique led to the identification of deep thalamic connections such as the ansa peduncularis of the thalamus, the auditory pathway, the cerebelothalamic afferences, the optic tract, the spinothalamic tract, and the trigeminothalamic tract (Serra et al., 2019). It clearly aided agreements in many discussions concerning the intrathalamic localization of endings of afferent pathways

(Jones, 1985). To get a more precise idea of how studies relying on Marchi's staining were constructed and how the connections of thalamic nuclei could be visualized under a microscope, the reader might see e.g., Le Gros Clarke and Boggon (1933a; 1933b). Le Gros Clarke made his mark on the history of the thalamus with an impressive review paper he published in 1932, and in which he summarized a large part of what had been done so far (Jones, 1985). While Marchi's method has been decisive in the study of corticothalamic connections, this is not true the other way round, i.e., in the thalamocortical direction (Jones, 1985). It was not before 1882 that myelinated fiber tracts could be nicely stained in the absence of lesions, when Carl Weigert (1845-1904) introduced his technique based on the use of potassium dichromate and hematoxylin to stain the myelin sheaths of axons. Conversely to reduced silver methods which stained axons themselves (e.g., Ramon Y Cajal, 1891), and thus led to a jumbled staining in which detail was difficult to follow, Weigert's methods produced a nice staining with weak background.

Further progress in fiber tracing appeared with the revolutionary contribution of Walle Nauta (1916-1994), his colleague Loyd Ryan, and the former's doctoral student Paul Gygax, near the middle of the 20th century. Already as a student, Nauta had been attracted by the hypothalamus, a structure in which most fiber tracts are unmyelinated. This characteristic unfortunately precluded the use of Marchi's method to study fiber tracks. In Nauta's view, an ideal stain would, therefore, highlight the axoplasm affected by the degenerative process, but without interfering with the other cells of the hypothalamic parenchyma. At the time Nauta tried to develop a technique meeting these specifications, he was a lecturer in Zürich, where he spent four years (1947-1951) in the laboratory headed by Gian Töndury, a renowned anatomist also interested in embryology. Nauta's method derived from the silver impregnation method of Max Bielschowky (1908). Bielschowsky's method had two major drawbacks: a high staining background and an unstable reproducibility. A third drawback was its time-consuming implementation, as, before additional improvements brought later on by other users, it took about 49 hours before the tissue was observable under a light microscope.

What Nauta tried to set up was a technique marking degeneration of amyelinic fibers in association with a reduced background from the co-staining of intact fibers. It is

beyond the scope of this article to present the many attempts Nauta made, and how he tried to meet this challenge, but success finally relied on perseverant empiricism and some serendipity (Jones, 2006). The method (Nauta and Gygax, 1954) was subsequently used in many places throughout the world to discover or reinvestigate all major connections in the central nervous system (Jones, 2006), including those of the thalamus. The principal gain of this method was an – at the time –, unachieved resolution quality, which made it remain the principal method for investigating neuroanatomy until the 1970s. For instance, in 1960, Nauta published a paper on the neuroanatomical organization of the spinothalamic track. Minderhoud (1971) used Nauta's method in order to visualize the efferent connections of the reticular nucleus in the rat, and Jones (1967) described cortical and thalamic connections of the somatosensory cortex. Johnson (1961) used it to study the connections between the thalamus and the striatum in the cat. Other descriptions concerned the cerebellar projections to the thalamus in the monkey (Kusama et al., 1971). These are just a few examples. Thanks to Nauta, the visualization of degenerating fibers had much improved, but the problem concerning the control of the localization and extent, and thus of the exact delimitations of the lesions and the latter's reproducibility remained unchanged. The progress nevertheless came at a point where neuroanatomy, which for a long time had been content with being barely a little more than structural, began to question itself more and more firmly in terms of functional analyses, because investigation tools then allowed acceptable tracking of connectivity.

Almost at the same time as intensive connectivity studies were performed with the Nauta technique, the introduction of glutaraldehyde (a molecule first produced in 1951) by Karnovsky (1965) and the availability of pure paraformaldehyde allowed improved brain fixation, compatible with reliable electron microscopy observations. These new approaches were considered a true revolution in the investigation of the nervous system anatomy. Indeed, electron microscopy gave access to the recognition of axons, dendrites, dendritic spines, synaptic structures, organels… (e.g., Peters et al., 1976). Amongst major advances was the finding that the majority of thalamic fiber terminations in the cerebral cortex were upon dendritic spines, implying that the fibers did not only end on cells other than the not well-understood 'stellate cells' of layer IV, but also on pyramidal neurons. This would not have been a surprise for Cajal, who proposed at the end of the 19th century that the pyramidal

cells were the major recipients of thalamic afferents. In the 1960-1970s, the German school of cytoarchitectonic saw thalamic afferents ending on vaguely-defined 'granule cells' of layer IV. The latter were supposed to relay the afferent impulses to the pyramidal cells (Sholl, 1956; Jones, 2007 for a review). The renewed gain of interest for the Golgi technique that could be used on fixed tissue permitted the (re)examination, with both light and electron microscopy, of the classification of cortical neurons in the primate visual and somatosensory cortex. Thalamocortical projections were also (re)investigated, often in conjunction with terminal degeneration of specific pathways (Fairen et al., 1977; Jones, 1975). In particular, it became possible to identify with precision the cortical neurons in lower layer III and in layer IV, with which thalamic afferents establish synapses (e.g., Peters, 1979).

For instance, Jones (1975) used a combination of **i)** axonal degeneration (Nauta technique, see above), **ii)** autoradiography ([^{3}H] proline, see below), both targeting the ventrobasal and/or the ventrolateral thalamus, and **iii)** rapid Golgi coloration and light microscopy in order to precisely study the terminations of thalamic afferents in the sensorimotor regions of the primate cerebral cortex. He showed, for the first time in somatic cortical areas, a great proportion of thalamic terminals in a part of layer III, in addition to the expected dense projection in layer IV, previously evidenced using degeneration techniques. He also showed that this part of layer III encompassed large pyramidal cells and corresponded to layer IIIb of Brodmann. A second major finding concerned qualitative and quantitative differences of thalamic terminations in area 3 as compared to areas 1 and 2 of the somatic cortex: terminals were found to be largely denser in area 3 than in the two other ones.

Another example is by Morest (1975), who studied the synaptic relationships of Golgi type II cells (neurons with a short ramifying axon) in the medial geniculate body of the cat using combined Golgi and silver-degeneration methods, and electron microscopy. He showed that these cells form dendro-dendritic synapses with the principal neuron in terminal aggregates called synaptic nests, and that Golgi type II cells that receive endings from the afferent axons send processes to principal cells, which are also contacted by the same afferent axons. Furthermore, Morest's analyses pointed to the origin of different types of afferent axonal endings and defined the morphological types of axonal endings and their relationship with the Golgi type II cells. Finally,

Morest provided a fine description of the cytological varieties of synapses according to their origins and their relationships to the surface of the Golgi type II cells. To give a last example, Somogyi (1978) also used combined degeneration, Golgi staining and both light and electron microscopy methods to study thalamocortical connections. After damage to a lateral geniculate body, he visualized thalamocortical projections synapsing on two neurons located in layer IV, presumably a spiny interneuron and a small pyramidal one, which established connections with the dendrites of two cortical stellate neurons. Thus, he was able to describe three links in a thalamo-cortical-intracortical neuronal chain.

12. Direct tracing methods

The aforementioned problem related to the fact that lesions were mandatory would be solved with a tracing technique that does not require its combination to a lesion approach, and thus a method enabling direct visualization of fibers connecting two brain structures. In the early 1890s, as mentioned above, silver impregnation techniques (e.g., Ramon y Cajal, 1891) enabled the staining of neurons, but they stained all axons, which negated any precise investigation in the jungle of axons. It is also noteworthy that a gain in precision of the lesions was achieved by the use of stereotaxic apparatuses that were adapted from primates to other animal species (e.g., Blomstedt et al., 2007), but even with an inframillimetric assistance to the 3D positioning of lesion devices in the brain, there remained between-subject variability.

In the late 1960s and early 1970s, methods relying on the axonal transport of injected molecules to either axonal processes (anterograde tracers; Figure 6, top) or cell bodies (retrograde tracers; Figure 6, middle) began to develop. Kristensson and Olsson (1971a, b) found that the intrinsic cellular transport mechanisms can be used to spread stains within neurons and label the origin, the course and termination of axons. This discovery, combined with immunohistochemistry, genetics, and neurophysiology, signaled the beginning of a new era in neuroanatomy (Lanciego and Wouterloud, 2011 for a review, Figure 6, bottom), including for the thalamus. The first tracers (anterograde) were radiolabeled amino acids ([^{3}H]leucine or [^{3}H]proline). They were injected into the cerebral tissue, whence they were incorporated in polypeptides, transported to axons and terminals, and subsequently identified by autoradiography (Droz and Leblond, 1962, 1963; Grafstein, 1967). They were used

826 systematically by Cowan, Hendrickson and collaborators (e.g. Cowan et al., 1972,
827 see also Woolsey, 2016). The Cowan et al. review article summarized publications
828 using autoradiography alone or in combination with degeneration methods and
829 became a standard reference.
830
831 For example, the retino-thalamic pathway in the monkey and chick was labelled
832 without any lesion after injection of a radioactive tracer in the eye vitreous
833 (Hendrickson et al., 1970; Hendrickson, 1972; Schonbach and Cuénod, 1971;
834 Schonbach et al., 1971). Likewise, the cortico-thalamic pathways were defined after
835 radioactive tracer injection in the mouse somatosensory or motor cortex (Price and
836 Woolsey, 1971). In the macaque, using [^{3}H]proline, Nelson and Kaas (1981)
837 described the somatotopic pattern of the connections between the ventroposterior
838 nucleus of the thalamus and areas of the somatic cortex. In dogs, using a
839 combination of [^{3}H]leucine or [^{3}H]proline, Person et al. (1986) traced the contralateral
840 projections of the fastigial nucleus to – among other targets – the thalamus, including
841 the paraventricular complex and the medial dorsal nucleus, as well as the central
842 medial, paracentral, parafasicular central lateral, ventral medial, and ventral lateral
843 nuclei. The eye-specific segregation of the retinal input in the dorsal lateral geniculate
844 nucleus was identified with [^{3}H]proline combined to local eye lesions or eye removal
845 (e.g., Guillery et al., 1980). Notice that ocular dominance columns were identified by
846 Wiesel et al. (1974) with the same technic, which was also used to study the
847 development of these columns (e.g., Ruthazer et al., 1999).
848
849 Such autoradiographic techniques provided a very sensitive method for tracing fine-
850 fibered systems and for visualizing pathway terminals. Furthermore, these methods
851 did not suffer from retrograde transport of labeled materials or from the fiber *en*
852 *passage* problem (Figure 7) observed with the previous degeneration-based
853 techniques. This approach supplanted the Nauta techniques (Jones, 2007 for a
854 review). Tracers such as [^{3}H]leucine were also used in functional imaging studies.
855 For instance, Pohle and Matthies (1974) injected [^{3}H]leucine intraperitoneally to
856 identify the structures implicated in the consolidation of a brightness discrimination
857 task; an increased radioactivity was found in the hippocampus and cortex, not in the
858 lateral thalamus, disqualifying the latter from a possible contribution to consolidation
859 of that type of memory. Although the autoradiographic method had a huge impact on

the field of neuroanatomy, radioactive tracers were not used for very long. Different reasons included: **i)** the long duration of the experiments, **ii)** the nature of the labeling (indirect, i.e., silver grains outside of the labeled neurons vs direct, i.e., immunohistochemistry labeling inside the neurons), and **iii)** the restrictions and measures associated with the short- and long-term management of radioactive isotopes (Lanciego and Wouterloud, 2011).

In the 1970s-1980s, the use of retrograde tracers arose. Macromolecules injected around a set of axon terminals were transported back to the cell bodies of origin. These tracers are compatible with light or electronic microscopy using histological or immunohistological processing, fluorophores, or conjugation of a fluorophore with an enzymatically active probe (Saleeba et al., 2019 for a review). The first – mainly – retrograde tracer, namely the glycoprotein and enzyme horseradish peroxidase (HRP), started to be used in the early 1970s. It could be visualized by a simple histochemical reaction. The first reports with HRP labeling concerned the peripheral nervous system (Kristensson et al., 1971a,b,c). These authors introduced HRP in the gastrocnemius muscle of rats and after a few days detected HRP activity in spinal motoneurons. A similar experiment with HRP injected into the tongue of rats showed labeled motoneurons in the hypoglossal nucleus. LaVail and LaVail (1972) demonstrated retrograde transport of HRP in the CNS itself after intraocular injection in young chicks; 23 to 30 hrs later, they visualized peroxidase in cell bodies of the isthmo-optic nucleus.

The HRP method was also applied to the study of thalamic connections. For instance, in the cat, Nakano et al. (1980) traced projections from the cerebellum to the ventral nuclei of the thalamus. Still in the cat, Somogyi (1978) described fibers innervating the three anterior nuclei of the thalamus from the hippocampal region, septum, cingulate cortex, and mammillary nuclei. In the rat, Herkenham (1978) provided the first evidence of direct thalamo-hippocampal connections using HRP injection in the reuniens nucleus and anterograde fiber tracing by autoradiography. Indeed, efferents from the reuniens nucleus were shown to innervate the entorhinal and parahippocampal cortices, and Ammon's horn. Entorhinal afferents were localized to layers I and III, while hippocampal ones were restricted to the stratum lacunosum moleculare of the CA1 field and the corresponding stratum in the ventral

subiculum. Hayes and Rustioni (1979) developed a method based on the combined use of enzymatically active HRP and inactive radioactive [^{3}H]apo-HRP for exploration of axon collaterals in the somatosensory systems of adult cats. They showed the topography of double labeled and single labeled neurons in the ventralis posterolateralis thalamus (VPL) after ipsilateral HRP injection in the somatosensory cortex I and [^{3}H]apo-HRP in the somatosensory cortex II , with 10% of cells combining both labels. Finally, Hayes and Rustioni (1979), using the same approach, showed that the projections from the rostral cuneate nucleus to the VPL and cerebellar cortex arise from 2 separate cell populations.

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Insert Figures 6 & 7 about here

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Although HRP suffered from the "fiber *en passage"* problem (Figure 7), as did the Nauta technique, its far more limited technical demands as compared to autoradiography made it the next '*revolutionary*' method for connection tracing in the nervous system. Some refinement of the HRP technique came from its possible combination with the lectin wheat germ agglutinin (WGA), which improved both uptake and transport within neurons, increasing the sensitivity by up to 40 times (Gonatas et al., 1979; Köbbert et al., 2000). WGA binds to N-acetylglucosamine and the plasma membrane-bound sugar sialic acid, and it is rapidly and actively transported in both anterograde and retrograde directions providing more extensive labeling of the neuron as compared to HRP alone (Levy et al., 2017). For instance, WGA-HRP was used to study the olfactory representation in the rat thalamus. Price and Slotnick (1983) injected the tracer into the mediodorsal thalamus and showed labeled cells in the polymorphic cell zone deep to the olfactory tubercule, the ventral endopiriform nucleus deep to the piriform one, and in a similar position deep to the periamygdaloid and lateral entorhinal cortices. After an injection into the submedius nucleus, the number of labelled cells was smaller in similar areas, except deep to the lateral part of the piriform cortex. These results were confirmed using the anterograde tracer [^{3}H]leucine injected between the anterior piriform cortex and the olfactory tubercle.

928 Cholera toxin subunit B (CTB) was introduced in 1977 (Stoeckel et al., 1977) and
929 started to be used for retrograde tracing, either alone or combined to HRP, the HRP-
930 CTB conjugate showing better staining quality than free HRP (Trojanowski et al.,
931 1981, 1982). For example, Krout et al. (2002) provided a very fine description of all
932 the projections from the brainstem to the midline and intralaminar thalamic nuclei.
933 CTB was injected in each of the 6 nuclei constituting the intralaminar thalamus, the 6
934 nuclei of the midline thalamus, as well as in the anteroventral parvicellular part of the
935 ventral posterior and caudal ventral medial nuclei. It was found that each of these
936 thalamic nuclei receives input from a selective set of brainstem nuclei. Previous data
937 showed that the efferent projections from these thalamic nuclei to the cerebral cortex,
938 striatum and amygdala are highly specific (Berendse and Groenewegen, 1990, 1991;
939 Moga et al., 1995; Turner and Herkenham, 1991). Krout et al.'s findings contributed
940 to change the concept of the midline and intralaminar thalamic nuclei as forming a
941 *non specific* relay center that globally activates the cerebral cortex in response to
942 signals from the brainstem reticular formation.
943
944 During the 1980s, more sensitive inorganic fluorescent retrograde tracers such as
945 FluoroGold™, '*the gold standard*' tracer in rodents, were developped (Lanciego and
946 Wouterlood, 2011). Fast Blue, Diamidino Yellow, True Blue, and the carbocyanines
947 DiI and DiO (Figure 8) are other such tracers (e.g., Bentivoglio et al., 1980; Kuypers
948 et al., 1980; Puigdellivol-Sanchez et al., 1998). Importantly, these fluorescent dyes
949 can be combined in the same experiment for multiple retrograde labeling, allowing
950 the demonstration of collateral projections. Typically, a dye fluorescing at one
951 wavelength is injected into one terminal site, and a second dye fluorescing at a
952 different wavelength into a second site, so that both are transported to the same
953 parent cells (Akintunde and Buxton, 1992; Jones, 2007).The carbocyanine dye
954 tracing method allowed the visualization of the topography of the thalamocortical
955 connectivity and interactions with subplate (Molnar and Blakemore, 1995), as well as
956 of its development in human post-mortem specimens (e.g., Molnar et al., 1998). The
957 subplate is a zone containing neurons supporting several crucial steps of cortical
958 development and which largely disappears thereafter.
959
960
961 **********

962 Insert Figure 8 about here
963 **********
964

965 Hoover and Vertes (2012) injected fluorogold into the mPFC and fluororuby into the
966 hippocampus, or *vice versa*, and found that 3-9% of the neurons of the nucleus
967 reuniens had collaterals in both regions. At the same period, the vision of a traditional
968 anatomy essentially focused on connectivity and cytoarchitecture in a one-dimension
969 scale, rapidly changed. Indeed, reports described two- or multi-dimensional
970 procedures associating tracing connectivity with double- or multiple
971 immunohistochemical (functional, e.g. neurotransmission- or neuroactivity-related
972 markers [calcium binding proteins, enzymes, transporters…]) identification of neurons
973 and brains areas (e.g. van der Kooy and Steinbush, 1980; rev Lanciego and
974 Wouterlood, 2020); multi-fluorescence microscopy was used for determining
975 colocalization. Thus, Gerfen and Sawchenko (1985) used the uptake and transport of
976 leucoagglutinating subunits complexes of the red kidney bean (Phaseolus vulgaris)
977 lectin (PHA-L, Gerfen and Sawchenko, 1984), one of the earliest and most widely
978 '*conventional anterograde tracer'* with high sensitivity and specificity.
979

980 PHA-L introduction marked the beginning of multi-dimensional anterograde tracing
981 (rev Groenewegen et al., 1990; Lanciego and Wouterlood, 2011). PHA-L, like WGA,
982 binds to membrane bound carbohydrates (N-acetyl D-glucosamine and mannose) to
983 enter cells and is detected via antibodies against PHA-L. Gerfen and Sawchenko
984 (1985), using PHA-L injection in the substantia nigra and tyrosine hydroxylase (TH)
985 immunoreactivity, showed that most nigrostriatal and nigrocortical projections were
986 dopaminergic (Figure 6, bottom) and unexpectedly, some nigrostriatal afferents were
987 non-dopaminergic and morphologically distinct, with a much thicker caliber than the
988 dopaminergic afferents (0.1 vs 0.5 µm diameter). These authors pointed to the
989 importance of the combination of anterograde tracing with immunohistochemical
990 identification of labeled fibers to get new information on the neurochemical tag of the
991 neuroanatomical pathways. Originally most of these approaches used non
992 fluorescent reported markers.
993

994 One of the first studies to use PHA-L for tracing thalamic connections visualized
995 thalamocortical axon terminals in vibrissae area of the mouse somatosensory cortex

(Keller et al., 1985). Aarnisalo and Panula (1995) were among the first to introduce fluorescence reporting targeting neuropeptide FF-containing efferent projections from the medial hypothalamus. They combined PHA-L tracing with labeling of a small population of target neurons with neurobiotin (dendrite labeling) and opened the modern "*fluorescence age*".

Approximately at the same time as the development of the anterograde PHA-L tracer, Glover et al. (1986) introduced conjugates of dextran-amines and fluorescein and rhodamin as neuroanatomical tracers. Two key methodological papers using the systemic application of biotinylated dextran amines (BDA) in rats and monkeys were published by Veenman et al. (1992) and Brandt and Apkarian (1992). This tracer, which can be conjugated to fluorescent dyes or biotinylated, was rapidly adopted and still remains widely used, due to its high quality of cell processes labeling and suitability for electron microscopy without prior alteration. Although first considered as an anterograde tracer, many studies indicate bidirectional travel that is exploited for conventional tracing (e.g., Sivertsen et al., 2014) or for the delivery of calcium-sensitive indicators for optical recording of neurons selected by axonal trajectory (O'Donovan et al., 1993).

One interesting example of its use to study the thalamus is by Craig et al. (1994). In the macaque monkey, these authors described a thalamic nucleus specific for pain and temperature sensation. They used: **i)** a combination of retrograde PHA-L-Texas Red to label the spinothalamic tract and single unit recordings, **ii)** injections at the trigeminal and cervical levels and at cervical and lumbar levels of FITC- and TRITC-labelled dextrans, and **iii)** calbindin immunoreactivity (IR) with FITC. They showed that a distinct nucleus in the posterior thalamus received a dense topographic input from spinothalamic lamina I neurons, and that this nucleus is constituted of nociceptive or thermoreceptive-specific neurons. Calbindin IR showed that this nucleus, named VMpo (posterior part of the ventral medial nucleus) is defined by a dense calbindin-positive fiber plexus. Applied to human thalamus sections, Craig et al. (1994) found a nearly identical fiber plexus localized in a nucleus that is cytoarchitectonically homologous to the lamina I relay nucleus in the macaque monkey. Two other examples of the same group used the BDA and CTB methods to study the thalamus connectivity, and especially the cortical afferents of the zona

incerta (ZI) (Mitrofanis and Mikuletic, 1999), as well as its efferents to the dorsal thalamic nuclei (Power et al., 1999). In the first study, the BDA tracer was injected into various parts of the cortex (frontal, cingulate, parietal, occipital…) or the CTB tracer into the ZI. The authors showed that the cingulate cortex has the heaviest projection to ZI, the occipital cortex the weakest, and that all retrogradely-labelled cells in the neocortex were limited to layer V. The second paper showed that there are large projections from the ZI to various dorsal thalamic nuclei, mostly the association and intralaminar ones.

In addition, biotin derivatives, such as BDA, but also biocytin and neurobiotin, are used to fill neurons as a neutral marker after intracellular recording. Biocytin and neurobiotin were introduced by Horikawa and Armstrong (1988) and Kita and Armstrong (1991), respectively. The quite short survival time of these dyes (1-4 days) is due to a quick metabolism and renders them useful for short-distance anterograde/retrograde tracing, notably in combination with electrophysiological recording (Taverna et al., 2004) and chemical phenotyping (Toney and Daws, 2006). Pinault (1996) elegantly described a novel single-cell staining procedure *in vivo* under electrophysiological control, targeting thalamic cells with biocytin or neurobiotin via juxtacellular injection. He showed that the reticular cells projected to restricted regions of a single thalamic nucleus, including anterior thalamic nuclei, and that the thalamus and reticular complex have reciprocal connections.

Finally, despite their durable popularity, it is to note that for most of the '*conventional tracers*' cited above (whether retrograde or anterograde), there are some well-described drawbacks. These include: **i)** a direction of axonal transport that is rarely exclusive, complicating circuit analysis, **ii)** that these tracers are taken up by fibers *en passage*, **iii)** the spread around the injection sites that induces intense and diffuse labeling (see more details in Saleeba et al., 2019).

13. Viruses as tracing tools

The mid-1970s began a new era with the introduction of much more sensitive techniques than those using '*conventional tracers*', based on the use of viruses as markers. These techniques exploited the natural capacity of some neurotropic viruses to be transported along the axons and to travel across neuronal pathways. In

addition, viruses have a unique ability to self-amplify by replicating in recipient neurons, producing intense transneuronal labeling (Kuypers and Ugolini, 1990; Ugolini, 2010 for a review). It is out of the scope of this historical review to list all viral transneuronal tracing methods, the corresponding viruses, their combination, as well as the great variety of transgenic mice designed to serve some of these neuroanatomical tracing approaches. For more details, see the reviews by Lanciego and Wouterloud (2011, 2020), Nassi et al. (2015), Ugolini (2010) and Wouterloud et al. (2014). Here, we focus on the main viral approaches and how they were used to reveal/confim/validate aspects of thalamic connectivity.

The origin of viruses has its roots in human medicine with a so-called 'mysterious agent', transmitted via kissing or sexual contact; it causes blisters on the lips, in the oral region or genitals. Hippocrates and the emperors in Rome seemed to be aware of this phenomenon, and Shakespeare may have mentioned it in his 'Romeo and Juliet' tragedy (excerpt: *"O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plagues, because their breaths with sweetmeats tainted are"*). The vector of the disease was identified in the 1920s as Herpes simplex virus (HSV). Goodpasture and Teague (1923) described the type 1 virus, HSV-1, as an infectious agent in skin epithelial cells, which migrates to nerve ending and then travels along sensory nerves to settle in neuronal perikarya inside spinal cord and cranial nerve ganglia. Hill et al. (1972) confirmed by electron microscopy the presence of HSV nanoparticles inside axons. Evidence that such viruses might move through neuronal circuits has been found in quite old literature (e.g., Howe and Bodian, 1942 [poliovirus]; Kristensson, 1996 [Borna disease virus, influenza virus…]; Sabin, 1938 [herpes virus]). These authors established the fundamental principles of the use of these viruses for analysis of brain circuitry. For example, in mice, Sabin (1938) examined the different routes of entry into the CNS of a variety of neurotropic viruses following intranasal infection. He showed that all produced infection, but some viruses infected multiple neural systems (e.g. pseudorabies virus, PRV) except the olfactory one, while others (e.g. vesicular stomatis virus, VSV) only infected the olfactory system. This pointed to some specificity and thus differential applications according to the virus type.

In the 1970s, with the pioneering work of Krister Kristensson and collaborators, began studies using a neurotropic virus for pathway tracing in both the CNS and PNS. Kristensson et al. (1974) provided the first clear example of transneuronal infection of sensory neural pathway using Herpes Simplex virus type 2, HSV-2, injected intra-ocularly. A few years later, the same authors used HSV-1 to trace olfactory sensory pathways after unilateral snout infection (Kristensson et al., 1982; Kristensson, 1996). However, it is not until the early 1990s that the use of viruses became a consistent part of neuroanatomy literature. In the rat, Ugolini et al. (1989) nicely described HSV-1 labeling of multisynaptic circuits in cortical and brainstem neurons after HSV-1 injection in peripheral (forelimb, hindlimb) nerves. One example targeting the thalamo-cortical pathways in the cebus monkey was provided by Zemanick et al. (1991), who used 2 types of HSV-1 viruses: the HSV-1 (McIntyre-B) virus as a retrograde tracer, and HSV-1 (H129) as an anterograde tracer. After injection of HSV-1 (McIntyre-B) in the primary motor cortex, they showed densely labeled neurons in all the cortical and subcortical regions that project to the arm area of the primary motor cortex and notably, in two subdivisions of the ventrolateral thalamus, the ventralis lateralis pars oralis (VLo), and ventralis posterior lateralis pars oralis (VPLo). In addition, densely labeled neurons were present within specific portions of the reticular thalamus that projects to the VLo and VPLo. Globally, within various brain areas, a very different pattern of transport was observed with HSV-1(H129) injected into the primary motor cortex, but concerning the thalamus, the ventrolateral part was labeled as with the HSV-1 (McIntyre-B). For the authors it was unclear whether these thalamic neurons were labeled by retrograde or anterograde transport of HSV-1(H129).

In the beginning, viruses were used as '*conventional tracers'* with the clear advantage of improving the retrograde filling of neurons with Golgi-like retrograde labeling, notably with rabies viruses. In addition, RNA viruses (e.g., rabies) showed a low cytopathogenicity and there was no uptake through 'fibers *en passage'* (Nassi and Callaway, 2006; Ugolini, 1995, 2008). Some viruses are particularly well-adapted for transynaptic labeling and allowed the visualization of entire functional neuronal networks (first order neurons, i.e. the initially infected one, 2nd order, i.e., after the first synapse, and so forth, Kuypers and Ugolini, 1990). Since the 1990s, two main classes of viral transneuronal tracers have been available, the Herpes Simplex virus

type 1 (HSV-1) and pseudorabies (PRV), both derived from alpha-herpes viruses, and the rhabdovirus (e.g., RV) belonging to rabies virus (Kuypers and Ugolini, 1990). The introduction of the retrograde transneuronal tracing methodology based on the use of HSV-1 or PRV viruses was the major step in the development of sensitive transneuronal tracers (e.g., Middleton and Strick 1994; Ugolini, 1995). An example of its application to rat thalamus connectivity is described in the study of the visual pathways labeled with 2 strains of PRV, the PRV-Be (wild type and virulent strain), or the PRV-bartha (attenuate vaccine strain) after intravitreal injection (Card et al., 1991, 1998). The data showed a marked infection of the geniculate complex of the thalamus. However, if the 3 sub-areas (dorsal, intermediate, or ventral) of the geniculate nucleus were labeled with the wild type PRV (PRV-Be), only the intermediate and ventral geniculate nuclei were infected after PRV-bartha injection. This pointed to the differential sensitivity of brain cells according to the virus strain. In addition, advances in the use of virus rapidly led to the introduction of less aggressively spreading strains of virus. Another example is by McLean et al. (1989) who injected the HSV-1 in the primary visual cortex of rats and showed intense labeling (Golgi-like) in several ipsilateral visual relay region, notably the dorsal lateral geniculate nucleus of the thalamus and back to the retina.

Some improvement of tracing methods was obtained by combining virus transneuronal tracing with other methodologies such as: **i)** rabies virus and neurotransmitter or cell markers: these approaches are possible because infected neurons remain metabolically viable for a long time (unlike the narrow window by alpha-herpes viruses) (Ugolini, 2010); among the immonomarkers are antibodies against choline acetyl transferase (ChAT, for motoneurons and autonomic preganglionic neurons), oxytocin, calbindin, or parvalbumin, and **ii)** mixing rabies virus with CTB fragment to allow a precise definition of the injection area and simultaneous identification of first order (CTB) and higher-order neurons (rabies virus); see Figure 10 for definition of and distinction between first and higher order nuclei. To illustrate the latter method, Prevosto et al. (2010) were interested in cerebellar pathways involved in the control of eye movements and coordination of arm/eye/head movement. They injected unilaterally both rabies virus and CTB in the ventral lateral intraparietal area (LIPv) or in the medial intraparietal area (MIP) in 3 monkeys to trace their direct thalamocortical and polysynaptic inputs. They notably

showed that the first order (CTB) inputs from the thalamus to MIP and LIPv were largely topographically segregated. For example, the pulvinar inputs to MIP derived mostly from the ipsilateral dorsal and lateral parts as well as from the anterior pulvinar, while inputs to LIPv originated from the more caudal medial and ventral portion of the pulvinar complex. The rabies transneuroal disynaptic transfer (at 2.5 days) showed labeling in the ipsilateral reticular thalamus and in some additional thalamic nuclei. At this delay, rabies virus labeling also occurred in some contralateral thalamic nuclei that mirrored the distribution of first order ipsilateral labeling (CTB). At 3 days, the rabies tracer crossed an additional synaptic step, as shown by the labeling of the contralateral reticular thalamic nucleus.

At the end of the 20th century, the development of molecular biology and virology allowed the construction of viral vectors at will. As a result, combinations of preferred features from different viruses and assembled into new, unique, and highly specific vectors (including the ability to switch on gene expression in infected cells), rapidly developed. One of the first reports used a recombinant virus primarily to act as a delivery agent to transfect neurons in the CNS with a gene that codes for green fluorescent proteins (GFP) (Chamberlain et al., 1998). These authors, using an adeno-associated virus (AAV)-GFP vector injected into the parabrachial nucleus, showed a robust GFP immunoreactivity in cell bodies, axons and terminals in the forebrain, brainstem, and spinal cord, attesting of the specific anterograde transport of GFP.

The utility of neurotropic viruses was greatly improved by controlling what cell types are initially infected or are permissive for viral infection, or by controlling the number of synaptic steps that are crossed. De Falco et al. (2001) were originators of the use of a modified form of PRV-Bartha (Ba2001), unable to replicate unless its genome is recombined by a cre-recombinase. The strategy is based on the fact that viral thymidine kinase (TK), required for viral replication, was deleted from the PRV genome and replaced with a ‘floxed stop’ sequence followed by coding sequences for both TK and GFP. This approach also takes advantage of the existence of mouse lines which express cre-recombinase only in specific cell types. Thus, in Ba2001, cre-recombinase will excise a ‘floxed stop’ sequence and allow TK+GFP expression only in Cre-recombinase expressing cells. So, the GFP-expressing virus replicates only in

neurons that express the Cre recombinase and in neurons that are in synaptic contact with the originally infected cells. DeFalco et al. (2001) injected the virus into the arcuate nucleus of mice that express Cre only in NPY neurons (NPY Cre mice). They showed that 4 days after injection, the virus spread notably to the posterior hypothalamus and the mediodorsal thalamus, but also to the dentate gyrus, the piriform cortex, and ventral basal amygdala.

More recently, still with AAV tracing, the projections from cortical layer 6b to thalamic nuclei could be studied. Hoerder-Suabedissen et al. (2018) used cre-dependent viral tracing in a mouse line expressing cre-recombinase in a subpopulation of layer 6b cortical neurons. Unlike neurons from layers 6a and 5, those from layer 6b were found to project on higher order thalamic nuclei. Similarly, still with cre driver mouse lines, a hierarchical organization of corticocortical, corticothalamic and thalamocortical connectivity could be established, leading to the construction of a feedforward or feedback connection model between these brain regions (Harris et al., 2019). Crosshierarchical corticothalamic rewiring after loss of visual input (monocular enucleation) could also be identified and described with such methods (Grant et al., 2016).

Over the same period, glycoprotein G, a key ingredient in multisynaptic retrograde transport, allowed the development of a new tool for first-order-neuron-only-retrograde tracing (rev Callaway, 2008; Callaway and Luo, 2015). Mazarakis et al. (2001) demonstrated that the rabies-G envelope of the rabies virus is sufficient to confer retrograde axonal transport properties to a heterologous virus, both *in vivo* and *in vitro*. They particularly showed, in the rat, strong labeling in several thalamic areas (i.e., centromedian, submedius, pericentral, subthalamic) 1 month after intra-striatal injection of the pseudotyping lentiviral vector (pONY8.0Z) with rabies-G. The substitution by Wickersham and collaborators of the glycoprotein G gene by a gene coding for EGFP produced powerful first-in-line-neuron-only retrogradely transported vectors that, by virtue of expressing EGFP in infected cells, made retrograde labeled infected neurons directly visible under fluorescence (SADΔG-EGFP derived from the SAD B19 rabies virus; Kim et al., 2016; Wickersham et al., 2007a). In the Wickersham et al.'s paper, the retrograde virus (RABVΔG-EGFP) was injected in the mouse thalamus and many bright EGFP-expressing pyramidal neurons in the

overlying cortex were apparent. Finally, Wickersham et al. (2007b) developed a method in which the initial viral infection is restricted to particular cells. They deleted the RG gene from the SAD-B19 rabies genome, and replaced it with GFP. Then, they produced viral particles with a different envelope protein by pseudotyping the RG-deleted virus with the avian virus envelope protein EnvA; this new virus was called Env-A-SADΔG-GFP. When this virus is injected into the brain of a normal animal, it does not infect any neuron because the mammalian brain has no receptors for EnvA. By misexpressing the EnvA receptors, TVA, in particular cells, it was possible to selectively infect those cells (Callaway, 2008 for a review).

This system, tested and validated in cultured slices of neonatal rat brain has the advantage that rabies behaves as a mono-trans-synaptic tracer and permitted the first unambiguous identification of retrogradely connected cells from an initially infected cell. Haberl et al. (2015) used a similar approach and injected the anterograde RABVΔG (VSV-G^{RtmC}) into the mouse ventral posteromedial (VPm) thalamic nucleus or the posteromedial (POm) one. This allowed a 3D reconstruction of 3 excitatory neurons, 1 inhibitory interneuron, and 3 astrocytes within the imaged volume of the thalamus and cortex.

Monosynaptic restriction of trans-synaptic tracing also occurred in the anterograde direction with the first demonstration by Beier et al. (2011). These authors demonstrated that recombinant vesicular stomatitis virus (VSV) vectors can be endowed with anterograde or retrograde transynaptic tracing ability by providing the virus with different glycoproteins (RABV-G or lymphocytic choriomeningitis virus, LCMV-G). By targeting the visual system with eye injection of the anterograde VSV (LCMV-G) virus, labeling was restricted to the visual system including primary retinorecipient areas (lateral geniculate nucleus, superior colliculus, and suprachiasmatic nucleus), and secondary (V1) visual centers (Beier et al., 2011). This paper provided definitive evidence that directionality of a virus is a property of the G protein, i.e., RABV-G for retrograde and LCMV-G for anterograde properties. In 2013, these authors extended the characterization of the transmission and gene expression of recombinant VSV either with the rabies virus glycoprotein (rVSV(RABV-G)) for retrograde labeling, or recombinant VSV-virus glycoprotein (rVSV(VSVS-G)) for anterograde labeling (Beier et al., 2011). Using these two

transsynaptic virus vectors injected into the motor cortex area 1, they showed that the thalamic neurons expressed either the retrograde- or the anterograde virus, with no co-labeling (Beier et al., 2013).

Another example of the contribution of these virus-based neuroanatomical techniques to the thalamus is by Broms et al. (2017). In mice, these authors very nicely described microcircuits within the thalamus and habenula using GPR151, a G protein-coupled receptor whose expression is enriched in specific diencephalic structures. They traced the afferent connectivity of habenular and thalamic neurons defined by their GRP151 expression, using a virus/vector combination in a transgenic Gpr151-cre-mouse. These mice received a mixture of AAV8-Ef1a-FLEX-TVA-mCherry and AAV8-CA-FLEX-RG unilaterally into the habenula, the paraventricular- or the lateral posterior thalamic nuclei. After 21 days, the stereotaxic procedure was repeated for the SAD∆G-eGFP(EnvA) vector injection in the same brain areas. The data showed that thalamic vs habenular GPR151-expressing neurons differed substantially. As an example, the habenular neurons primarily received inputs from basal forebrain structures, the bed nucleus of the stria terminalis, the lateral preoptic area, the entopeduncular nucleus, and the lateral hypothalamic areas. The GPR151 neurons of the paraventricular thalamus were contacted primarily by the medial hypothalamic areas as well as by neurons from the zona incerta.

Finally, Xu and Südhof (2013) mapped the synaptic projections from the medial prefrontal cortex (mPFC) and confirmed a specific neural pathway from the mPFC to the thalamic reuniens nucleus (Re). The neuroanatomical tracing approach used an anterograde synaptoTag-AAV which co-express red fluorescent mCherry protein and EGFP-Synaptobrevin 2 (a synaptic vesicule protein). The SynaptoTag-AAV-infected neurons were filled with diffusible mCherry and selectively localized GFP-synaptobrevin-2 to efferent synapses formed in the target regions. This allowed a quantitative assessment of the number of synapses in the target areas by the SynaptoTag-AAV-infected neurons. Eight weeks after stereotactic injection of the SynaptoTag virus into the mPFC of adult mice, the authors observed that, within the thalamus, most projections were targeted to the mediodorsal (MD) and Re nuclei. Then, to determine if the same mPFC neurons project to both targets, Xu and Südhof injected a cholera toxin B (CTB)-Alexa-Fluor-488 or -594, the former in the Re and

the latter in the MD. Retrogradely labeled neurons were observed in the 3 major mPFC subregions, i.e., the prelimbic, infralimbic, and anterior cingulate cortex. However, most fluorescent mPFC neurons contained preferentially only one fluorophore indicating that these neurons project to only one of these thalamic regions. The following experiments consisted in inactivating either the mPFC-Re- or the mPFC-MD pathway, using double-floxed inverted TetTox AAV in the mPFC and a WGA-Cre AAV in the Re or MD nucleus. Four weeks after viral injections, fear conditioning tests were performed and data showed that the mPFC-Re pathway controls memory specificity (Xu and Südhof, 2013; see also Ferraris et al., current issue).

One key aspect of the development of neuroanatomical tracing concerns the study of the brain of transgenic animals in which cells of a particular neurochemical phenotype express a fluorescent protein, e.g., the green fluorescent protein (GFP) under Cre-mediated recombination (e.g., GFP-cre fusion gene, Sauer, 1998). Then, came neurotransmitter-specific transgenic mice with Cre-recombinase expression in dopaminergic (DA) neurons (Bäckman et al., 2006; Witten et al., 2011; Zhao et al., 2004), GAD67 neurons (Tamamaki et al., 2003), serotonin (5HT) neurons (Zhuang et al., 2005), choline acetyltransferase (ChAT) neurons (von Engelhardt et al., 2007), and parvalbumin(PV)-expressing GABAergic neurons (Tanahira et al., 2009). However, the drawback of this first generation of mice was that all members of a particular species of cells light up green simultaneously (as in the brainbow mice, Weissman et al., 2011). The key vector to achieve trace axonal connectivity in a small portion of neurotransmitter-specific neurons is an adeno virus (AV) or adeno-associated virus (AAV). In that case, the role of the virus is to deliver a gene coding for one of the fluorescent proteins and expression of GFP (or channel rhodopsin) can be forced in Cre-recombinase mice through focal injection with the viral vector. Atasoy et al. (2008) developed a Cre-recombinase-dependent viral vector for targeting channel rhodopsin 2 (ChR2) (e.g., rAAV-FLEX-rev-ChR2mCherry and rAAV-ChR2EGFP) in order to map long-range circuits. As an example, they injected a rAAAV-FLEX-rev-ChR2mCherry virus in the arcuate nucleus of an agrp-cre;rosa26-loxSTOPlox-eyfp mice and observed strong axonal labeling in the projections of AGRP (Agouti-related protein) neurons in the paraventricular hypothalamus as well as in the paraventricular thalamus. Kuhlman and Huang (2008) also found a solution

by using a transgenic parvalbumin (PV)-cre mouse strain to specifically target neocortical GABAergic interneurons by switching GFP expression in a small population of cells. They engineered AAV that express GFP or other markers (dsRedExpress, Channel rhodopsine 2…) upon Cre/loxP recombinant-mediated removal of a transcription-translation STOP cassette (Figure 9). They injected an AAV-lox-STOP-lox (LS1, L) GFP virus into the neocortex of a PV-cre mouse to specifically label neurons expressing cre-recombinase. They followed the dynamic of parvalbumin-containing (PV) GABAergic cortical interneurons over 1 week and showed in young adult mice that inhibitory PV circuits maintained the potential for structural rewiring via boutons on axonal shafts (Kuhlman and Huang, 2008).

Tang et al. (2015) developed a new method allowing the cell type-specific manipulation with GFP-dependent Cre recombinase (CRE-DOG) to directly induce Cre/loxP recombination using plasmid electroporation and AAV viral vectors. They tested their system in the retina and various brain areas using several GFP mouse lines. As an example, using a Tg(TRHR-GFP) mouse (a line expressing GFP in a specific retinal ganglion cell subtype), they showed, after retinal injection of the 2 viruses, rAAV-encoding CRE-DOG OPT and rAAV-FLEX-tdTomato(tdT) (the first virus allowing he turn on of the 2nd one in a GFP-dependent manner), a strong tdT expression in the retinal ganglion cell projection targets, the dorsal lateral geniculate thalamic nucleus, and the superior colliculus.

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Insert Figure 9 about here

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Finally, a last paper illustrating the powerful tool of genetic virus-based tracing methods using Cre driver mouse lines is one by Harris et al. (2019). These authors described a major expansion of the Allen Mouse brain connectivity Atlas resource (Oh et al., 2014) involving thousands of experiments in the cortex and thalamus to map brain-wide connections (cortico-cortical, thalamo-cortical, and cortico-thalamic). For this, they used a cre-dependent AAV vector that express EGFP in the cytoplasm of cre-expressing infected neurons, the AAV2/1.pCAG.FLEX.EGFP.WPRE. They also used a Cre-dependent AAV virus expressing synaptophysin-EGFP fusion

protein to specifically label presynaptic terminals, the AAV2/1.pCAG.FLEX.sypEGFP.WPRE. Injections were made into lines with regulatable versions of Cre, the tamoxifen- or thimethoprim-inducible Cre line, (CreR) mice or (dCre) mice, respectively. The huge amount of data collected allowed the mapping of projections originating from unique cell populations in the same cortical area and from distinct projection classes in the thalamus. Harris et al. were then able to lay the foundation of some anatomical rules of cortical and thalamic connections, i.e., cell-class specific connections are organized in a shallow hierarchy within the mouse cortico-thalamic network.

In conclusion, the introduction of viruses as tracing tools in the 1970s allowed spectacular progress in the field of neuronatomy, particularly thalamus connectivity. From one-dimensional retrograde tracing with herpes simplex virus 1 (Kristensson et al., 1974), a rapid evolution was noticed with: **i)** virus-based tools being combined with genetics and molecular biology, and **ii)** the introduction of adeno-associated viruses (AAV). The latter served as vehicles for altered gene delivery to force neurons to express green fluorescent proteins (GFP), a major discovery in neurosciences. The reader interested in more details about virus-based tracing method is invited to take a look at the recent review by Lanciego and Wouterloud (2020).

All of these combined approaches markedly transformed neuronanatomical tracing techniques into a large multi-dimensional molecular-genetic tracing spectrum: a great tool, that was also applied, for instance, to research on the organization of thalamic microcircuits (Broms et al., 2017), on brain-wide corticothalamic and thalamocortical connections, and on thalamic cell classes (core, matrix, intralaminar, Harris et al., 2019).

The progress of tracing techniques, combined to functional studies supported by electrophysiological approaches, led Guillery (e.g., 1995) to make an important distinction between two kinds of thalamic nuclei based on the nature of their inputs, namely first order nuclei and higher order nuclei (Figure 10). First order nuclei (e.g., lateral and medial geniculate nucleus, ventrolateral and ventrobasal thalamus, and anterior nuclei) receive their sensory inputs from subcortical structures as well as

cortical afferents originating in layer 6, which have modulatory functions. Higher order nuclei (e.g., mediodorsal and lateroposterior thalamus, pulvinar), which have substantially expanded during primate evolution, receive most of their afferents from pyramidal cells located in layer 5 and 6 of cortical regions and send projections mainly to cortical regions which are different from those providing afferents. These nuclei are kind of a thalamic relay for corticocortical communication that often parallels a direct corticocortical one (see also Wolff et al., 2021, current issue). With his long date collaborator Murray Sherman (e.g., Sherman, 2019), Guillery also distinguished two types of glutamatergic pathways, one he called drivers and one he called modulators (Figure 10). Briefly, driver neurons carry the principal information. As such, they are the "main conduits of information" (Sherman, 2019) arriving to thalamic nuclei from where they are passed to the cortex. Conversely, modulator neurons affect the way the information provided by driving neurons is processed. Interestingly, if both types of neurons are glutamatergic, they differ in several structural and functional instances. Indeed, driver neurons act on ionotropic receptors (modulators on metabotropic ones), have large EPSPs (they are small in modulators), have thick axons (small ones in modulators), synapse on proximal dendrites (on terminal ones in modulators), etc (Sherman, 2019). It is noteworthy that the aforementioned classifications, as well as other ones of pathways (e.g., core vs. matrix neurons that coexist in defined nuclei) have been questioned recently (Halassa and Sherman, 2019).

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Insert Figure 10 about here

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14. *In vitro* approaches

Interactions and connections between the thalamus and other structures, predominantly cortical ones, have also started to be investigated with *in vitro* approaches near the end of the 1980s. These approaches included organotypic co-cultures, thalamocortical slice cultures and co-cultured organoids. A cell culture consists in preparing a usually large amount of tissue from which cells are kept operational in an isolated environment under conditions enabling their survival. An organotypic culture is a culture of a tissue or an organ (or pieces thereof) compatible

with structural and functional characteristics closer to *in vivo* conditions than cell cultures have. Regarding the brain, organotypic co-cultures, or slice cultures or slice co-cultures are variants of this methodology applied to portions of the brain. These techniques have the advantage of offering not only possibilities to investigate connectivity and its development/degeneration/regeneration within or between brain regions, but also track many functional characteristics therein (e.g., Humpel, 2015). Regarding the thalamus, the aforementioned approaches have considerably extended the understanding of the development and specificity of thalamocortical interactions. Furthermore, slice culture preparations are highly compatible with functional approaches using localized stimulations and either localized recordings, including patch clamp recording, or calcium or voltage-sensitive dies (e.g., Higashi et al., 2005).

To give just a few examples in relation with the thalamus, Higashi et al. (2005) studied the thalamocortical connectivity in thalamocortical slice preparations from immediately postnatal rats showing reversed cortical layering. Bolz et al. (1990), using visual cortex slices co-cultured with thalamic slices observed that the cortico-thalamic projections developed according to a pattern similar to the one observed in vivo, cortical axons growing directly to their appropriate thalamic target, most probably under the influence of a chemotropic attraction. The same is true the other way around (Bolz et al., 1992; Yamamoto et al., 1989). Agmon and Connors (1991) have developed a slice preparation containing the ventrobasal nucleus of the thalamus and the sensorimotor barrel cortex of the mouse. In this slice culture, they could study the physiology and pharmacology of the thalamocortical synapse, and map the connections. Lotto et al. (1999), again on thalamic and cortical slice co-cultures, could observe that axons from the thalamus to the cortex grew to both superficial and deep cortical layers indifferently, but axons inappropriately located (i.e., in the superficial layers) were subsequently lost, most probably because of repressive vs. permissive intracortical, contact-mediated interactions. Finally, still with thalamic and cortical slice co-cultures, Yamada et al. (2010) labeled individual thalamocortical axons and reduced firing therein. They found that the reduction of activity of thalamocortical neurons decreased branching, demonstrating that such activity also contributes to the correct development of this connectivity.

15. Magnetic resonance imaging

Magnetic resonance imaging (MRI) consists in generating images by three-dimensional detection and computer-assisted reconstruction of the post-excitation (by application of an electromagnetic field at a given frequency) realignments of the magnetic moments of spins of particular atoms (e.g., hydrogen). The first images this technique generated were those of two water-filled capillary tubes (Lauterbur, 1973), and, for the human body, it was that of a finger in 1976 (Mansfield and Maudsley, 1977; in fact it was one of Maudsley's fingers). Damadian, who had understood that MRI would become an investigation tool of very high clinical relevance (Damadian, 1971), is the first to have scanned the entire body (Damadian et al., 1977). Regarding the human brain, Young and Clow obtained the two first MRI images in 1978 (e.g., Hounsfield, 1980).

In 1991, John Belliveau communicated about his dynamic imaging of activation of visual brain areas in response to visual stimulation. This is how functional MRI made the first of many subsequent steps (for an exhaustive account of the story and all its actors, see the special issue of Neuroimage edited by Bandettini, 2012). The principle of fMRI is relatively simple. When a brain region is active, its blood flow increases. When oxygenated, hemoglobin is diamagnetic (meaning that there is no unpaired electron in an orbital of the atom), but once deoxygenated, it becomes paramagnetic (there are unpaired electrons), the contrast between both being detectable in a scanner. Thus, by collecting the three-dimensional distribution of venous blood oxygenation level-dependent (BOLD) magnetic resonance along a time line, it is possible to image the three-dimensional map of brain activation patterns, which correlate with local blood flow.

Diffusion weighted MRI (DWI; e.g., Baliyan et al., 2016) is another development of MRI. It uses the fact that water molecules move in tissues, that their motion is anisotropic, and that the movement coefficients of these molecules depend on the nature and composition of the tissues. Detection of these anisotropic movements is what DWI is doing to construct three-dimensional images of organs and tissues. Diffusion tensor imaging (DTI) is a particular way to model DWI data sets. It visualizes axonal organization in nervous system tissues, and more specifically orientation of fiber tracts. The technique arose in the mid-1990s (e.g., Basser et al.,

1994). It uses the fact that the movement of water molecules is easier along the axonal fiber tracts than when perpendicular to it. DTI exploits this water-motion orientation bias as a probe to infer the neuroanatomical paths of fiber ensembles, this motion undergoing more influence by static anatomy than by physiological factors (Le Bihan and Johansen-Berg, 2012).

All these technics, which have the huge advantage of being non-invasive and compatible with small animal explorations (generally with a higher magnetic field than in humans), contributed to a better knowledge of the anatomy and connectivity of the thalamus in both humans and other animals. In January 2021, a Pubmed search crossing the keywords 'thalamus' and 'MRI' generated a list of > 12,500 hits (and almost as many when 'fMRI' was substituted for 'MRI'). Suffice to say that it would be herculean work to summarize them in a few short paragraphs. Therefore, as often done above, we will only consider a small number of representative examples.

Using DWI, Johansen-Berg et al. (2005) explored the structural connectivity of the human thalamus with the cortex. They established that the connectivity pattern (white matter) between thalamus and cortex was an acceptable criterion to segregate thalamic regions that corresponded to nuclei (gray matter). Before them, Behrens et al. (2003) had reported on similar observations demonstrating a strong similarity between the organization of the human thalamic gray matter according to its cortical connectivity and the different thalamic subregions as shown by histological studies. Furthermore, when compared to the connectivity map of non-human primates, the DTI images revealed very similar. Still with DWI and using a whole-brain approach, Grodd et al. (2020) recently reported on the connectivity of several thalamic nuclei (e.g., anterodorsal, anteromedial, anteroventral…) with other structures of the limbic system. These authors could establish high precision maps of this connectivity and identify some differences with tracer studies. In particular, they distinguished direct projections of the mediodorsal nucleus to brainstem, and indirect projections of this nucleus interconnecting temporal and mediofrontal regions. Interestingly, DTI was also used to follow the timing of the growth of thalamocortical fibers in the human fetal brain (Krsnik et al., 2017).

The connectivity maps such as the ones mentioned in the previous paragraph were also explored at a functional level by resting-state fMRI. Resting-state fMRI is a spatial and temporal signal acquisition approach in which no specific stimulus, no particular instruction and no cognitive task is used. In other words, recordings occur while the subject is resting. It relies on the BOLD signal and, by establishing correlations between the activation patterns, it provides useful information about the macroscopic organization of systems of neural processing. Using such an approach, Kim et al. (2013) could establish a detailed map of functional network connectivity between the basal ganglia, the thalamus, and different regions of the cerebral cortex. This led to a nice description of the cortico-striato-thalamo-cortical circuits in the human brain.

Combination of DWI and fMRI enables direct comparison between structural and functional connectivity patterns. Such combined approaches have also been used to explore the thalamus. For example, using DWI and fMRI, Zhang et al. (2010) have focused on the thalamocortical system. They could establish excellent overlapping of their MRI maps with the maps based on human and non-human primate histology. Whereas histological, functional and connectional maps were concordant in most instances, there were also differences, the greatest of them concerning motor and premotor regions of the cortex. Such differences do not point to the limits of each technique, whether considered separately or in combination. They rather indicate that each technique may measure different aspects of connectivity, which can be mutually enriching and thereby permit a better tuning in the interpretation of relationships between connectivity and functions. Kumar et al. (2017) also combined DWI and fMRI to generate a functional map of thalamic parcels. They found these parcels to correspond to cytoarchitectonally-defined nuclei, of which they characterized the structural connectivity. Interestingly, for some of these thalamic parcels, there seemed to be a good correspondence with thalamocortical maps described in mice according to histological studies.

Anatomical and functional connectivity ‘units’ can also be modulated functionally with non-invasive tools, and this is true for thalamic nuclei. For instance, in the study by Zotev et al. (2018), real-time fMRI was coupled to neurofeedback and electroencephalographic (EEG) recordings. The principle of neurofeedback is to

provide a subject with information about ongoing brain activity so that self-control of this activity can occur. Zotev et al. assessed correlations between alpha EEG activity (in the parieto-occipital region of the brain) and BOLD activity in the mediodorsal and anterior thalamus. At the same time, subjects were recalling autobiographical memories or doing control tasks. The results established an enhancement of the functional connectivity between the mediodorsal thalamus and the inferior precuneus during the fMRI neurofeedback episodes. In addition, the alpha EEG power was correlated with the BOLD activity in the mediodorsal thalamus. Finally, the temporal correlation between the alpha EEG power and the BOLD activity in the mediodorsal and the anterior thalamus was enhanced during the neurofeedback episodes. Based on these observations, Zotev et al. suggested that the functional connectivity between the precuneus and the mediodorsal thalamus contributed to the enhancement of the correlation between thalamic BOLD and cortical alpha EEG activity during the neurofeedback procedure.

Another exploration technique may consist in using deep brain stimulation (DBS) in patients. Usually, DBS is primarily used for a therapeutic purpose. It is invasive, as it requires a surgical procedure consisting in the intracerebral implantation of a device delivering pacemaker-like controlled electrical stimulations to specific brain regions. DBS is a surgical treatment of e.g., Parkinson's disease or some intractable forms of epilepsy. The effects of DBS can be followed with EEG recordings or fMRI. In epileptic patients equipped with DBS devices targeting the anterior and medial thalamus, Zumsteg et al. (2006) observed that the low frequency stimulation of the anterior and medial thalamus induced rhythmical cortical EEG synchronization in some patients, indicating an influence of these thalamic nuclei on cortical activity.

Thus, over the last 20 years, nuclear magnetic resonance, quickly called MRI because people were reluctant to the word 'nuclear', DWI, DTI, fMRI, combined or not with other approaches (e.g., EEG, neurofeedback, DBS), largely supported the recent progress of our knowledge of the functional connectivity of thalamic nuclei.

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Insert Figure 11 about here

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General conclusions

In his first chapter of "The Thalamus", Edward Jones (1985) provided a detailed account of how knowledge about the organization of the thalamus progressed during the 19th century and the first two thirds of the 20th century. With the refinement level of tissue and fiber staining techniques constantly improving, driven by the omnipresent concern to rid them of their disadvantages, the thalamus could be subdivided into a set of nuclei characterized by both their cytoarchitectonic peculiarities and their connection patterns, whether afferent or efferent. This is exactly what Edward Jones insisted on when he wrote *"The past history of thalamic studies, (…), has been characterized by periods during each of which the work was dominated by a particular methodological approach."* (Jones, 1985). Such methods were, for instance, those developped by von Gudden (retrograde degeneration), Marchi (stain of myelin in degenerating fiber tracks), or Nissl (with a stain binding to nucleic acids).

Of course, as is often the case, there was no consensus on the limits of regions or thalamic nuclei, no more than on where fiber tracks were coarsing, starting, ending, or on the nomenclature. One needs only look at the recent article by Mai and Majtanik (2019), for instance, to be convinced that subdiving the thalamus into coherent nuclei or ensembles of nuclei has generated much debate. Indeed, this article, which its authors have limited to the human thalamus, lists no less than twelve proposals for neuroanatomical organization and nomenclature of identifiable thalamic nuclei on a frontal section passing through the posterior commissure, and all these proposals arose between 1971 and 2017. This is less than half a century! Mai and Majtanik (2019) have also risked a suggestion to achieve better consistency within the scientific thalamus community. Now let us wait and see what will happen to it.

Providing an exhaustive account of why and how the conceptions about the macroscopic and microscopic anatomy of the thalamus and of its complex connectivity has evolved lies beyond the scope of the current contribution, but the reader may find some relevant information in the first chapter of Jones' *The Thalamus* (1985), along with an abundant literature. Instead, using a series of selected examples in an already plethoric literature, we have, in centuries of

thalamus history, set milestones at times which, according to our own knowledge of historical and scientific literature, seemed to us to correspond to determinant advances.

When it comes to the future, the next burst of progress will probably rely on furthering explorations supported by 'omics' approaches. In fact, this type of studies has already started to develop. In a relatively recent research article, Nagalski et al. (2016) have used gene expression data from the Allen (mouse) Brain Atlas database in order to ground a proposal about the molecular anatomy of the thalamus complex on the combinatorial expression of a series of transcription factor genes. This combinatorial distinguished nine thalamic nuclei or ensembles of nuclei, each of them having a particular molecular signature. For instance, the lateral thalamus, characterized by high *Tcf7/2* and *Lef1* expression, and less pronounced *Gbx2* expression, encompass the mediodorsal, laterodorsalventral anterior/ventral lateral, the ventral posteromedial and posterolateral, the submedius, the ventromedial, the parvicellular part of ventral posterior, the posterior, the dorsolateral and medial geniculate nuclei or nuclear groups. The reticular thalamic nucleus, which plays a role in sensory processing, attention and cognition, is characterized by a high expression of *Six3* and *Esrrg*, and none of the indentifying transcription factors corresponding to the lateral or other thalamic nuclei.

An even more recently published study focused on this reticular nucleus using a single-cell transcriptomic approach combined to electrophysiological recordings (Li et al., 2020). Li et al. established a cellular heterogeneity in this nucleus. Indeed, they identified four subpopulations of cells: one in which the *Spp1* gene was highly expressed, one in which it was the *Ecel1* gene, another one which expressed both genes, and a final one in which neither was expressed. Interestingly, in coronal sections of the reticular nucleus, *Ecel1* cells formed kind of a shell surrounding a core of *Spp1* cells. The next step established that *Spp1* neurons projected predominantly to first order thalamic nuclei, which receive information from ascending pathways, whereas *Ecel1* neurons projected predominantly to high order nuclei, which gate the cortical feedback to first order nuclei and monitor/modulate cortico-thalamo-cortical communication, especially from one cortical area to another (e.g., Guillery, 1995; Figure 10). *Spp1* neurons, which have a simple, short-extending dendritic

arborization, exhibit greater excitability and a lower action potential threshold than *Ecel1* neurons. They are of the first type. *Ecel1* neurons, which have a complex dendritic pattern with longer ramifications in all directions, have a more robust bursting activity than the *Spp1* ones. Li et al. then examined whether the two types of neurons had differential contributions to electroencephalographic activity in the somatosensory cortex. By genetic manipulations, they reduced Ca^{++} currents in one or the other population and observed that the cortical delta power during non-REM sleep was reduced by the manipulation in *Spp1* neurons, not by a similar manipulation of *Ecel1* neurons. When spindle oscillations during non-REM sleep were analyzed, the reduction of Ca^{++} currents in *Spp1* neurons reduced the oscillations in number and length, whereas the same modification in *Ecel1* neurons increased the oscillations in length (not number).

This type of finely tuned approach certainly foreshadows what the functional anatomy of the thalamus (and of other brain structures) will be in the next years. Technically, it is now feasible to track the organization of neurons within a thalamic nucleus, their precise connectivity patterns with e.g., cortical regions, and their functional characteristics, all of this in close relationship with their transcriptomic peculiarities, and it is possible to do it at a single-cell resolution level (see e.g., Li et al., 2020).

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure captions

Figure 1: The cerebral ventricles as illustrated by Albertus Magnus in his *Philosophia Naturalis* published posthumus in 1506. This drawing corresponds to an anatomo-functional conception in which common sense and basic imagination are determined in the most anterior ventricle, creative imagination, phantasy, rational thought, and evaluation in the ventricle next behind, and memory and reminiscence in the most posterior ventricle. Drawing (Indian ink) is a faithful interpretation by Audrey Chateaux according to a reproduction of the original material.

Figure 2: Ventricular theory of brain functions. Another illustration of the ventricular theory of brain function which points to a connection between the most anterior ventricle and four organs of senses (eye, nose, tongue, and ears). The drawing is from Gregor Reisch's (ca. 1467-1525), Margarita philosophica compilation. In fact, this was the first encyclopedic compilation published so far. Drawing (Indian ink) is a faithful interpretation by Audrey Chateaux according to a reproduction of the original material.

Figure 3: Thalamus according to Vesalius. Drawing of a horizontal section through the head showing the lateral ventricles, the striatum (S) and the right and left (T) thalami. This drawing by Audrey Chateaux (Indian ink) is a faithful interpretation of a photograph of a copy by the spanish anatomist Juan Valverde de Amusco (Lanska and Lanska, 2013) of the original woodcut print of Andrea Vesalius' *Fabrica*. Valverde de Amusco's *Historia de la Composición del Cuerpo Humano* was published in 1556. It included 42 anatomical illustrations from copperplate engravings, of which 38 were copies of Vesalius' work (Bahsi et al., 2020). Valverde de Amusco is considered the greatest Castilian anatomist of this time.

Figure 4: Thalamus according to Burdach. Drawing of a coronal section passing through the human brain at the level of the thalamus. The drawing is a faithful interpretation by Audrey Chateaux (Indian ink) according to Burdach (1822) in *Vom Baue und Leben des Gehirns*. This frontal section is a posterior view. Two original annotations are not shown in this drawing. Above, on the left, one could read: "Burdach .vom Gehirne" (meaning: "*Burdach. Of the brain*"; on the right: "*IV. Tafel.*"

(meaning: “Board IV”). The visible structures in the middle of the section are: **a**, the thalamus; **b**, the medial geniculate nucleus; **c**, the lateral geniculate nucleus; **d**, the pineal gland (with the 3rd ventricule above and behind it); **e**, the superior colliculus; **f**, the inferior colliculus; **g**, the cerebral peduncle; **h**, the superior cerebellar peduncle. On the original board, these indications were not provided. Underneath the section shown here, however, was an annotated drawing outlining of the different structures, to which either letters or numbers were associated. This picture is the first to mention the pulvinar in the literature (as *Polster*; in german, the word means ‘pad’ or ‘pillow’). It is indicated by the asterisk on the current drawing.

Figure 5: Ablation methods. A illustrates the principle of the ablation method to identify existing connectivity between brain structures. Regions ‘a’, ‘b’ and ‘c’ project to their target regions ‘1’, ‘2’ and ‘3’, respectively. In order to identify the origin of the projections to region ‘2’, this region is damaged (top) and, usually, the animal is left for a relatively long survival time after which it is killed for neuroanatomical investigations. The neuroanatomist will seek the regions in the brain that have undergone shrinkage (bottom) due to loss of their target, which would then point to a connectivity between ‘b’ and ‘2’. **B** illustrates the method developed by Vittorio Marchi to visualize degenerating nervous fibers. It consists in placing tissues in a mixture of osmium tetroxide and potassium chlorate. So exposed, degenerating myelin becomes black. As with the ablation method, a lesion or a fiber track section (top) was mandatory for successful tracing, but the staining allowed perfect localization of the pathways that had undergone Wallerian degeneration (bottom), which the ablation method did not. A major drawback of Marchi’s technique was its restriction to myelinated fibers.

Figure 6: Principles of anterograde, retrograde and multidimensional (anterograde+@b) neuroanatomical tracing methods. A Anterograde: a tracer undergoing anterograde transport is injected into a nucleus, from where it is transported to the axonal terminals. Usually, dendrites, cell bodies (of both projection neurons and interneurons) and axons are labelled. The usual tracers are biocytin, biotinylated dextran amines (BDA), phaseolus vulgaris leucoagglutinin (PHA-L), rhodamine-conjugated dextran amine (RDA), wheat germ agglutinin-horseradish peroxidase (WGA-HRP). These tracers do not distinguish the neurochemical identity

of the neurons. **B** Retrograde: a tracer undergoing retrograde transport is injected into a nucleus from where it is transported to the cell body and dendrites of the projection neurons that innervate this nucleus. The usual tracers are Cholera toxin subunit B (CTB), fluoro-gold, pseudorabies virus (PRV), and rabies virus (RV). **C** Anterograde + @b: an anterograde (or retrograde; not illustrated) tracing method is combined to immunohistochemistry with an antibody that will contribute to precise the e.g. neurochemical identity of specific neurons and their terminals within the larger population of labelled ones. Thereby the dopaminergic, cholinergic, GABAergic… nature of the connections between two structures can be identified. This figure has been redrawn according to Lanciego and Wouterloud (2020), figures 2 and 5.

Figure 7: Illustration of the fiber *en passage* problem with the ablation method (A) or the method of Marchi based on myelin staining of degenerating fibers (B). **A** In the case of ablation of region 'b' (on top panel), when fibers pass through the damaged region, they are also disrupted, which leads to a shrinkage of their region of origin ('a' in bottom panel) along with a shrinkage of the region in which fibers innervating 'b' originate ('2'). A consequence could be to consider that both regions 'a' and '2' innervate 'b'; in fact, the innervation of 'b' by 'a' is a false conclusion. **B** In the case of Marchi's method, there is a similar problem. If myelinated fibers originating in region 'a' and innervating region '2' pass through region 'b', 'b' being a region innervating region '1', and region 'b' is destroyed (top panel), these fibers en passage will also be disrupted. The consequence of this is that Marchi's method will reveal tracks corresponding to fibers en passage in region 'b' and fibers going from region 'b' to region '1' (bottom panel). A consequence is to consider that 'b' innervates both region '1' and region '2'. Whereas the conclusion for region '1' is correct, the conclusion for region '2' is false.

Figure 8: Principle of the carbocyanine staining. Carbocyanine dyes were first introduced by Honig and Hume in 1985 (Honig and Hume, 1989). They are lipophilic substances undergoing fast axonal transport in both directions (anterograde and retrograde). They label the whole neuron (soma, dendrites, axon). Transcellular labeling has also been observed (Godement et al., 1987). The dies (e.g., DiL, DiO, DiA, DiAsp) diffuse along the plasma membrane of living tissue and can be even used in formalin-fixed tissue (Godement et al., 1987), and this is a hudge advantage

in comparison with other staining techniques requiring *in vivo* injections, and thus stereotaxic surgery. They provide intense fluorescence with little fading (meaning the staining is persistent) and are particularly well-adapted to post-mortem human brain tissue. They are compatible with double labeling (e.g., DiI in one structure and DiA in another one).

Figure 9: Virus-mediated tracing. A Principle of neuroanatomical tracing combining a genetic modification of the animal model in order to get an expression of a reporter gene in neurons having a specific neurochemical identity. The example illustrated here is that of a parvalbumin-cre-dependent mouse in a brain structure where a viral vector is injected, introducing into transfected neurons a stop codon with loxP sites and the reporter gene (here GFP). With such an approach, only GABAergic neurons (red circle) of the parvalbumin type will express GFP and appear entirely stained. The others will not express the reported gene. This figure has been redrawn according to Lanciego and Wouterloud (2020), figure 10. **B** Principle of retrograde transynaptic staining with a rabies virus. In the absence of genetic manipulations, rabies are naturally retrograde, polysynaptic viruses with a very high tropism for neurons. This is due to their full competence to replicate, including the code of the G protein, which is a glycoprotein necessary and sufficient to support retrograde transynaptic infection. When the virus binds to one of its membrane receptors (there are several types), it undergoes endocytosis-driven internalization and is transported to the soma. Rabies have the capacity to retrogradelly cross a potentially unlimited number of synapses; it just takes time. **C** Principle of tracing with a (modified) monosynaptic rabies virus. Monosynaptic rabies virus spread is obtained by a modification of the wild-type virus such as to delete the gene coding for the G glycoprotein. By this way, the still G-protein-coated virus infects cells via the G receptor of the afferent neurons. When the virus is released from an infected cell (the starter cell), it is not taken up by adjacent nerve terminals. The fact that the virus does not cross more than one synapse is due to the absence of the code corresponding to the G protein transcription when the virus is replicating (e.g., Ghanem and Conzelmann, 2016).

Figure 10: Schematical representation of the connectivity differences between first order thalamic nuclei (dark grey) and higher order nuclei (light grey). First

order nuclei receive driving afferents from the periphery (peri) and modulating afferents from the cortex that exert a gating role (layer VI). They send efferents to the cortex (layer IV). Higher order nuclei also receive afferents from the periphery, though to a lesser degree than first order nuclei. Their driving afferents come from the cortex (layer V). They also receive modulating afferents from the cortex (layer VI). In addition, efferents going to the cortex (layer IV) are implicated in the transthalamic corticocortical information flow that may parallel the direct corticocortical flow. First order nuclei are implicated in sensory functions while higher order nuclei participate in cognitive functions. CTX1, CTX2, and CTX3 represent cortical regions.

Figure 11: Principle of fMRI (A,B). When an ensemble of neurons is activated, the metabolic activity of the region encompassing these neurons increases (compared to rest, **A**) and induces a correlative increase of blood flow (**B**). This increased blood flow provides neurons with an additional supply of oxygen and glucose. These modifications are termed neurovascular coupling. Neurovascular coupling is supported by chemical signals and astrocytes, what induces a localized dilatation of blood vessels (**B**). In the activated brain structure or region (stimulation), the deoxyhemoglobin/oxyhemoglobin ratio undergoes a transient modification (because of the transfer of oxygen to neural tissue), which is detected by the MRI scanner at a resolution achieving the mm^3 in space and an order of 1 s in time. **Principle of fiber tract tracing by DTI (C).** In gray matter, diffusion of water molecules is unrestricted, so that these molecules can diffuse in all possible directions. This diffusion is called isotropic diffusion. Restricted (anisotropic) diffusion corresponds to a situation in which water molecules are limited by obstacles such as membranes, macromolecules or white matter. Indeed, when such an obstacle is a fiber track, the water molecules tend to diffuse along the track, more rarely perpendicularly to it. Furthermore, when they diffuse along the track, this diffusion is faster than in case of a perpendicular one. The track and its orientation can be visualized by detection of the directionality of the water diffusion process. Such detection make it possible to create, voxel by voxel, fiber orientation maps. This is called white matter tractography. On the left part of figure **C**, the short lines in each square indicate the orientation of a virtual fiber track, here illustrated in a two-dimensional scale, thus for each virtual pixel (each of the squares delimited by blue lines). In each pixel, the red line indicates a virtual average orientation of diffusion. Notice that the alignment of

these red lines is influenced by the orientation of the fiber track. For DTI, the average diffusion vector is computed for each voxel. On the right, the reconstructed image in the same scale.

Figure 1


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Figure 2

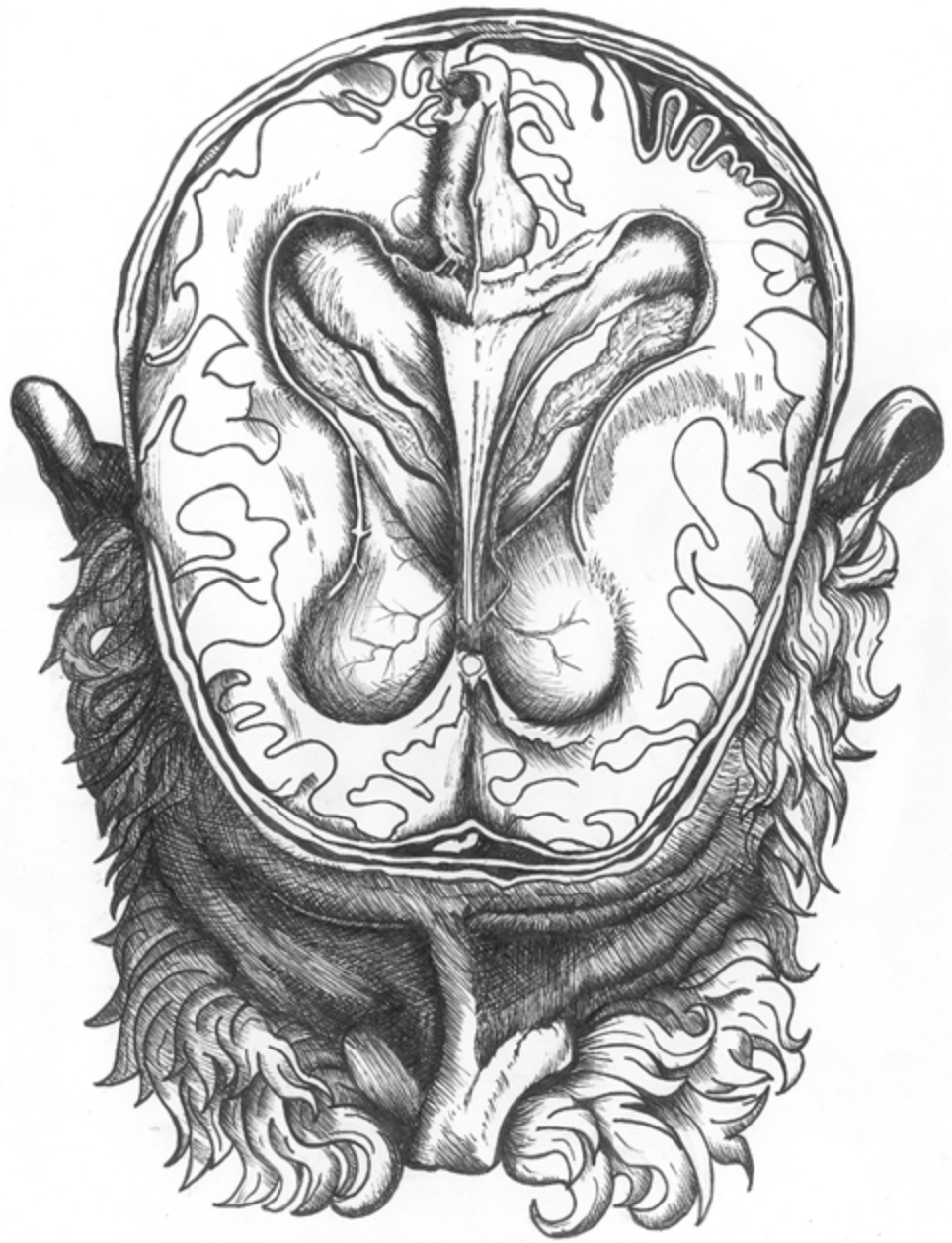

Figure 3

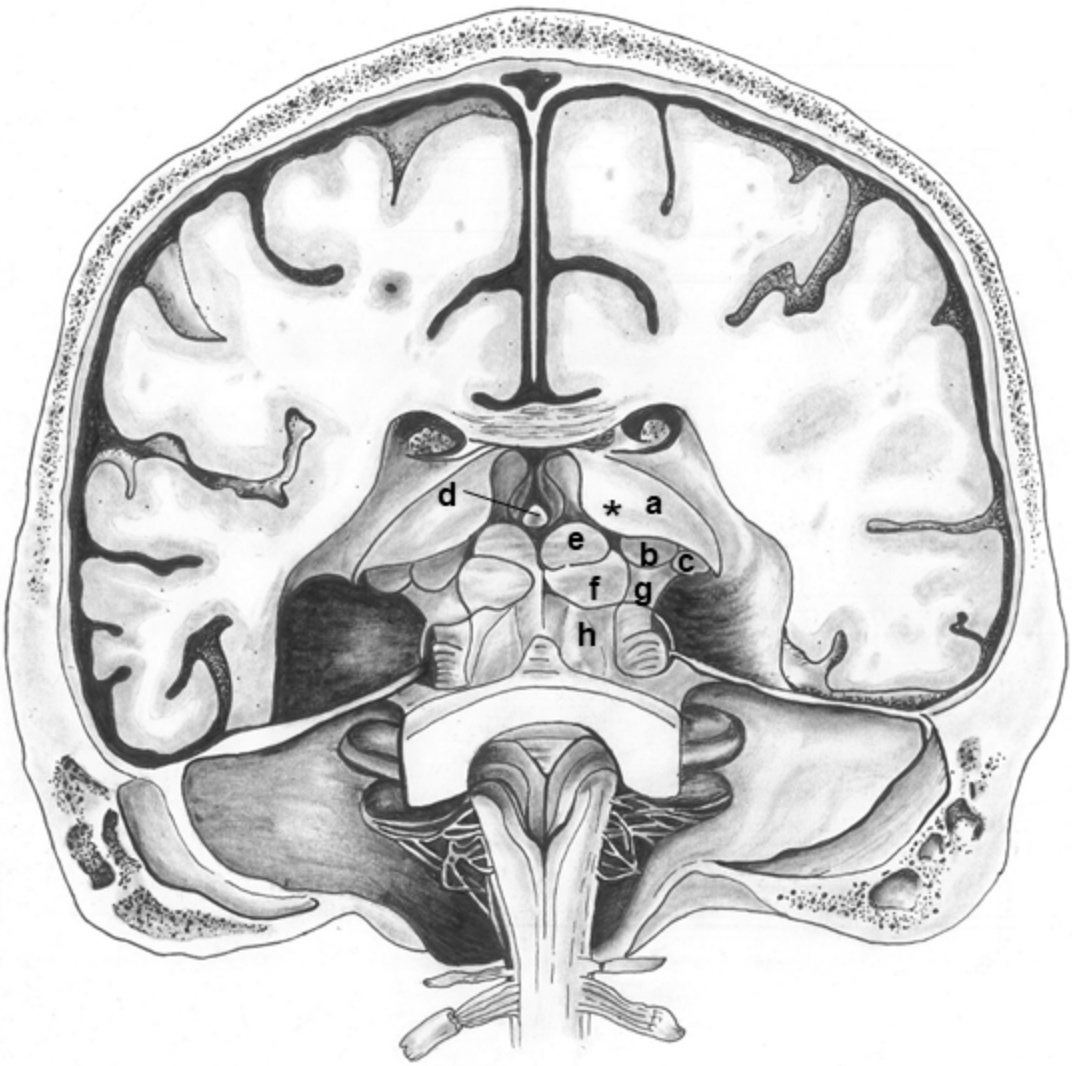

Figure 4

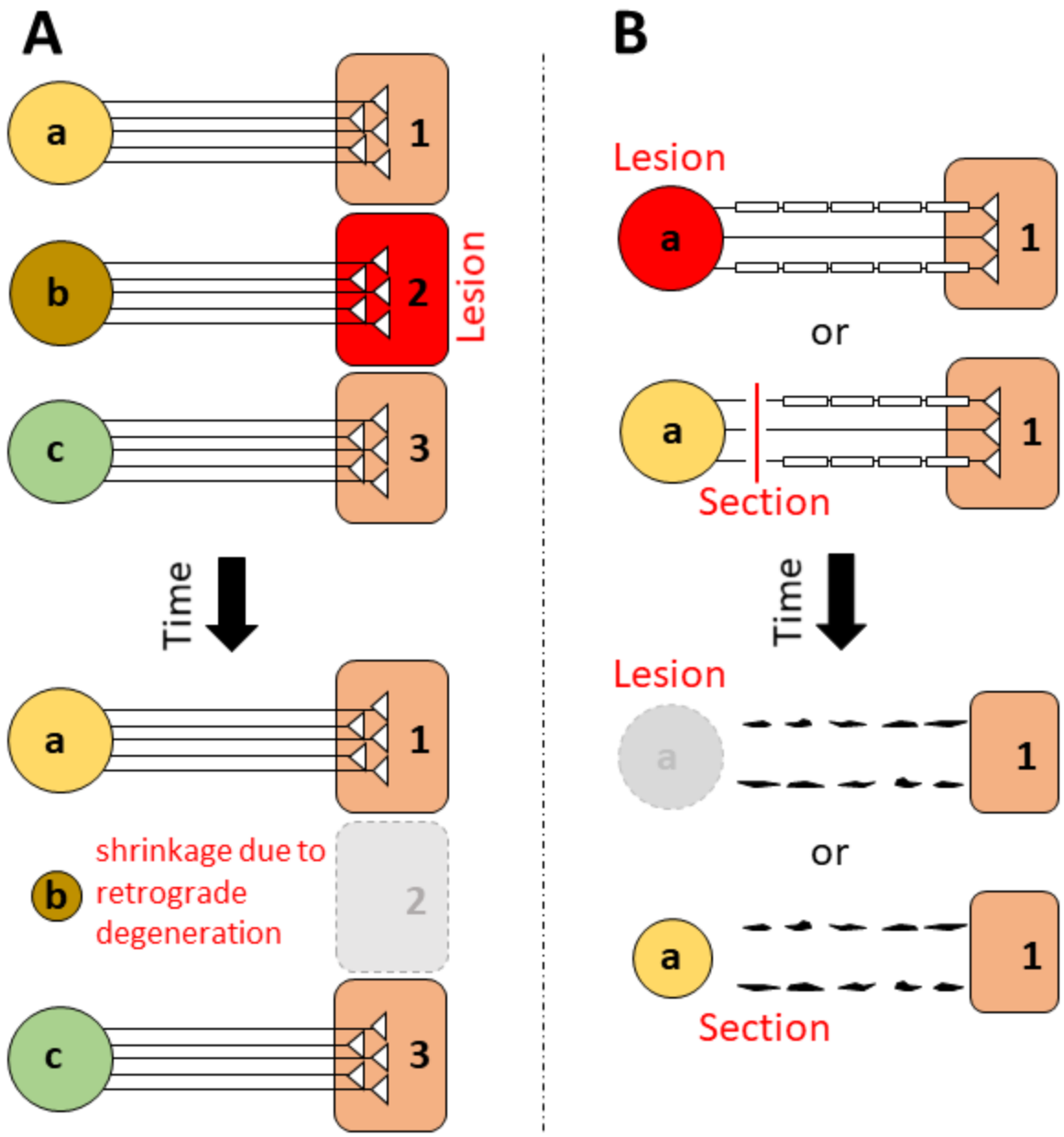
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Figure 5

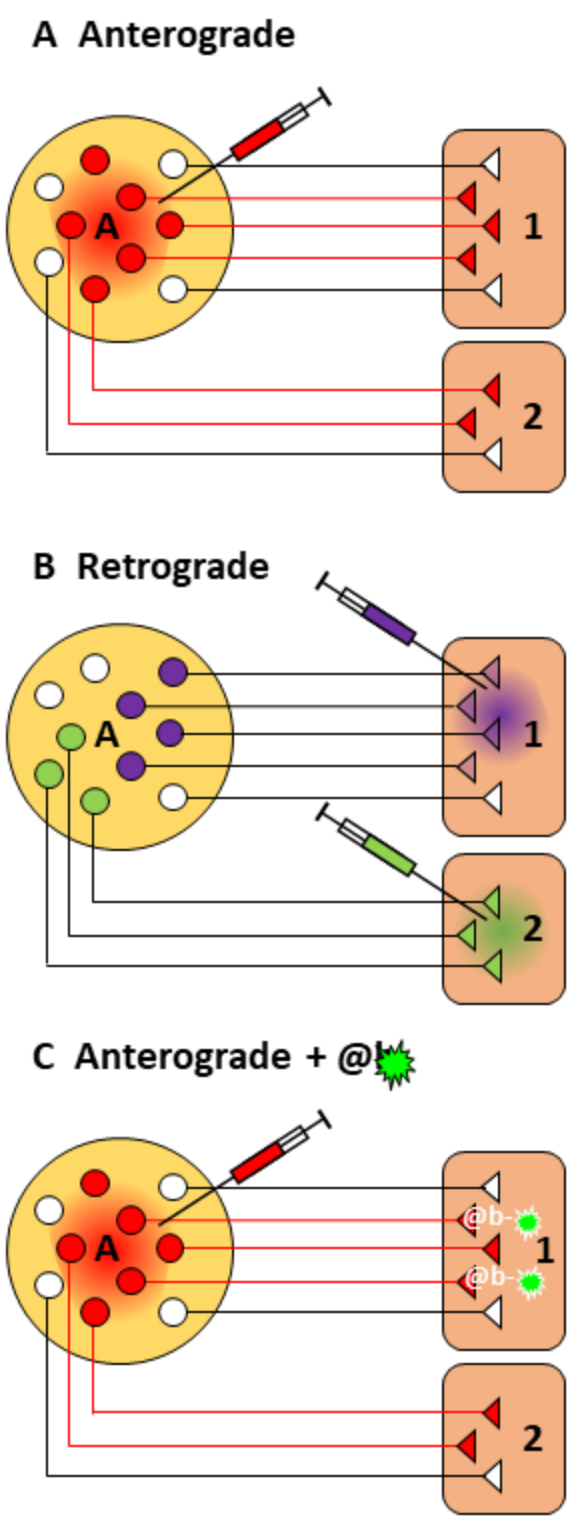

Figure 6

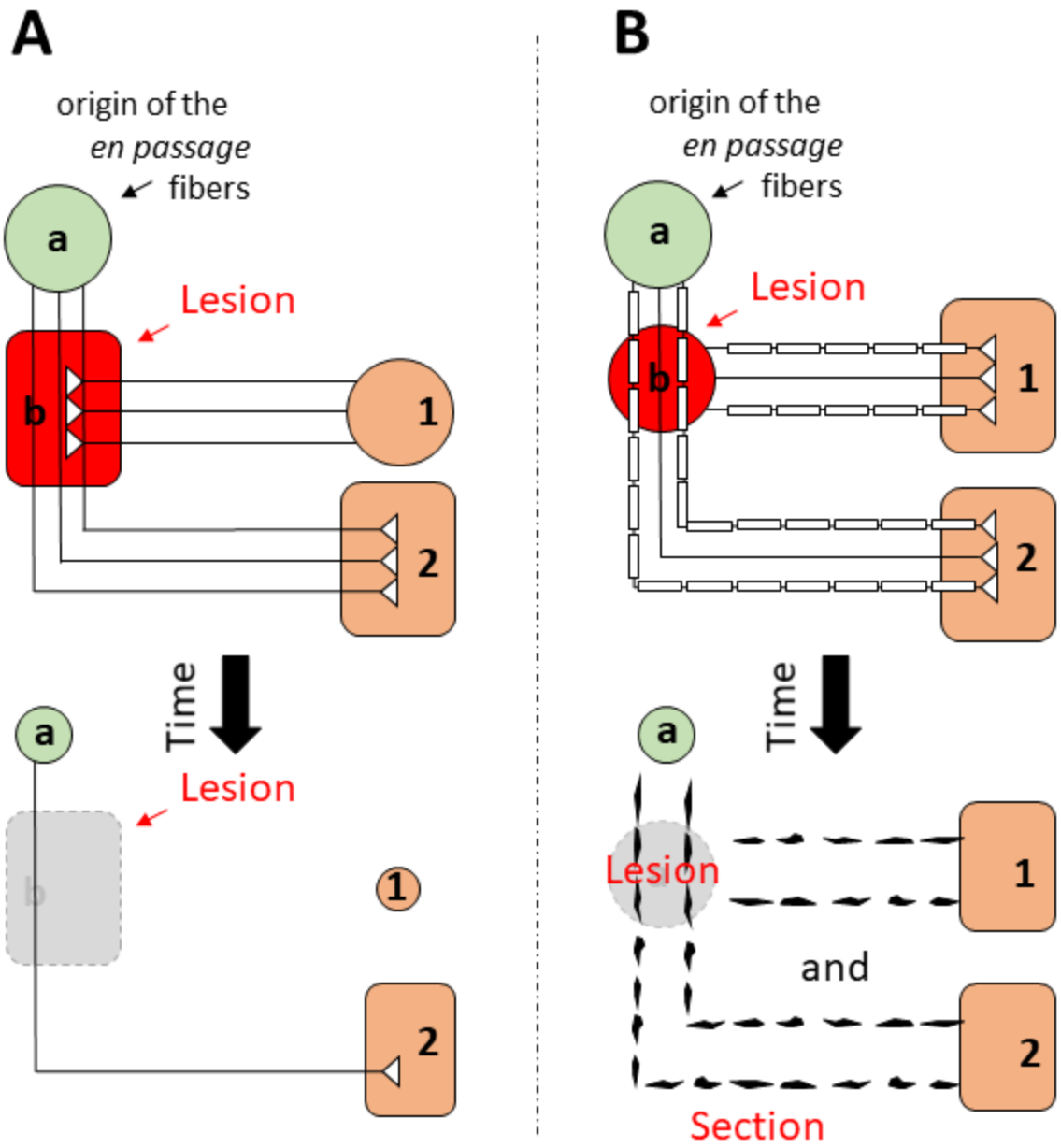
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Figure 7

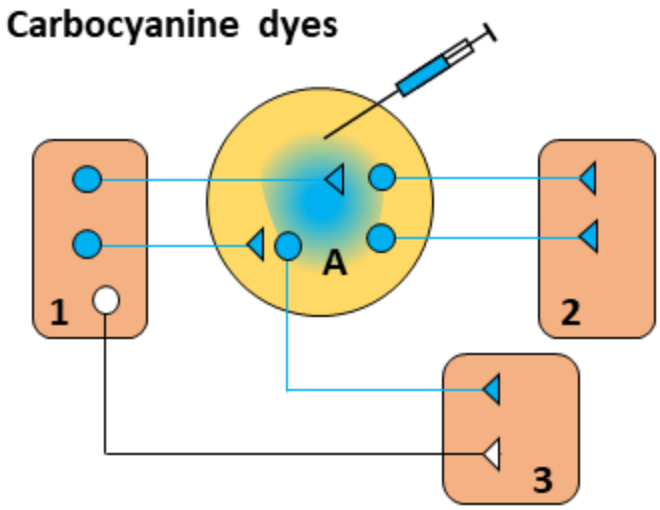
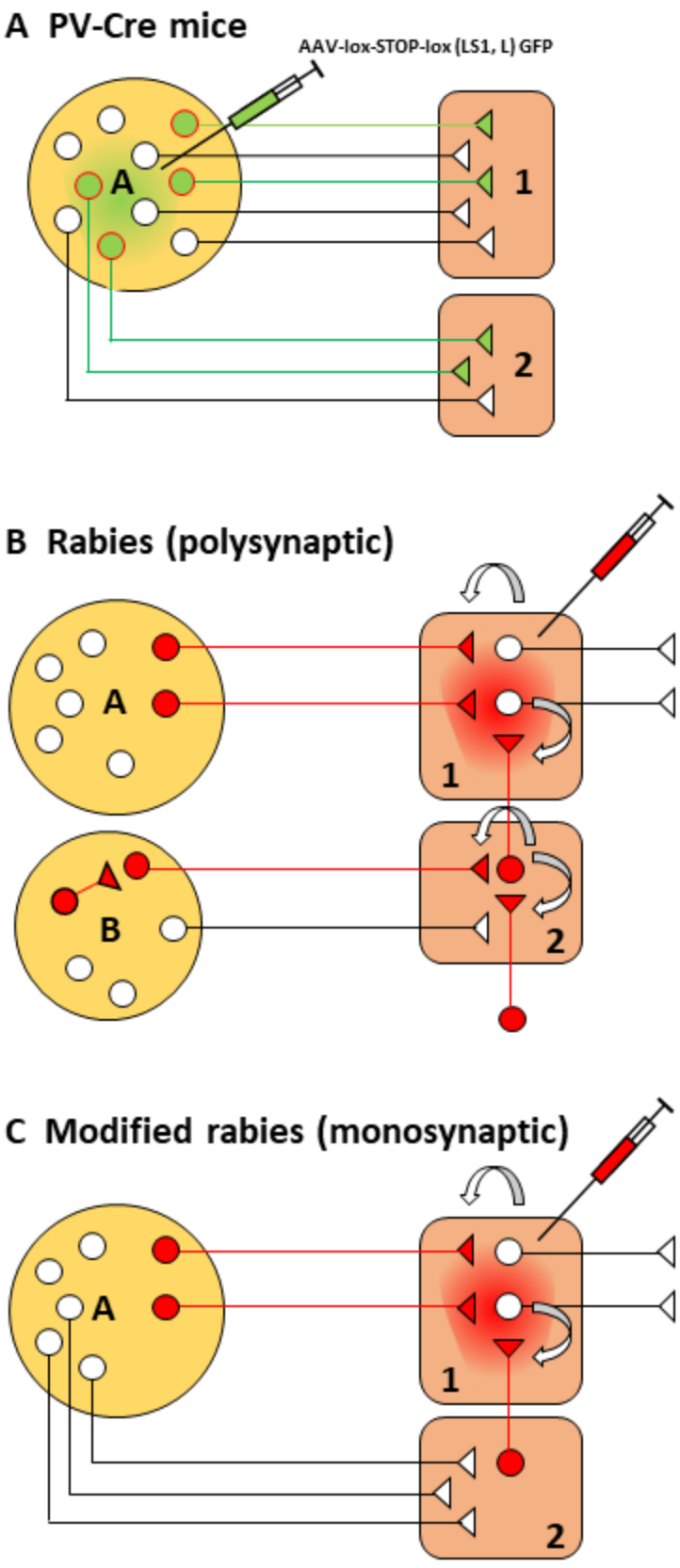


Figure 8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AAV-lox-STOP-lox (LS1, L) GFP
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B Rabies (polysynaptic)
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C Modified rabies (monosynaptic)
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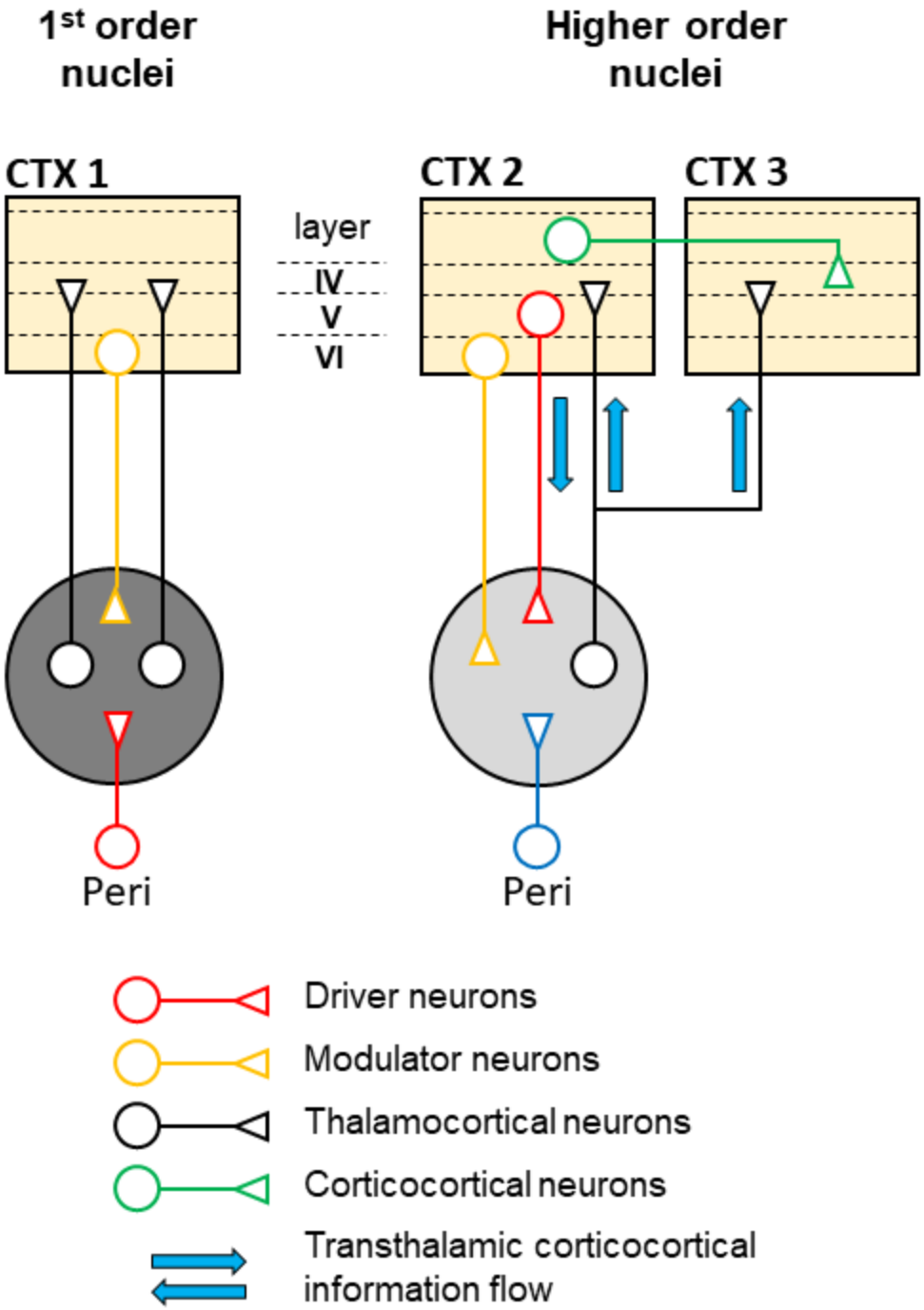
Figure 9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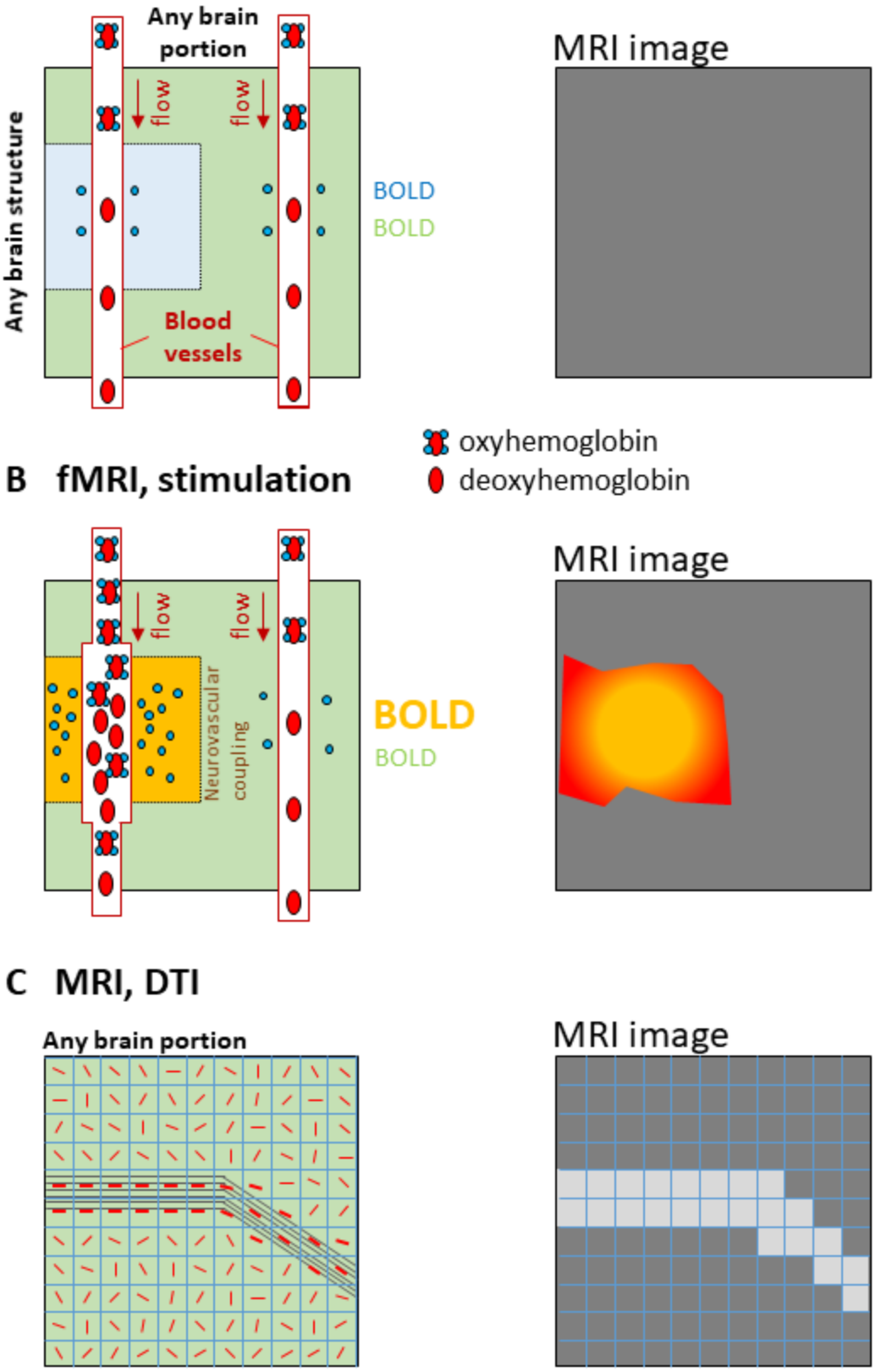
Modulator neurons
Thalamocortical neurons
Corticocortical neurons
Transthalamic corticocortical information flow

Figure 10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deoxyhemoglobin
B fMRI, stimulation
flow
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Neurovascular coupling
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C MRI, DTI
Any brain portion
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Figure ##

Graphical abstract

A selection of milestones (in brown) on the route of the thalamus in the history of neuroanatomy from antiquity to the XXth century, with apologies from the authors for all omissions.