Quantification of Spine Density in the Rat Default Mode Network

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Laura Marian Valencia Pesqueira

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Summary

Using 3D reconstruction of dendrite segments of Golgi-stained neurons in the rat brain, spine density was quantified in different cortical regions of the default mode network. Segments of basal dendrites in layer V pyramidal neurons were sampled. Multiple comparisons pointed to the cingulate cortex with the highest spine density. Previously, fMRI studies have shown that the cingulate cortex has dense connectivity levels with different brain systems, suggesting that the cingulate cortex may act as a transmodal cortical region. The cingulate cortex is one of the functional hubs of the default mode network. Mind wandering is an event of internally directed cognition that drifts freely through diverse thought contents, correlated with activity in the default mode network. Mind wandering has been hypothesized to have a role in cognitive processes that require highly integrated contents and the ability to change and adapt, such as self-regulation and social behavior. The current study provides evidence for the structural support of the high connectivity (i.e. integration capacity) in the cingulate cortex.

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Chapter 1

Introduction

> Realize that everything connects to everything else.
>
> Leonardo da Vinci

When we see a person walking relaxedly down the street, with no apparent urgent needs or uneasy feelings, we know by personal experience that his or her mind is not silent. We have found ourselves in the same position and surely have experienced more than mental silence. Our minds get flooded by thoughts, mental voices and images. We have internal monologues that take inspiration from many sources, from the current environment to episodic memories, plans and desires. This kind of thought is called self-generated thought, and can be elicited voluntarily or spontaneously. *Mind wandering* is a form of self-generated thought characterized by a spontaneous drift in attention from the current moment to internal thoughts or concerns (Andrews-Hanna et al., 2014), and a continuous change between different contents without discomfort (Irving, 2015). Mind wandering contents have information about themselves and others (Stawarczyk et al., 2013), and is thought to have a role in problem-solving and creativity (Baird et al., 2012).

Internal cognition and thoughts form mental representations about current, past and future events, and have a productive value that gives the possibility of creating mental simulations for problem solving (Barsalou, 1999). Mind wandering is triggered when a need of future planning is announced and can have contents with a tendency to remind the individual of personal goals (Morsella et al., 2010), helping to solve problems creatively (Baird et al., 2012; Baars, 2010). Williams (2008) has suggested that the mind has different modes of mind, and that one of them is a problem-solving mode that operates on goals, current states and strategies to achieve goals. However, when this mechanism of problem solving becomes too judgemental, discrepancies between personal goals and current states can signify failure or the presence of negative outcomes and induce sadness (Higgins, 1987). *Rumination* is a symptom of depression that is similar to mind wandering, characterized by an attentional focus in the self and repetitive negative thoughts (Treynor et al., 2003). Mind wandering has been correlated with sadness (Mrazek et al., 2013; Killingsworth and Gilbert, 2010), and even though mind wandering episodes in dys-

phoric subjects seem more accessible (Smallwood et al., 2007), mind wandering can be distinguished from rumination (Irving, 2015). Whether mind wandering itself can cause depression is a current debate. The study of mind wandering has an importance for the understanding of daily human thought and the mechanisms that underlie maladaptive forms of thought, but is a complex mechanism that requires an inter-level approach.

Functional brain imaging is a non-invasive method that has the benefit of being able to observe activity in the brain during different mental states and tasks (Papanicolaou, 1998). It was the discovery of the *default mode network* (DMN) (Raichle et al., 2001), a set of brain regions that become active during mind wandering episodes (Christoff et al., 2009), which placed the study of mind wandering in the field of neuroscience. Different regions of the default mode network are associated with different, but complementary highly complex functions, related to the self and social interaction (Andrews-Hanna et al., 2010b; Spreng and Grady, 2010). Benson's hierarchical classification of brain regions (Benson, 1993) is an scheme that can be useful to describe the different kinds of regions that comprise the default mode network. In the same scheme, regions can be described as regions of high integration and low integration (Jacobs et al., 2001).

Mesulam et al. (1998) proposed the existence of a kind of cortical region, the "transmodal association cortex", that acts as a nexus that accesses to distributed information across different brain subsystems. The default mode network has two main core regions that are transmodal, the cingulate cortex and the medial prefrontal cortex (Andrews-Hanna et al., 2010b). The core regions of the default mode network have become known as functional hubs of the network and are implicated in a cross-talk between different functional brain systems (Braga et al., 2013). The fact that the DMN is comprised by cortical regions with high integration capacities is consistent with the notion that mind wandering operates on thought contents with high integration, for example self-referential memories, future plans, creative thoughts (Baird et al., 2012; Stawarczyk et al., 2013; Baird et al., 2011).

Given that the DMN has activations correlated with self-referential and social behaviors (Spreng and Grady, 2010), the DMN has been suggested as part of the "social brain" (Mars et al., 2012). Smallwood et al. (2012) proposed that the DMN may be exposed to complex social environments that change continuously, requiring high adaptation demands that could be expressed as high integration capacities and synaptic plasticity. Despite the fact that fMRI studies have been very useful to describe and scrutinize the functional properties of the DMN in different tasks (Andrews-Hanna et al., 2014), fMRI studies cannot describe the architecture of neural cortical circuits in detail (Grillner et al., 2005).

Processing demands can increase the expression of spine density in basal dendrites of cortical circuits (Globus et al., 1973), and the increase of spine density can improve processing itself (Moser et al., 1997). Dendritic spines are cellular structures located on the dendrites of neurons, and spines are where neurons form connections with other neurons and receive signals. Each neuron is an integrator unit that takes different sources of information or inputs, and produce an all-or-none output that will be sent to other neurons. Neurons connect different functional systems in the brain, working as relays of information (Kandel, 2013a, 343-348). Therefore, the measure of spine density could be measure of the input connectivity and the processing/integration capacity in the local neural architecture of the DMN and represent a demonstration of the structural basis of current theories of connectivity and function in the DMN.

Chapter 2

Background

The default mode network is a set of brain cortical regions that activates during internal cognition and in a spontaneous form of thought called mind wandering. The study of mind wandering and its neural correlate requires a multidisciplinary approach, from psychology to neurobiology. In this chapter, the theoretical background is reviewed in three main sections, starting with a description of the phenomenology of mind wandering from the perspective of psychology and cognitive science (section 2.1), followed by a description of the neural correlate of mind wandering, the default mode network, and its functional and clinical relevance (section 2.2), and finally a review of the relevant literature for the functional implication of the study of morphology and anatomy of synapses, the neural correlate at the cellular level of information processing and integration (section 2.4).

2.1 Mind wandering

Thinking (or *thought*) is the activity to produce *thoughts* or ideas. The Cambridge Dictionary of Psychology (Matsumoto, 2009, 543) defines *thinking* as any product of the mind, from the stream of consciousness with all of its contents, silent vocalizations, mental images, the perception of the external and internal world, knowledge, judgement, opinions, beliefs, to all the mechanisms underlying behaviour and experience. From this definition, it is clear that thought is a complex experience that is diverse and dynamic. Mind wandering is an experience of thought that well depicts the dynamic nature of thought: mind wandering is the spontaneous switch of attentional focus from a current task to unrelated thoughts and feelings of internal origin. Mind wandering is part of daily life and has been estimated to happen approximately 20% of the daily thinking time (Killingsworth and Gilbert, 2010; Song et al., 2012).

Mind wandering is both a form of internal cognition and self-generated thought. *Internal cognition* refers to the kind of thought that has an origin from within the mind of an individual, rather than a thought elicited by an event happening in the external environment. *Self-generated thought* is a term used to describe the form of thought that arises from within an individual, rather than external cues from the environment. For example,

a thought that is produced by deduction or is part of an imaginary simulation, instead of a thought elicited by the current contents of perception. Self-generated thought can be part of a task that requires access to internal knowledge, deduction or imagination. Mind wandering is a kind of self generated thought characterized by being elicited spontaneously and having task-unrelated content (Smallwood and Schooler, 2015). For Metzinger (2013), the spontaneous happening of mind wandering can be described as non-agency or an instant of loss of autonomy. The loss of control over the mind, making us unable to decide whether the mind should wander or not, is a defining feature of mind wandering.

A mind wandering episode not only brings in mind task-unrelated thoughts that can be distracting, but reduces the capacity to pay attention to the current external events (Smallwood et al., 2008) and an attenuation of sensory and motor capacities termed sensory decoupling (Kam and Handy, 2013). In neuroscience, attention deficit and sensory decoupling has been described as competition between the networks implicated in mind wandering and attention (Smallwood et al., 2012), and is discussed in section 2.2.3.

Attention to the external environment varies with the happening of mind wandering episodes, and vice versa. An individual that has a mind wandering episode can be aware or unaware of the state of his own mind, and this awareness can be influenced by attention as well. Meta-awareness is the mental state elicited when attention is focused in recognizing the current contents and states of mind, for example realizing that one is having a mind wandering episode (Smallwood and Schooler, 2015). Whether a mind wandering episode is accompanied by meta-awareness can cause a difference in how significant are the negative effects of the episode. *Zoning-out* and *tuning-out* are terms used to distinguish between two kinds of mind wandering, according to the involvement of meta-awareness. Zoning-out happens when an individual has a mind wandering episode and is unable to be aware of it and report about it later. As a contrast, tuning-out happens when an individual has a mind wandering episode and is aware of it while it happens, being able to report and avoid negative effects of attentional loss in a current task (Smallwood et al., 2008).

So far, mind wandering has been described as a thought process that is relatively independent of the current task of an individual, and in close relationship with attention. What about the contents of the thoughts elicited during mind wandering? Is the content of these thoughts relevant at all? The contents of the thoughts of a mind that wanders may shed light on the phenomenology of mind wandering and possible adaptive functions.

2.1.1 The contents of mind wandering

What are the contents of mind wandering? Are they relevant to the individual who wanders? Killingsworth and Gilbert (2010) used a smart phone application that at random times of a day would ask whether the user was having a mind wandering episode at the moment, and if there was an episode, included a questionnaire that explored the contents of their thoughts. They found that almost 50% of their sampled population had mind wandering episodes, and for at least 30% of their subjects, the episodes happened in most activities. In all cases, mind wandering caused unhappiness during negative thoughts, and no more happiness during positive and neutral contents when compared to their emotional state when subjects' attention was focused on current tasks. The notion of mind wandering being a trigger of sadness calls to another study by Smallwood et al. (2009), who found that induced bad mood caused longer periods of mind wandering, rising the question of

whether mind wandering causes bad mood, or is a response to it. In any of both cases, these studies suggest that mind wandering has a correlation with the emotional state of the individual, even though it is not clear if it is the mind wandering process itself that cause the bad mood or vice versa.

Mind wandering could be triggered by problems or future plans. Another study by Morsella et al. (2010), proposed that intrusive thoughts (i.e. mind wandering) are triggered by the announcement of future tasks that require planning. They tested the hypothesis by comparing the content of self-generated thoughts of a group of subjects, that had to prepare for a future memory task (geography quiz), compared to a control group. Subjects that had to prepare for the memory task, had increased mind wandering and difficulties to concentrate, while the contents of their self-generated thoughts were related to the upcoming task in 70% of the sampled thought reports. A third study by Baird et al. (2011) found that the content of most self-generated thoughts during mind wandering episodes were future oriented and that 55% of such thoughts were in fact directed to the goals of the individuals who were sampled. The findings of Morsella et al. (2010) and Baird et al. (2011) suggest that the contents of spontaneous thought during mind wandering episodes can affect the mental state of the individual in question, and are shaped by future plans and goals.

Irving (2015) proposed a theory of "mind wandering is unguided attention", that defines mind wandering as an unguided event in which attention drifts from one topic to another without discomfort, while motivation in the individual influences the tendency of the mind to wander towards contents related to personal goals. As a contrast, an individual in state of attention or "guided attention" would feel discomfort when an exogenous event appears as distraction and forces the individual to change his focus of attention. Irving (2015) mentions as an example the study by Morsella et al. (2010), where people who were going to have a geography quiz, would have a tendency to mind wander towards the possible answers of the exam (i.e. geography). It is worth noting that the "discomfort" that Irving (2015) uses to contrast mind wandering is different from the negative effects that mind wandering can have (e.g. sadness and stress) (Mrazek et al., 2013), and rather it refers to the event of exogenous attention (a sudden external stimuli that triggers the orienting of attention (Purves et al., 2008, pp. 272-298).

Stawarczyk et al. (2013) characterized the phenomenological differences between future oriented and non-prospective thoughts during mind wandering episodes. A large part of their contribution consisted the characterization of the phenomenological dimension of mind wandering. Using a task and thought sampling questionnaires, they found that the spontaneous thought of mind wandering is structured in four factors: representational format (inner speech or visual imagery), personal relevance, realism/concreteness and structure. Spontaneous thought varied across these factors, distinguishing between future-oriented and non-prospective thoughts. Future oriented mind wandering had a predilection for inner speech, personal relevance, and realistic/concrete structured sequences of thought. Conversely, the average sequence of thoughts during mind wandering did not have an overall structure and not always had a tendency towards personal goals. The findings that spontaneous thought has different contents and the frequent lack of structure, are consistent with two suggestions by Irving (2015). The first, that mind wandering as an episode of unguided attention is experienced as the drift of the mind through several

thoughts of different contents, and the lack of discomfort in this drifting is what characterizes mind wandering. And the second, that even if mind wandering can have a tendency to drift towards personal goals, goals themselves do not guide mind wandering.

The facts that the contents of mind wandering episodes can be goal related and triggered by upcoming future events (Baird et al., 2011; Morsella et al., 2010), suggest that mind wandering may have an adaptive function. A function is adaptive because it offers a mechanism with benefits for adaptation and survival, and mind wandering could have such function because it could spontaneously remind of current goals and upcoming events. In the following section, some theories of adaptive function will be reviewed, from cognitive science to self-regulation and social behaviour.

2.1.2 The adaptive function of mind wandering

One of the first in notice the adaptive importance of mind wandering was (Singer, 1974), who gave the name *happy dreamers* to individuals who described their mind wandering episodes as pleasant and useful for planning. Mind wandering can be a source of creativity (Baird et al., 2012) and an useful tool for future planning (Baird et al., 2011; Morsella et al., 2010). To explain how mind wandering can have such adaptive value, I will begin with a general introduction to cognitive theory and related definitions (section 2.1.2), followed by a discussion of the cognitive theory of thought productivity and mental simulation, and where mind wandering has a place in this theory (section 2.1.2). The implications of mind wandering for self-regulation, depression and social behaviour will be discussed in section 2.1.3.

Cognitive Theory and Reasoning

Cognitive science is the study of cognition, or the mechanisms pertaining knowledge and its acquisition. *Cognoscere* is the etymological Latin root of the term cognition, and means to know something (Anshakov and Gergely, 2010). From a mechanistic point of view, cognition can be a *mental mechanism* with operations that act on information (Bechtel, 2012, p. 69). In this definition of cognition, the mind is viewed as having internal mental states (beliefs, wishes, intentions) that can be explained by information processing in cases when abstraction and knowledge are involved (Anshakov and Gergely, 2010).

In general terms, information is a registry of a relationship to something absent. As a referent, information represents something at any point in time, whether present or not, gone or yet to come, abstract or physical (Deacon, 2013, pp. 371-391). In the perspective of information processing, cognition consists of the reduction of uncertainty that is achieved by obtaining missing information and gaining new knowledge. Sometimes the information available in the environment alone is not enough to solve a problem, thus there is a need for acquisition, completion or generation of new information. *Cognitive reasoning* is the activity of generating new information. Reasoning consists of the organization of several basic information processing operations or steps (i.e. judgements, estimations or inferences) that have as ultimate goal to connect information *premises* (i.e. known facts or observations) to information conclusions (predictions). The extraction and generation of new information from the environment is highly valuable for the adaptation of an individual because with that information, internal representations or models of the environment

can be constructed and guide the agent's behaviour. Improvement of such models allows for the understanding of how situations and problems might be, both in the present and past, and to predict the future. Problem solving as a cognitive process results in finding a solution that was not directly attainable (Anshakov and Gergely, 2010).

For the realization of cognition, individuals require complex functions, such as learning (encoding of information), integration (i.e. a form of processing), and reasoning (deduction). *Learning* is the acquisition of new information or abilities, and is needed in every step of cognitive action, from the input (i.e. encoding new information) to the output (i.e. updating an internal model) (Anshakov and Gergely, 2010). In biology, learning consists of modifications to the information processing mechanisms in the neuronal networks of the brain. Modifications happen at different levels, for example in morphology (i.e. increased dendritic spine density) and physiology (e.g. synaptic potentials, neurotransmitter release) and have impact in behaviour (Purves, 2012, pp. 163-185).

From the point of view of information processing, integration is the process by which information of different sources is put together and processed to obtain an unified view (Lenzerini, 2002). *Integration* in cognitive science is a fundamental information processing mechanism and is a basic step for reasoning, where different premises are unified to produce a single *integrated* concept (Anshakov and Gergely, 2010). Integration in the brain, is a basic function of neurons (brain cells) (Etherington et al., 2001), and the simplest structure able to perform it is the dendritic spine, for which it is also called "unit of integration" (Yuste and Denk, 1995), and modifications in the neural mechanisms for integration are the basis of learning in the brain (Purves, 2012, pp. 163-185). In other sections of the background, the biological correlates of information processing (section 2.4), the role of dendritic spines for integration as well as processing capacity (section 2.4.3) and how spines can be quantified and their number interpreted (section 2.5) will be discussed.

Internally guided thought can be considered a correlate of *cognitive reasoning*. Internally guided thought operates on the absent: making use of imagination and the creation of internal representations to reconstruct or simulate situations, understand uncertain stimulus, pattern completion or generate an answer to a question (Andrews-Hanna et al., 2014). In this paradigm, mind wandering is a mechanism that spontaneously shifts the individual's attention to internal thoughts, and can be influenced by motivation to increase the tendency to mind wander towards personal goals (Morsella et al., 2010; Baird et al., 2011). Nevertheless, as Irving (2015) described, mind wandering not necessarily consists only of goal reminders, but is a dynamic unguided process. How is that the mind can create simulations, use mental representations and imagery is the next step to tie cognition and mind wandering as an adaptive function.

The productive value of mental representations

Mental imagery is a kind of mental representation that is accompanied by a virtual sensory experience of diverse modalities (i.e. an experience within the mind) in the absence of sensory stimuli. External events can induce mental imagery, involuntarily, and even if one does not want to experience the mental image (Pearson et al., 2015). Mind wandering episodes come in different representational formats, including mental imagery of different modalities (visual and auditory) and inner speech (Klinger and Cox, 1987). Additionally, a study by Delamillieure et al. (2010), has shown individual differences regarding the dom-

inant modality of mental imagery during spontaneous thought, with types such as visual, auditory or somatosensory dominance, as well as dominance in numerical processing or internal musical experience. In this section, theories of how thought and mental imagery can be productive are discussed, as well as how mind wandering as an episode of unguided attention Irving (2015) can increase this creative possibility.

Mental representations can act as concepts that are interchangeable and be used to build complex and structured thoughts. To explain this possibility, Barsalou (1999) proposed that concepts can be considered units with meaning, that can represent objects and events, and compose thoughts. Perception is the source of mental representations that are bound to knowledge of the real world. The representations obtained by perception can be used later in the absence of the original source (i.e. the referent object or event) for off-line processing. Barsalou's perspective has been portrayed by the "language of thought hypotesis" (LOTH) by Fodor (1975), who argued that thought formation has two properties of natural languages, including productivity and systematicity.

Productivity refers to the possibility of forming a thought with a combination of concepts that nobody has done before, as a language with productivity could create a sentence with a unique combination of words, yet making sense out of meaning and grammar. *Systematicity* means that a cognitive system that can think of a string of concepts with sense and meaning, is able to understand variations derived of such initial thought. For example, a main sentence in english could be "The boy loves the girl". A derived sentence "the girl loves the boy" could be understood if an individual can understand english.

Fodor and Pylyshyn (1988) suggested that productivity and systematicity are properties of systems that use mental representations composed by syntactic rules. Compositional syntax consists of the construction of complex strings of concepts, while maintaining the structure of each component concept. In this way, the concept "boy" would not change when embedded in the string or sentence "The boy loves the girl". Barsalou (1999) extends the definition of language of thought to mental imagery, as tools for mental simulation, in a way that an individual can execute the same operations that would be involved in actually experiencing or perceiving something. Furthermore, Barsalou points to the dynamic condition of simulation processes having a temporal character, such as sequences of acts that happened in the past or that have to be planned for future events. The fact that mind wandering episodes have contents with a temporal focus and a tendency for personal goals, that has inspired the idea that mind wandering has an adaptive value as a problem-solving mechanism (Baars, 2010).

Mental simulations are not limited to repeating experience, but have a productivity, in the same way that Fodor and Pylyshyn (1988) suggested. Mental simulations can take several mental representations and build an experience in different ways and perspectives. Perceptual symbols or mental representations obtained by a physical event (perception) or by deduction (introspection) have modularity, thus can be put together in different combinations to form different complex simulations that constitute the ground for abstract concepts (Barsalou, 1999). If thought has productivity as Barsalou and Fodor suggested, internal cognition can be used to produce mental simulations and to connects premises to conclusions (see *cognitive reasoning* in section 2.1.2) (Anshakov and Gergely, 2010).

In accordance with the "unguided attention" theory of mind wandering (Irving, 2015), mind wandering could contribute to off-line processing by spontaneously driving a train of

thought in unexpected ways. The phenomenology of the contents of mind wandering also suggest that off-line processing could be the possibility, since in average mind wandering episodes involve different modalities of mental imagery and even though contents did not have a structured sequence of thoughts (i.e. seemingly random strings of concepts), the contents often were realistic and concrete, with varying importance and relation to personal goals Stawarczyk et al. (2013). The implication of mind wandering in problem solving and creativity has been assessed in a study by Baird et al. (2012). The performance in a creativity task was evaluated after incubation periods with different conditions. Incubation periods included one task of two options, the first a demanding task with working memory that is thought to recruit attentional tasks, and the second an undemanding task in which mind wandering could happen.

After the incubation period, self-reports were conducted to sample mind wandering thoughts. Finally, participants were tested again with the creativity task, involving both repetitions and new problems. Their findings showed that the individuals that had an incubation period with undemanding tasks had an increased performance in the creativity task, but only for repetition of already tested problems. The fact that performance in the solution of new problems did not improve is a demonstration that improvement of the performance was not a general creativity improvement. The fact that only performance in repetitions improved suggested that mind wandering could have had a role in solving already known problems, and to test this possibility, Baird et al. (2012) analysed the thought contents of the mind wandering episodes that happened during incubation periods. Conversely, the contents of mind wandering episodes during undemanding tasks were not associated explicitly with the creativity tasks, even though mind wandering episodes had a high frequency during the undemanding tasks.

The contents of mind wandering can be a source of information for the phenomenology and possible benefits of mind wandering, but also for the possible reasons behind the costs of mind wandering in mental health. Mind wandering episodes have a high frequency of thoughts related to the self across time (Song et al., 2012; Stawarczyk et al., 2013). Self-regulation has implications in social behaviour, as the self is the central player in a society (Triandis, 1989), and the role that mind wandering has in cognition of the self may shed light on its implications for self-regulation, social adaptation and disease.

2.1.3 The self in mind wandering and rumination

Cognition of the self is thought to be relevant for self-regulation (Heatherton, 2011) and is closely linked to depression (Higgins, 1987). Importantly, a symptom of depression is rumination, a process very similar to mind wandering that consists of episodes of recurrent negative thoughts that is centered in the self (Treynor et al., 2003). Self-referential contents are frequently present in mind wandering episodes (Stawarczyk et al., 2013) and in the individual's inner speech (Morin et al., 2011). *Inner speech* is a form of though that is verbal, and consists of the activity of expressing silent words in the mind directed by oneself to oneself (Perrone-Bertolotti et al., 2014). The close relationship between the self and depression, the frequency of self-referential thoughts and the similarity of rumination with mind wandering, are facts that together suggest that mind wandering may have an implication in cognitive processes of the self and depression.

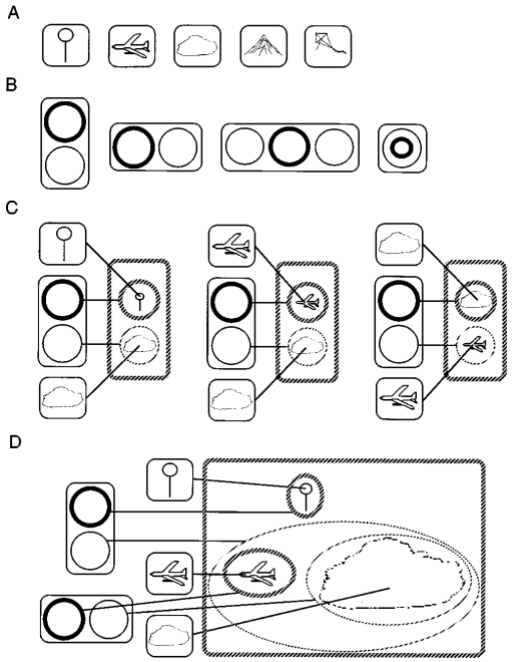

Figure 2.1: Barsalou (1999) proposed theory of perceptual knowledge describes perceptual symbols in a cognitive system. The cognitive system has productivity, which means it can form multiple combinations of perceptual symbols to form complex statements and simulations. A. Examples of perceptual symbols, including in order from left to right, baloon, plane, cloud, mountain and kite, B. boxes with thick lines represent spatial relationships that can act as ground for simulations, C. Perceptual symbols can be allocated with spatial relations to form simple simulations. Boxes with dashed lines are simulations, D. Simulation in the cognitive system can use previous simulations in different levels to form new simulations with higher complexity. Figure source is Barsalou (1999).

In philosophy, there are two main approaches to describe and study the *self*: the narrative self and the minimal self. The narrative self is concerned with personal identity and the temporal developement of an individual. A narrative self has a self-concept or self-image, past experiences in the form of narrative memories and a future with plans and desires (Gallagher, 2000). Selves have persistence, which means that despite changes in personality, the individual is the same (Olson, 2010). The minimal self approach tries to describe what are the essential elements of a self and the experience of oneself, in a closer way to a biological explanation of the self (Gallagher, 2000). For Deacon et al. (2011), the minimal self is a structure that can self-assemble, self-organize, self-maintain and self-repair, and that it is *itself* the purpose and beneficiary of the continuous effort for remaining consistent (Deacon et al., 2011). In other words, selves are the *purposes* in themselves (Popper, 1985). Deacon gives to the minimal self the same properties that living organisms have: living organisms are self-producing and self-maintaining systems (autopoietic), and they keep a continuously dynamic order (homeostatic) in order to produce and maintain (Roth, 2013, pp. 39-48). Individuals use of all senses and motor capacities from the perspective of oneself. Thus, from the first-person perspective, individuals navigate space from an egocentric point of view (Vogeley and Fink, 2003).

In psychology, the definition of self includes the informational component (e.g. experiences, plans) of the narrative self and the constructive part of the minimal self. The self takes a central role in society and differentially samples information that is beneficial or not, even though some variations in sampling appear in different cultural contexts such as those that tend to prioritize society over the individual (Triandis, 1989). Cognition of self-concepts and self-regulation are processes necessary for the adaptation and survival of an individual in society (Heatherton, 2011). According to the *self-discrepancy* theory, different representations of the self exist (self-concepts), and actions taken to improve the current self are taken in accordance to idealized concepts or goals. However, when self-discrepancy tests are highly judgemental and often signify lack of success or presence of negative outcomes, the discrepancy between self concepts can induce depression (Higgins, 1987) and a maladaptive process similar to mind wandering, called rumination (Treynor et al., 2003).

Depressive patients with rumination are characterized for a fixation on a thought or goals that have not been able to be attained (Treynor et al., 2003), which indicates that rumination can be goal oriented. Miller et al. (1960) proposed the model of planning *Test-Operate-Test-Exit* (TOTE), which is based in discrepancy, similarly to the theory by (Higgins, 1987).In the TOTE model of planning, behaviour pursuits goals (i.e. desired states, ought or ideal self-concepts), and actions to diminish the difference between the current state and the desired state(i.e. the goals). The TOTE format consists of an initial discrepancy test between the current and goal state, followed by an operation to reduce the differences between both states, then a second discrepancy test to verify results, and in the cases of obtaining successful results or abandoning the goal, an exit of the problem-solving mode (Miller et al., 1960, 21-39). When a depressive subject who is unable to abandon goals, he or she cannot exit the TOTE process and goes through the cycle over and over again. As a result, a "holding pattern" is established in place of a pattern that changes and adapts with time. The ideas and representations about the goal and the problems are maintained, or fixated, like in rumination Williams (2008).

Even though rumination shares several characteristics with mind wandering, such as task-unrelatedness, attentional loss and sensory decoupling, it can be distinguished from mind wandering. Irving (2015) proposed in his theory of "unguided attention", that mind wandering differs from rumination in terms of attentional focus. Mind wandering episodes consist of "unstable" trains of thought that drift through several contents without "discomfort". Rumination focuses only in one topic and attention is constantly drawn to the same thought, leading to relatively "stable" trains of thought (Irving, 2015). There is a possibility that mind wandering, initially drifting from content to content in an "unstable" train of thought, could access a memory or a thought that has personal value for the depressive individual, and then in accordance with a model of depression proposed by Williams (2008), the "sad" contents could stabilize the train of thought in a form of *rumination*.

The model proposed by Williams (2008) , the "differential activation" model, tries to explain the observation that in patients with depression, risk of relapse is increased by a *cognitive reactivity* to mild negative changes in mood (Teasdale et al., 1995). The model suggests that negative feelings about the self (i.e. failure, self-devaluation, hopelessness, rejection), initially arise as the result of a negative self-discrepancy test (Higgins, 1987; Miller et al., 1960). In addition to the presence of genetic traits that influence the worsening of depressive symptoms (Caspi et al., 2003), the co-occurrence of depressive symptoms in patterns (e.g. negative feelings, physical sensations, avoidance and rumination) produce changes in the subject. The changes result in a pattern of activation: A mild exposure to any of the depressive symptoms can trigger the co-activation of any other symptoms. Williams (2008) adds that the co-activation of symptoms brings thoughts of negative content and a "problem solving mode of mind" in a TOTE format, and even though is intended to help, fails and backfires. In accordance with the differential activation mode, it is possible that during a mind wandering episode, if a patient with vulnerability to depression is exposed to a negative thought or feeling, despite them being mild or part of an unstable train of thought, the thought could be able to trigger the co-activation of other symptoms, including a maladaptive problem solving mode that results in rumination.

The concepts of attention and awareness are closely related to the concept of consciousness, but they are different concepts often confused. Mind wandering and rumination are two different thought processes that differ in the way attention is focused or guided, the presence of meta-awareness and task-relatedness, and happen during conscious states. In the following section, the distinctions between attention, awareness and consciousness, and the way mind wandering relates to them will be discussed.

2.1.4 Relating consciousness, awareness and attention to mind wandering

Mind wandering is a thought process that consists in the shift of attention from current events to internal cognition. When an individual has a mind wandering episode, attention to external events is reduced to an extent that even sensory inputs seem to be ignored, a term called sensory decoupling (Kam and Handy, 2013). Additionally, people can be aware of their own mind wandering episode or not (meta-awareness) (Smallwood et al., 2008). The terms attention, awareness are closely related to the concept of consciousness, and often there is a risk of confusion between them. In this section, the distinctions be-

tween the processes of attention, awareness and consciousness are described, and the way mind wandering relates to them is discussed.

Mind wandering is experienced. People mind wander, are able to notice how ideas appear and drift in their minds. Mental representations from the external world and from internal thoughts, including those occurring during mind wandering episodes, are experienced in the conscious field (Smallwood and Schooler, 2015). A proof is that people are able to report the contents of their thoughts (Song et al., 2012). The whole construct of mental representations of the external world, integrated and experienced together, make up a collective mental phenomena known as *conscious experience*. *Consciousness* refers to the mechanisms by which conscious experiences come to be (Gray, 2004, 2-3).

Ongoing representations present in the conscious field range from inner private bodily feelings to external phenomena that can be experienced and shared with others. In general terms, the world that we navigate and experience *as it is* consists of the interpretation of ongoing information and the internal representation of it *within the brain*. That world *out there* that we experience is *within* our minds (Gray, 2004, 2-3). Cognitive reasoning, externally and internally guided thought are experienced as well in the conscious field.

There are different kinds of conscious experiences that can be distinguished from each other. First, experiences of the external world, which have a subjective point of view, but are shared with others as a *public* space, where not only perception of the environment is experienced, but social interactions, language and communication take place. Second, sensations of which only the subject in question is aware of, and make up a *private* space. The second kind can have another subdivision, which consists on the nature of the private experience, whether the experience depends on bodily senses or not. Thoughts, mental imagery and episodic memory are relatively independent of bodily sensations. Experiences that do depend on bodily sensations (i.e warmth, hunger, illness, tiredness) form part of the experience of the external world (Gray, 2004, 2-3).

Phenomenal consciousness describes the different features of different experiences (e.g. *red or green*); and *access consciousness* focuses in the information that arrives to the brain's processing systems (e.g. memory, categorization, planning, decision making and attention). Both kinds of consciousness are regarded as overlapping in the real world but can be separated for their study (Lau and Rosenthal, 2011).

Even though mind wandering can be experienced and reported, the truth is that often, some people is not even able to notice that their mind wandered: when they lacked the meta-awareness of their own mind (Smallwood et al., 2008). The lack of awareness, however, does not mean that the person was unconscious or under anaesthesia during the mind wandering episode, but that her mind was in a certain conscious state. Consciousness comes at different degrees, also called *conscious states*. Awareness comes in different levels, and a mind wandering episode is itself classified as a conscious state with decreased awareness of the external world (Bell, 1980). An extreme example of conscious state is *minimal consciousness* (Giacino et al., 2002) refers to the state when a subject is able to understand limited information whether or not is able of responding.

In condition of disease, medicine defines unconsciousness as the state of loss of capacity to process entrant information. Unconscious subjects are often unresponsive, but not every unresponsive subject is unconscious (Sanders et al., 2012). The definition of unconsciousness in medicine is of current debate due to its ambiguity, and because con-

scious patients can lose their ability to express their responses, there is an important need of being able to identify and quantify consciousness in patients due to ethical, economical and social reasons. The processes by which the continuum of different states are differentially generated are widely studied by neurobiology of consciousness, but nevertheless remain complex and difficult to distinguish. One example of this problem is the state of sleep, which can be considered *unconscious* due to the unresponsive condition, but can be remarkably rich in mental representations and experiences (dreaming) (Gray, 2004, 2-3).

Another characteristic of mind wandering is the loss of attention, which is correlated with impairments in information encoding and retrieval (Smallwood et al., 2003). The fact that mind wandering interferes with task performance, is different from the facts that mind wandering can be experienced (conscious), and can be detected by the individual himself (meta-awareness), despite being closely related characteristics. The executive process by which the brain seems to be able to select which content can take part of the conscious field is *Attention*. This process is thought to have both conscious and unconscious components. The brain can focus attentional resources and link it to the conscious field, or can process information without the involvement of consciousness, but can analyse at a high detail pieces of information that don't enter to the conscious field. For example, in *blindsight* experiments, where subjects are presented to cues that are overlapping with distractory information. Even if the subjects are unable to report the content of certain cues, they are able to reconstruct their content. For example, if the word *flake* is presented and the subjects are unaware of it, later on when asked to complete the word *fla...*, the subjects are able to retrieve the correct word (Gray, 2004, 166-167).

Attention is not a synonym of consciousness but, rather a mechanism in its own that uses processing resources in order to *select* and *exclude* information (Purves et al., 2008, 278-298). The fact that mind wandering episodes seem to reduce attention, suggests that both mechanisms (i.e. mind wandering and attention) are complementary. (Smallwood et al., 2012) suggested that the neural correlates of attention and mind wandering compete to access the conscious field. The study of neural correlates of behaviour and mental processes opens a a new perspective in the study of mind wandering: the neuroscience of functional networks in the brain. Mind wandering is a complex mechanism, and its involvement with mental representations of complex thoughts that require information from several sources (i.e. memory, mental imagery, self-referential thoughts), indicates that different functional systems in the brain must be involved in a cooperative manner to produce episodes of mind wandering.

In the next section, the neural correlates of mind wandering, the methods used for their study and their interactions with different brain systems will be reviewed.

2.2 The study of mind wandering in neuroscience

The default mode network is the neural correlate of mind wandering. Mind wandering has been described in psychology (Singer, 1974) and has behavioural characteristics that can be studied by means of questionnaires for thought sampling (Baird et al., 2012), (Baird et al., 2011), (Morsella et al., 2010), and other methods that rely on the observable effects of mind wandering episodes, such as changes in eye movements (Uzzaman and Joordens, 2011). However, there was a need to find a linkage between the brain and mind

wandering. In neuroscience, *functional brain imaging* is a method widely used to study behaviour in living subjects and mind wandering has been studied with it. The discovery of the default mode network (DMN) by means of functional brain imaging (Raichle et al., 2001), together with the fact that this network is consistently active during mind wandering (Christoff et al., 2009), have positioned the study of mind wandering in the field of study of neuroscience.

Functional brain imaging is a technique used in neuroscience to study the brain *in vivo*. Functional brain imaging is concerned with patterns of brain activation. Metabolism and neural signalling are the causes of the signal and consist of physiological and biochemical processes in the brain. Metabolic processes are detected as differences in rate and volume of blood in certain areas, relative concentration of oxygen in a volume of tissue in a time frame, perfusion rates and oxygen or glucose consumption rates. Neural signalling processes are identified with neurotransmitter release and ionic currents. Altogether, these processes can directly or indirectly be associated with electromagnetic signals in the form of magnetic flux that can be detected and constructed by methods as functional Magnetic Resonance Imaging (fMRI). Electromagnetic signals do not influence the natural occurring biological processes that produced them. In other words, this methods do not require the destruction of the imaged subject (Papanicolaou, 1998). A non-invasive method is advantageous because the brain can be studied in a virtually natural state and it is possible to elicit its activity when subjects take part on tasks (Logothetis, 2008). Metabolism and signalling are constitutive processes with varying rates. Different rates are found in different brain areas, for example, the cortex has the highest rates, compared to the white matter. Functional imaging tries to find a specific baseline activation profile to be able to compare to pathological (Papanicolaou, 1998) or task-induced activation profiles (Logothetis, 2008).

2.2.1 The default mode network

The default mode network (DMN) is a set of brain regions that have a characteristic pattern of activity, in which the DMN is recruited during rest (a lack of task), and deactivated during tasks. (Raichle et al., 2001) was the first study to describe this network and its patterns of activity. After the description of the DMN, Christoff et al. (2009) found that the DMN is active when subjects mind wandered, which has turned the DMN into a neural correlate of mind wandering.

Using functional magnetic resonance imaging (fMRI), (Raichle et al., 2001) conducted the first study that described a set of brain regions that had a unique pattern of activation, the default mode network (DMN). In a *block design*, activity in the brain is compared between different blocks or steps with different conditions per block (Harmon-Jones and Beer, 2009, 318). In the design by Raichle et al. (2001), blocks of task and rest were alternated, but with continuous imaging during all the experiment, a paradigm now called *resting state imaging* or rsfMRI. This design showed that the DMN was active during blocks of rest, that is when the subject stayed in the fMRI scanner, without any task or additional treatment. Conversely, during the task block, the brain regions of the DMN would deactivate. This pattern of activity inspired Raichle and colleagues to call the network "default mode network" because resting state was regarded as the default state of the brain. Initially, activity in the DMN was suggested by (Raichle et al., 2001) as a baseline in

terms of a constant oxygen extraction fraction (OEF) as magnitude of uniformity of neural activity. The OEF is proportional to the ratio of used to delivered oxygen in the brain (Fox and Raichle, 1986). However, the definition of DMN activity as a baseline resulted problematic.

Morcom and Fletcher (2007) pointed out that there are problems with this proposition: first, in cognitive studies, activity in a region can be compared to several baselines to obtain a difference or "substraction", and such differences are detected regardless of the baseline selected; second, the OEF is independent of neural activity, since it can be manipulated with hyperventilation and not lead to any difference in neural responses. Morcom and Fletcher (2007) is very critical with the cognitive functions of the DMN that Raichle suggested in the first studies of the DMN (Raichle et al., 2001), specially because such functions (e.g. predictive coding, off-line proccessing) have been already proposed by cognitive theory for the brain and in specific for information processing in the cortex (the same functional propositions were discussed in section 2.1.2). (Andrews-Hanna et al., 2014) explains that viewing the DMN as a "task-negative network" (i.e. active during rest) can ignore DMN activity beyond rest. Seeley et al. (2007) proposed a new concept called "intrinsic connectivity networks" to include all possible functional implications of the DMN. Intrinsic connectivity networks have activity observable both in rest and some tasks. Regions of the DMN are active in diverse functions, not only during rest, for example during self-referential processes (Qin and Northoff, 2011) and social cognition Mars et al. (2012). The nature of these functions had lead to the idea of a strong relevance of the DMN in social cognition and to include the network as part of the social brain (Mars et al., 2012). However, inferring what functions the DMN might have beyond rest still cannot answer what is the DMN during rest, as a "default mode" (Morcom and Fletcher, 2007).

2.2.2 Functional implications of DMN activity

Another problem that Morcom and Fletcher (2007) pointed out is the difficulty to adjudicate functions to DMN resting-state activity by solely looking at individual regions and their involvement in cognitive functions. For example, if a region A is concerned with a function a during a specific task, the problem comes when the region A is active during rest and the an inference is done that says the function a being elicited doing rest. Morcom and Fletcher (2007) suggests that there is no way to be sure that the region A is doing the same function a during rest or that it is not doing a different function. To answer this problem, Morcom and Fletcher (2007) proposes that task manipulations are required to unravel the possible contributions of the DMN activity observed during rest.

Andrews-Hanna et al. (2010b) argued that despite the fact that task studies may not specifically explain the possible function of resting-state activity, a problem that (Morcom and Fletcher, 2007) pointed out, studies about functional correlations of the individual regions of the DMN are a source of information. The DMN anatomy consists of brain regions of the anterior and posterior midline (cingulate and retrosplenial cortices), lateral parietal cortex, prefrontal cortex and he medial temporal lobe. These regions are recruited in tasks of internal cognition, such as autobiographical memory, future planning, theory of mind, and decisions that involve the self and affection (Buckner et al., 2008). Using Intrinsic functional connectivity MRI (fcMRI) Andrews-Hanna et al. (2010b) analyzed the brain activity at rest of 11 regions of the DMN to find activity correlations in the resting

state. Intrinsic functional connectivity MRI (fcMRI) is an imaging technique that is based in detecting spontaneous activity in the brain measured at rest to find activity correlations between different brain regions (Buckner et al., 2013). After imaging, the functional correlation levels were analysed with the Kamada-Kawai clustering algorithm, which brings together data points with strong correlation and brings apart weakly correlated data points Andrews-Hanna et al. (2010b). Notice in the figure 2.2 the two regions in yellow. The size o the circle represents the *betweenness centrality*, which is a measure of how central is a node in a network or how often the node is used to transfer signals (Newman, 2010). Given that the two nodes, the anterior prefrontal cortex (aMPFC) and the posterior cingulate cortex (PCC), had a high betweenness centrality, Andrews-Hanna et al. (2010b) suggested that these two nodes represented the functional hubs of the DMN, where information transfer between all subsystems in the network is mediated. The remaining regions with less connectivity were clustered together in two subsystems: the "dorsal medial prefrontal cortex subsystem" and the "medial temporal lobe subsystem". The different regions of each subsystem are listed in table 2.1.

Having identified such subsystems, in second part of the experiment by Andrews-Hanna et al. (2010b), the behaviours suggested by literature on functional brain imaging of DMN brain regions (Buckner et al., 2008) were selected to conduct task manipulations in a fMRI study of each subsystem of the DMN. This study represents an assessment of one of the problems regarding inferences in theories of DMN function, as Morcom and Fletcher (2007) had pointed out, and makes use of task manipulations to determine more accurately whether the DMN as a network of subsystems is involved in the suspected functions. All subsystems were recruited in decisions related to self-referential decisions, but each subsystem showed a predilection for different temporal aspects of the decision. The core subsystem had a mean predilection for self-related thoughts without temporal context, the dMPFC subsystem was concerned with self-referential decisions of present concerns, and the MTL subsystem with decisions about future plans (Andrews-Hanna et al., 2010b). In another study, Andrews-Hanna et al. (2014) made a meta-analysis of several imaging studies using the database tool *Neurosynth* (Yarkoni et al., 2011), and with the cognitive functions correlated to the brain regions of each subsystem in the DMN. The figure 2.3 shows the results of the meta-analysis for each subsystem with the functions correlated in each group. Notice that most functions have an implication in self-referential processes and social behaviour. Further studies with task-manipulations are required to find whether all the functions listed by this meta-analysis happen during resting-state activity of the DMN or during mind wandering.

Andrews-Hanna et al. (2010b) also argued that, surprisingly, a second source of information about the possible function of the DMN during rest is the contents of spontaneous thought. As it was mentioned earlier, resting-state activity in the DMN has been found correlated with mind wandering Christoff et al. (2009). In a study (Andrews-Hanna et al., 2010a), when mind wandering was induced, the DMN was observed active and this activity was not present when an external attentional demand was present. In the same study, the contents of mind wandering episodes were sampled, finding that spontaneous thoughts about near past and future dominated the tendency of mind wandering while activity was elicited in the DMN.

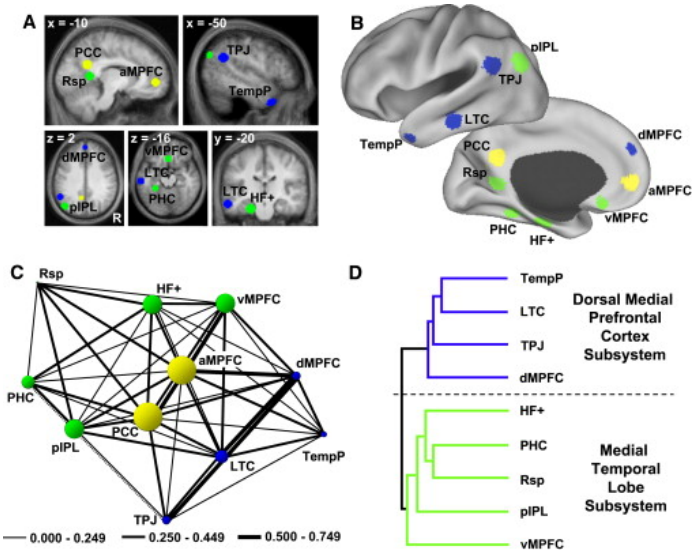

Figure 2.2: Subsystems of the default mode network identified by a clustering analysis. All four panels have the same color coding. Green: medial temporal subsystem, blue: dorsal medial subsystem, yellow: core subsystem. A. The localization of the DMN regions included in the study. B. Localization of the regions of interest in a 3D model. C. Results of the clustering analysis with the Kamada-Kawai algorithm, which brings close regions of high correlation, and locates away the regions weakly corrlated. Thicker lines represent higher correlation, and size of the node is the betweenness centrality, which represents how central is a node. The nodes with the highest centrality are the anterior prefrontal cortex (aMPFC) and the posterior cingulate cortex (PCC). All the regions with their complete names and subsystems are listed in table 2.1. D. Hierarchical clustering analysis demonstrated clustering of the remaining DMN regions in two distinct subsystems (Andrews-Hanna et al., 2010b).

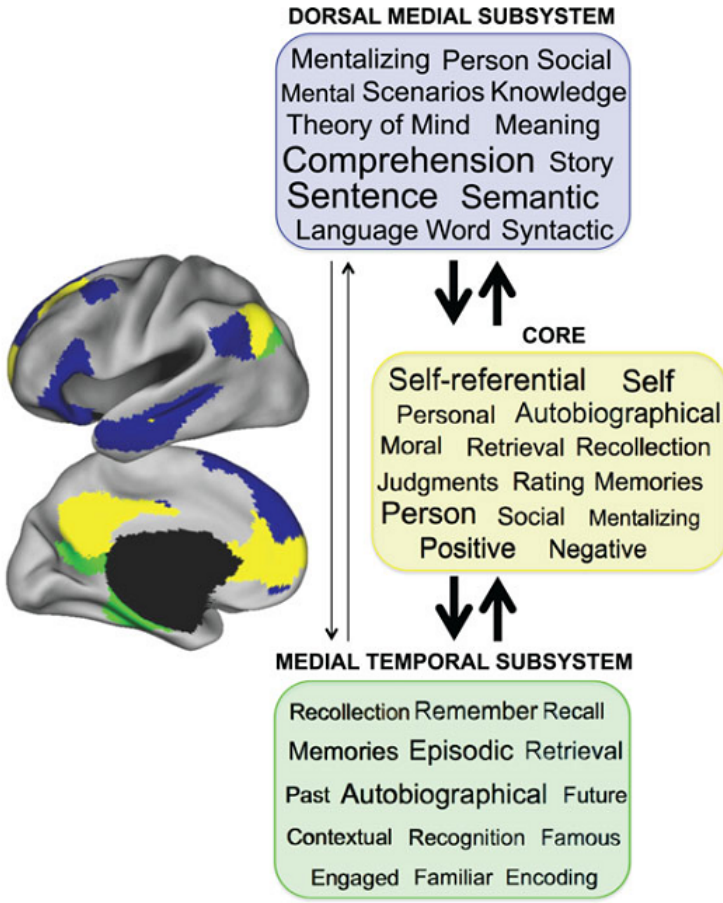

Figure 2.3: Meta-analysis of behavioural studies with functional brain imaging of the DMN subsystems. Each subsystem is implicated in different functions. The dorsal medial subsystem (blue) is implicated in functions important for social interaction, the core subsystem (yellow) with self-referential processes, and the medial temporal subsystem (green) with episodic memory (Andrews-Hanna et al., 2014).

Table 2.1: Different subsystems of the default mode network with their corresponding brain regions. Each subsystem was identified in a functional correlation imaging study with clustering analysis (Andrews-Hanna et al., 2010b). A total of 11 regions were classified per subsystem.

Subsystem	Regions
Core-aMPFC subsystem	Anterior medial prefrontal cortex (aMPFC)
	Posterior cingulate cortex (PCC)
Dorsal medial prefrontal cortex subsystem (dMPFC)	Dorsal medial prefrontal cortex (dMPFC)
	Temporoparietal junction (TPJ)
	Lateral temporal cortex (LTC)
	Temporal pole (TempP)
Medial temporal lobe subsystem (MTL)	Ventral medial prefrontal cortex (vMPFC)
	Posterior inferior parietal lobule (pIPL)
	Retrosplenial cortex (Rsp)
	Parahippocampal cortex (PHC)
	Hippocampal formation (HF)

2.2.3 Interactions between the DMN and other bran networks

The default mode network interacts with different subsystems in the cortex and in subcortical structures. DMN interaction with the fronto-parietal network (FPN), the dorsal attention network (DAN), and subcortical systems will be discussed in this section.

In the cortex, the fronto-parietal network (FPN) has been described as the neural correlate of the "global workspace". The global worspace theory says that in the brain, different subsystems have access to a "global workspace" with limited memory capacity. The separate brain subsystems have a degree of independence, thus in the global workspace the subsystems can interact together and produce more complex behaviours. The set of different brain regions act as parallel specialized processors that have to be coordinated and controlled, and information exchange happens in the global workspace (Baars, 2005).

The global workspace theory is one of the theories of consciousness, in which conscious cognition (i.e. what is being experienced) is the global workspace, and provides access to different brain functions. A metaphor is often used to explain how consciousness could act as a global workspace, the metaphor of the "theatre of mental functioning". The stage in the theatre is the *immediate memory*, and a bright spot in the stage is *consciousness*. What is illuminated by the bright spot is conscious, what is in the dark is unconscious. The bright spot is directed in different places by *attention* under executive guidance. For example, for sensory experience to be conscious, the spotlight of consciousness should include the sensory cortex, which could be activated "externally" for perception of ongoing stimulus, or "internally" for the experience of mental imagery and inner speech (Baars, 2005).

Mind wandering and attention are anticorrelated behaviours, which is observed when processing of the immediate perceptual input is impaired due to reduced attention during mind wandering episodes. Smallwood et al. (2012) proposed a model that includes the DMN and the dorsal attention network (DAN) in the theory of the global workspace. The model proposes that the DMN and the FPN cooperate for the production of a train of thought, and that the dorsal attention network has some degree of competition with the DMN to access the global workspace (Smallwood et al., 2012). Gao and Lin (2012) tested whether the FPN selectively would activate or suppress the activity in the DAN or the DMN, depending of the kind of task. They found that during an attention-demanding task of finger tapping sequences, the FPN had a significantly high correlation with the DAN, and an anticorrelation with the DMN. A completely opposite pattern was observed when a task of natural movie watching (MW) that can engage the DMN was tested. The FPN showed a significant anti-correlation with the DAN, and positively correlated with the DMN. The "anti-correlation" of the DMN and the DAN, however, is not an all-or-none relationship.

Whole-brain mapping of resting-state activity functional connectivity (Anderson et al., 2011), showed that the different networks with smooth gradients throughout their regions. The functional hubs had smoothly varying gradients of anti-correlation to correlation. Their study included subjects of different ages, which allowed them to observe differences between subjects and to detect a strengthening of the DAN-DMN connectivity gradients with age, from late adolescence into adulthood. Sharpening of the boundaries of the DMN, an integration of the cingulate cortex and the FPN, and an increase of the anti-correlation between the DMN and the DAN. The anti-correlation of the DAN and the DMN inspired a study of the anti-correlation between the DMN and the working memory network (Piccoli et al., 2015), where they showed that the DMN is not completely anti-correlated with the WMN during working memory tasks. At least during encoding and retrieval phases, the DMN and the WMN are coupled. As a contrast, during a memorizing task (i.e. when attention is focused in maintaining working memory), the DMN and the DAN are anti-correlated. The coupling and decoupling of the DMN and the WMN suggests the existence of a functional "switch" between networks.

Other regions of the DMN have interactions with distinct brain systems. The functional hubs of the DMN have implications with the processing of information with personal relevance, and both regions have connections with the limbic system (Öngür and Price, 2000; Devinsky et al., 1995), which processes emotional value and motivation (Devinsky et al., 1995). The posterior cingulate cortex (PCC) serves to integrate different types of information, and has been suggested as functional hub or transmodal cortex (Leech et al., 2012; Braga et al., 2013). The mPFC is considered part of the "social brain" (Mars et al., 2012) and is implicated in the processing of theory of mind (i.e. prediction of what others think), and the representations of the size of social networks (Lewis et al., 2011). The two functional hubs together are often referred to as cortical midline structures (CMSs) (Moran et al., 2013) and are active during processing of self-referential information (Gusnard et al., 2001; Macrae et al., 2004). Differences in the connectivity between the CMSs and the limbic system, specially the amygdala, suggesting that the connectivity between the DMN and the limbic system has relevance for depression (Sheline et al., 2009).

Differences in the connectivity within the DMN has been correlated with the presence

of different diseases. The DMN is comprised by different subsystems, and each subsystem is correlated with different functions relevant for social interaction and self-referential processes. If the DMN becomes disrupted or altered, one can expect to find differences in behaviour and general cognitive functioning. In the following section, the implications of altered activity patterns in the DMN for disease will be discussed.

2.2.4 Altered activity patterns in the DMN and implications in disease

Alterations in connectivity or activation in the default mode network (DMN) have been observed in altered function of mental diseases. The DMN comprises of several areas grouped by functional significance and connectivity in subsystems. The alteration pattern of activity and location in the DMN subsystems underlies the nature of the symptoms in the diseases. Hyperactivation and hyperconnectivity are often involved in disorders of content, such as depression, and disorders of regulation, such as attention deficit hyperactivity disorder ans schizophrenia. General deficits in generation of cognition are sufficient to generate diseases of integrity, such as Alzheimer's disease, where hypoactivation or hypoconnectivity of DMN components lead to abnormal general cognition (Andrews-Hanna et al., 2014). There are several theories of mechanisms of disease in the DMN.

The *integrity hypothesis of DNM function* consists of the causal relationship among neural degeneration in the DMN and loss or alteration of self-generated thought. If the DMN is disrupted, intrinsic activity is altered, thus abnormal self-generated is observed. General degeneration associated with normal and pathological ageing is associated with impairment in memory function. Neurodegenerative diseases, such as Alzheimer's disease and frontotemporal Lobar Degeneration (FTLD) may affect brain regions that are part of the DMN. Self-reflection, emotional processing, memory and future thought are impaired in such diseases (Andrews-Hanna et al., 2014).

The *content regulation hypothesis* suggests that content of self-generated thought can be extreme because of a failure of regulation and adaptation of the DMN. Overly negative thoughts and extreme "standards" or discrepancies (Williams, 2008) are a hallmark of depression, and in accordance with the content regulation hypothesis, depressive patients cannot easily abandon goals or have a flexible thinking, which is seen in rumination, a thought process where a negative thought is constantly processed without resolution (Treynor et al., 2003).

The *context regulation hypothesis*, which indicates that a failure of the DMN could lead to excessive episodes of mind wandering or sensory decoupling even in contexts when attention is required, causing adaptive problems, for example in hyperactivity disorder, where distractibility is increased (Andrews-Hanna et al., 2014). In cases like schizophrenia, hyperactivity of the DMN may reflect reality monitoring defects (Andrews-Hanna et al., 2014), where differences between internal thought and the external world cannot be identified (Langdon et al., 1997). The *task interference hypothesis* is closely related to the context hypothesis, and it proposes that there is a defect in the transition from task to rest states, causing interference with the task at hand and reduced attention. Abnormal anti-correlation patterns of activity between the DMN and other task-related networks, often reflect the inclusion of the DMN in the task (Broyd et al., 2009).

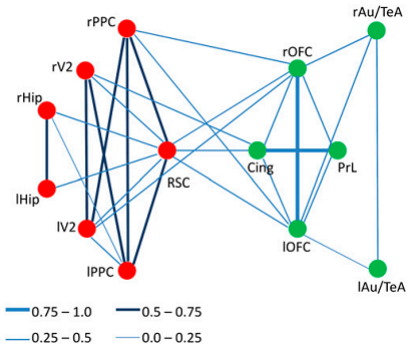

Figure 2.4: Functional correlation between the different subsystems of the default mode network in the rat. Line thickness and color represent correlation strength between regions. Cingulate cortex (Cing), prelimbic cortex (PrL), right and left auditory/temporal association cortices (rAu/TeA, lAu/TeA), right and left hippocampus (rHip, lHip), right and left orbital frontal cortex (rOFC, lOFC), right and left posterior parietal cortex (rPPC, lPPC), retrosplenial cortex (RSC), right and left secondary visual cortex (rV2, lV2).

2.2.5 Study of DMN in rats

Lu et al. (2012) identified a DMN-like network that is also active during rest. In this network, the retrosplenial cortex and a group of prefrontal cortices are equivalent to the human DMN functional hubs, and showed the highest degree of connections with other regions (fig. 2.4). Their network had two distinct subsystems. A prefrontal cortex subsystem, including the cingulate, orbitofrontal, and prelimbic cortices, with a strong correlation with a temporal group that included the auditory and temporal association cortices. The second retrosplenial subsystem had a strong correlation with the hippocampus, the posterior part of the cingulate cortex and the secondary visual cortex (V2).

Cytoarchitectonically, the rat prefrontal cortex is different to the human, because it has only agranular cortical areas (Öngür and Price, 2000) and unlike humans lacks the posterior part of the cingulate cortex (Vogt and Paxinos, 2014). Despite this difference, the rat DMN has activity correlation patterns within the network similar to those in the human DMN and can be a good experimental model (Sierakowiak et al., 2015).

The involvement of the hippocampus in the rat DMN has shown some discrepancies. Lu et al. (2012) observed a signal in the hippocampus correlated to the DMN activation, but in a different study (Sierakowiak et al., 2015), there is a lack of signal from the hippocampus during their resting connectivity analysis. The authors of the first study (Lu et al., 2012) mention the possibility of a partial volume effect from neighbouring activated areas (RSD/RSG).

The activity in the rat DMN has also been compared to other rat networks. Schwarz et al. (2013) identified a network with anticorrelated activity when compared to the rat DMN, the lateral cortical network (LCN). The LCN is a network that has homologue regions in the human brain that are also anticorrelated with the human DMN, including the supplementary motor area, precentral gyrus, frontal eye fields, secondary sensory cortex

and the insula (Fox et al., 2005). In rats, the LCN is concerned with control of gaze and orientation (Erlich et al., 2011). The LCN can be compared to the DMN because the first is a task-activated network, while the second shows task-induced deactivations.

Using carbon paste electrodes implanted in regions of the rat LCN and DMN, the anticorrelation between the networks has been studied in learning paradigms, and it has been shown that the characteristic pattern of activity in the DMN (i.e. task-induced deactivations and rest-state activity) is modified in extinction (i.e. when there is no more reward and learning paradigms are lost). When animals have learned a task, the DMN has task-induced deactivations, but in extinction, this pattern is lost. If learning paradigms are reinstated, the pattern of activity of the DMN is recovered. The changes in patterns of activity during task and rest blocks are shown in fig. 2.5 (Li et al., 2015).

DMN patterns of activity can be distinguished from the LCN patterns in terms of activity modulation. The LCN shows activity regardless of task, including rest, and is not affected by extinction (Li et al., 2015). The fact that the activity patterns in the DMN change with differences in a learning paradigm (i.e. extinction), suggests that the DMN may have more flexibility and is able to adapt. Smallwood et al. (2012) proposed the hypothesis that the DMN may require synaptic plasticity in order to adapt to changes in the environment, which is in line with the findings by Li et al. (2015). The comparison of different networks in the rat bring the possibility to detect changes in different experimental conditions.

2.3 Limitations of functional brain imaging in cognitive science

Imaging paradigms can provide basic knowledge for the study of pathologies to approach the highly difficult problems of cognition (Papanicolaou, 1998) such as mind wandering (Christoff et al., 2009), but it has limitations. Seeley et al. (2007) criticize the use of cognitive substraction paradigms for the study of brain activity in fMRI because such methods cannot distinguish between the activity of a single network and the coactivation of an ensemble of networks. In substraction paradigms, the activity of two regions is measured and then using different baselines (upper or lower bounds), a *substraction* or *difference* is calculated, which is thought to represent the activity elicited by a task (Logothetis, 2008). In fMRI, coactivation or correlated activity of two brain regions refers to how much the activation of one region influences the activation of the other, and in fMRI this is regarded as *functional connectivity* (Friston, 2011). In light of the observation that several brain regions coactivate in cognitive tasks , Seeley et al. (2007) propose the term "task-activation ensemble" (TEA) for a group of brain regions that coactivate. In fMRI, the study of TEAs naturally requires studies of coactivation, but an ensemble of several signals varying in time an space is difficult to disentangle. Independent component analysis is a mathematical approach to separate signals that fMRI studies use to decompose complex imaging datasets (Calhoun et al., 2009).

The study of the brain alone by imaging establishes *only* the localization of the areas activated during tasks, but their structure alone cannot explain the mechanisms of the neural processing during the activations Grillner et al. (2005), (Lamme, 2010). Grillner et al.

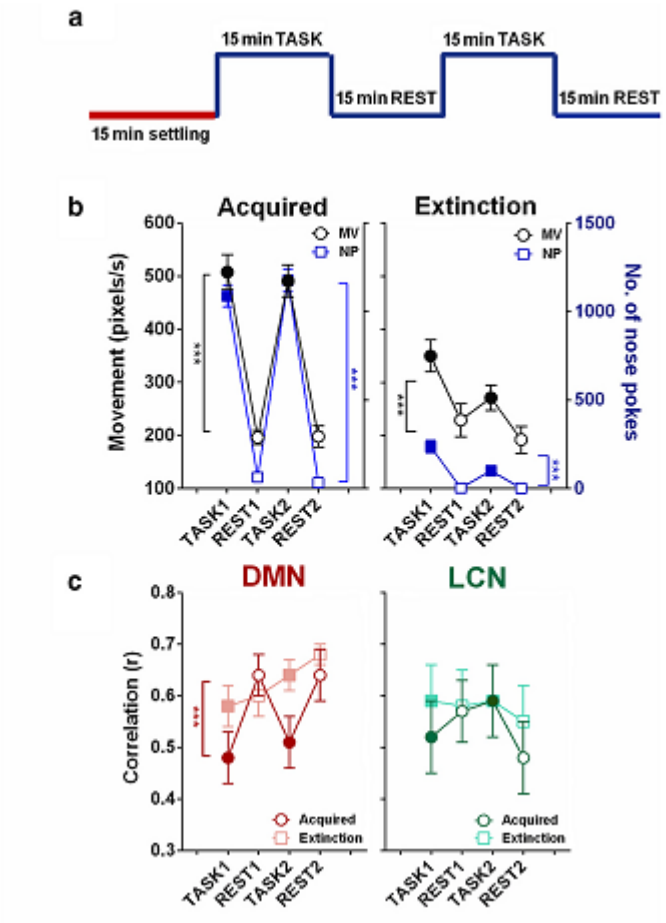

Figure 2.5: Different activation patterns of the rat DMN in extinction. Animals learned a task of nose-poking, in which a reward would be received with a correct response. When the learning paradigm is present, the DMN showed task-induced deactivations, while the LCN showed activity at all blocks. In extinction, the pattern of activity in the DMN is lost, but recovered with reinstatement of the learning paradigm. LCN: Lateral cortical network, DMN: Default mode network. A. Block paradigm with task and rest steps. B. Degree of movement of the animals during task and rest blocks to show that animals had more movement during tasks. C. Activity patterns of the DMN and LCN in the learning paradigm and during extinction (Li et al., 2015).

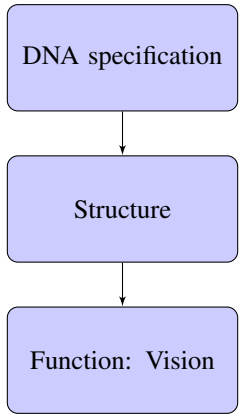

Figure 2.6: Sensory systems are best explained by the structural approach. For example, the visual system is directly generated by structure and do not have multifunctionality. Figure redrawn from (Poznanski, 2001, p. 8).

(2005) strongly argues that the overall function of cortical processing cannot be understood without a clear knowledge of neural processing within the microcircuitry.

Knowledge from all levels must be obtained, from synaptic interactions and connectivity to behaviour. Nevertheless, knowledge at different levels is still insufficient to describe the brain as a whole complex entity, and bridging knowledge (Bechtel, 2012, p. 129) and finding a clear distinction Brigandt and Love (2014) among levels is difficult. For example, molecular biology can describe the building blocks of neurons, not visible by brain imaging , but fails to explain how the brain *actually* engages in the behaviours observed (Grillner et al., 2005).

2.3.1 The interlevel study of the brain

Neuroscience has been studied largely by a structural approach: *structure defines function*. However, this approach is no applicable to every case. The structural approach (Buzsaki, 2006, p. 29) commands the scientific effort to focus on precisely understanding the neural circuitry and deciphering the rules of connectivity. Once rules of connectivity are understood, such knowledge can guide to understand the implementation of a function. The structural approach can be useful for cases like the visual system, as shown in fig. 2.6, because function in this system is directly generated by structure and there is no multifunctionality, but it is not applicable to every case. Cortical integration functions are too complex to be understood solely by structure (Poznanski, 2001, pp. 1-21).

Integrative neuroscience is a *functionalist* approach that advocates for a continuum of neural levels, hierarchically organized. Integrative neuroscience can bring together mathematical approaches and theoretical knowledge. The main motivation is the difficulty to explain effectively the brain as a complex system that varies continuously and non-linearly: A spatio-temporal continuous change of brain states (Poznanski, 2001).

Inter-level paradigms have started to be applied to the study of the brain and highly

complex cognitive processes, including the default mode network. The study of the brain in fMRI has undergone changes in recent years. An initial limitation of fMRI was that only population activity could be detected, thus there was a requirement of isolating the signals of separate networks. Independent component analysis, a mathematical method, became widely used to separate different signals from imaging datasets (Calhoun et al., 2009). Once networks achieved independence, to find how all these separate networks bound together into a single brain became the new challenge of cognitive neuroscience (Sepulcre et al., 2012).

2.4 Neural mechanisms of integration in the brain

The brain has several functional subsystems that are relatively autonomous among each other, but that need to cooperate in order to produce a fully integrated cognitive function. Different regions in the brain are highly interconnected, directly or indirectly. Each brain area receives activity as an *input* from other areas, and act as *synaptic relays*, where information may be amplified, attenuated or modified before being sent as an *output* to the next area (Kandel, 2013a, pp. 343-348). Different kinds of cells make up the brain population, which roughly can be classified as glial cells (i.e. thought to underlie metabolic support for neurons) and neurons (i.e. the cells that are able to transmit information through nervous signals). The activity of neurons is modulated by the presence of inhibitory signals from other neurons (Javier DeFelipe, 2010) and neuroactive molecules (modulatory systems such as serotonin, dopamine, noradrenalin, acetylcholine, etc) (Kandel, 2013b, pp. 290-298). A single neuron receives multiple inputs form multiple areas, and the summation of inputs is known as *synaptic integration* (Kandel, 2013a, pp. 343-348). Furthermore, glial cells (i.a. astrocytes), despite their slower signal temporal resolution compared to neurons, have an influence in neuronal network function, such as contributing to the maintenance of gamma-oscillations, a synchronized activity pattern in neurons implicated in novel object recognition behaviours (Lee et al., 2014).

In this section, the theoretical background for the biological correlates of integration are reviewed, from cellular to brain systems, and what place has the DMN in from this perspective. The section is divided in three parts. Section 2.4.1 consists of a review of the theoretical background of the biology neurons and synapses, their physiological properties that account for their function as integrators. Then, section 2.4.6 includes the background for the organization of neurons in the brain and the hierarchical functional organization of different brain areas to account for specialization, in what part of this classification the DMN and its functional hubs (the aMPFC and PCC) have a place, and the evidence of this kind of organization from fMRI studies. Section 2.5 is the last part of this section and includes the techniques by which morphology in neurons can be quantified to find differences in integration and computational capacities in the brain, and what this kind of measures could say about the DMN.

2.4.1 Integration in neurons

From the point of view of information processing, integration is the process by which information of different sources is put together and processed to obtain an unified view

(Lenzerini, 2002), in the same way as cognitive reasoning connects different information premises to information conclusions and lead to action or new knowledge (Anshakov and Gergely, 2010). For survival, integration of internal and external information is necessary for the coordination of different functions (e.g. several functions at the same time, in sequences, or the antagonistic selection of functions) (Chiel and Beer, 2001). The nervous system not only transmits information that comes in and out of the system, but needs integration to coordinate functions, enhancing the opportunity of a living being to generate reliably fast, globally coordinated behaviours to survive in complex external and internal environments (Chiel and Beer, 2001). In the nervous system, neurons are the integrators or cellular processor units of the brain. A single neuron receives several inputs from other neurons, and has the cellular mechanisms to sum such inputs, a process called *synaptic integration*, to produce an all-or-none output signal.

Figure 2.7 shows a section of a dendritic membrane which has pores or tunnel-like structures called ion channels. Ions can cross through the ion channels from one side of the membrane to the other, and as they move cause a change in electrical potential that produces a *graded potential*. The graded potential is a difference in potential that acts as input signal, called the *postsynaptic potential* (PSP). The term *synaptic* refers to the structural relationships called *synapses*, that connects neurons to each other, and as shown in figure 2.9, the previous neuron is the *presynaptic neuron*, and the next neuron is the *postsynaptic neuron* (Etherington et al., 2001). The mechanism of integration in neurons consists of the summation of different inputs in the form of PSPs.

At rest, when neurons do not receive inputs, ion concentrations between the inside and outside of the membrane are unequal, yielding a *resting membrane potential*, in which the inside of the cell is more negatively charged. Membranes with different permeabilities to different ions, act as *capacitors* or batteries, and have a *membrane capacitance*, or the capacity to store charge. Due to the membrane potential, when *ion channels* open (i.e. tunnel-like structure in the membrane, connecting the inside with the outside), ions move due to electrochemical driving force (2.7). Figure 2.8 shows the *action potential*, which is the mechanism of summation of PSPs. As PSPs arrive to the neuron, the membrane potential accumulates until a voltage threshold where voltage-gated ion channels are activated. When the voltage-gated ion channels open, the action potential is generated.

Ion channels can be activated and opened by neurotransmitters (chemical synapses) or directly connect neurons (electrical synapses). The movement of ions produces a change in the membrane potential, a change called *postsynaptic potential* (PSP), the basic neuronal input signal. Inputs to a cell can be excitatory (EPSP) or inhibitory (IPSP), depending on the kind of ion channels opened, increasing or decreasing the membrane potential. As the cell receives many inputs, both excitatory and inhibitory, the opening of different channels that allow different fluxes of ions according to their electrochemical driving force produces an overall membrane potential change, which is the summation of PSPs (Etherington et al., 2001).

The production of an output signal depends in the temporal frame of arrival of EPSP and IPSP. If several EPSP arrive in the same time window and accumulate in a neuron, the membrane potential is shifted to more positive values (i.e. more positive charge inside the cell). If EPSPs arrive quicker, their capacity to build up a signal is higher. When the membrane potential crosses a threshold value, voltage-gated ion channels activate and

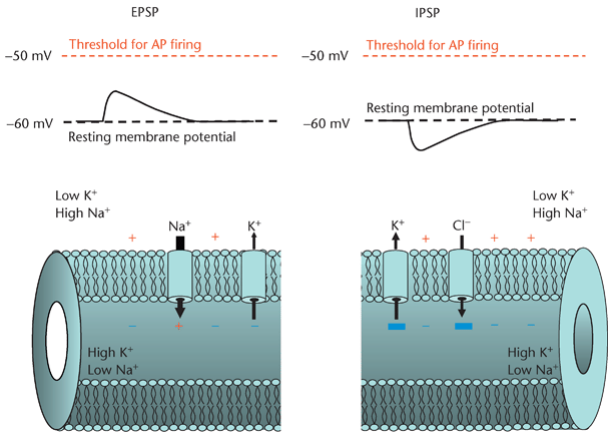

Figure 2.7: Mechanism for the generation of postsynaptic potentials in the neuron. As ions move through the membrane, the electrical potential across the membrane changes, producing a *graded* potential, or a difference in potential that acts as input signal, called *postsynaptic potential* (Etherington et al., 2001).

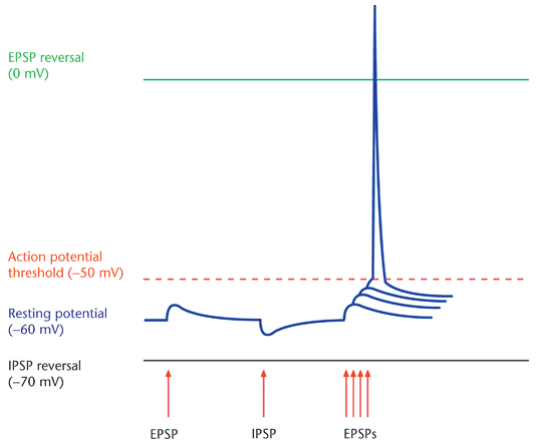

Figure 2.8: When several EPSPs arrive in the same time frame (i.e. when there is not enough time for them to decay), the membrane potential can accumulate until a threshold value that triggers the opening of voltage-gated ion channels and the production of an action potential (Etherington et al., 2001).

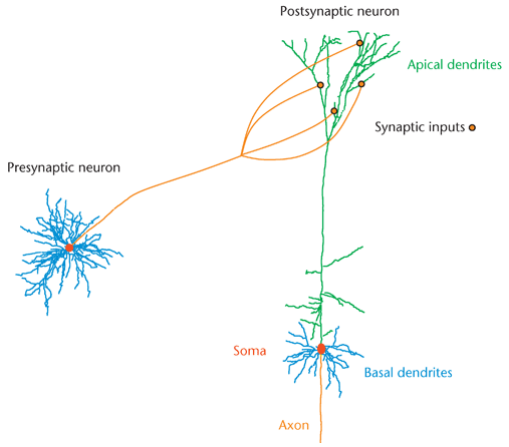

Figure 2.9: Schematic representation of a presynaptic neuron with synaptic inputs on the dendritic arbour of a postsynaptic neuron. (Etherington et al., 2001)

depolarize the membrane (i.e make the potential very positive due to continuous entry of sodium, positively charged ions), which is observed as a peak. At the membrane potential of the top of the peak, different voltage gated ion channels activate (potassium), while sodium channels inactivate, now shifting the membrane potential to resting states. Conductances of different ions (e.g. sodium, potassium, chloride), act in concert to produce the action potential, an all-or-none output signal with a characteristic shape. Once the action potential is produced, it acts as mechanism of regeneration of signals, because as it is transmitted down the axon, the signal is maintained as peaks, instead of as PSPs that can fade with time (Etherington et al., 2001)

2.4.2 Synapses

A *presynaptic* neuron is connected to a *postsynaptic* neuron by means of a synapse (2.9, where input signals can be transmitted from one cell to another, making the activity of neurons possibly correlated (Etherington et al., 2001).

Synapses convey signals from one neuron to another by two means: synapses can be electrical and transmit electrical current in the form of charged ions, directly through channels called *gap junctions*; or synapses can be chemical, translate the action potential into the release of neuroactive signals that activate the postsynaptic neuron to produce PSPs. Neurotransmitters activate different ion channels, having excitatory or inhibitory effects (eg. glutamate opens AMPA receptors yielding an excitatory influx of sodium or EPSP, while GABA activates chloride channels, yielding an inhibitory PSP) (Etherington et al., 2001).

Vertebrate neurons have different cellular processes that extend from the cell body or soma. One is the *axon*, the main output pathway, and the other is the *dendrites*, which form complex branching arbour-like structures where inputs are typically made. Dendritic arbours can extend widely for more than $1\,\mathrm{mm}$, increasing the available area to establish

synapses (Etherington et al., 2001). The complex morphology of dendritic threes and the localization of the synapse on it influence the likelihood of a synaptic input to be able to contribute to the production of an action potential.

Two main excitatory classes are identified in the cortex: pyramidal and spiny nonpyramidal cells. Both are glutamatergic and present dendritic spines. *Pyramidal cells* constitute ca. 80% of the total cell population and are found in all cortex, except layer 1. *Spiny nonpyramidal cells* (granule cells or stellate cells) are found in layer 4. They often have a short-axon and a characteristic star-like morphology. Inhibitory GABAergic interneurons are classified as *aspiny nonpyramidal cells* or *smooth interneurons* and are found in every layer, as 15%-20% of the total neuronal population . Classification of inhibitory interneurons depend on axon distribution, synaptic profile and type of expressed molecules, such as cotransmitters, peptides or calcium-binding proteins. They have short-axons and have few or none dendritic spines (Javier DeFelipe, 2010).

Synapses among neurons in the neocortex are classified as *asymmetric* (Gray's type I) and *symmetric* (type II), depending on the size of the postsynaptic density. Asymmetric synapses show a denser postsynaptic density and are mostly found in axons of spiny cells in extrinsic connections (among different areas), accounting for 80-90 % of the whole cortex. Symmetric synapses have a smaller postsynaptic density, are found on smooth interneurons in intrinsic connections (same area) and account for 10-20% of the cortex. It is often assumed that observed asymmetric connections are excitatory and symmetric are inhibitory connections.

Excitatory or asymmetric synapses are first made in proximal dendrites of pyramidal cells and spines of *spiny stellate cells*, close to the soma at $10\,\mu\mathrm{m}$ to $50\,\mu\mathrm{m}$. Among smooth interneurons, connections are established in the soma, dendrites and sometimes in the axonal initial segment. Inhibitory synapses are formed in all regions of pyramidal cells (Javier DeFelipe, 2010, pp. 5-14).

2.4.3 The dendritic spine

Excitatory contacts are made in the dendrites of neurons on a specialized morphological structure called spine. The dendritic spine is a small protrusion and in general is described in terms of its head shape, neck length and neck thickness. Spines are found in pyramidal cells and are the main input of excitatory connections. Nevertheless, spines are not mandatory structures for excitatory connections, since non-spiny neurons also have excitatory connections. Therefore, dendritic spines must have a function specific excitatory inputs on pyramidal cells (Arellano et al., 2007).

Different morphological features of the dendritic spines are correlated with different functional parameters. The spine-head size is correlated with the number of postsynaptic receptors (Nusser et al., 1998) and the number of docked neurotransmitter vesicles (Schikorski and Stevens, 1999). Spines act as compartments of calcium ions and can control its diffusion to the neuron. In neurons, calcium ions have a function different of the maintenance of membrane potential. Calcium has a function as signalling molecule that activates enzymatic cascades involved in several cellular functions, such as receptor trafficking (Arundine and Tymianski, 2003). The small spine head size has a relatively faster diffusion of calcium, while as the spine neck changes in thickness, calcium diffusion to the neuron is limited (Yuste et al., 2000). Spines not only act as input receivers, but can

modulate the input, and the function of neurons of modulating inputs and responses is called *synaptic plasticity*. Spines come in different shapes and undergo dynamic changes in number and shape, which have brought attention to the possible functional properties of dynamic changes in spines.

The morphology and cellular functions change under several circumstances, including neural stimulation alone. The study of the changes in spine morphology, function and number (i.e. spine density) have been studied in presence of synaptic plasticity. The first studies focused in the induction of long-term potentiation, a form of synaptic plasticity. LTP consists of a change in excitability of neurons induced by a repetitive synaptic stimulation, and was described first by Bliss and Lømo (1973). The change in excitability has been interpreted as a neural correlate of information storage and memory (Teyler and DiScenna, 1987). The fact that dendritic spines are a substrate of connectivity between excitatory neurons, one could suspect that an enhancement of excitability could be correlated with changes in the dendritic spines. Indeed, LTP induces an increase of spine density and the formation of bi-furcated spines (Trommald et al., 1996). Spine density can be a measure of connectivity in neurons, but connectivity alone is not the only function of spines. Spines have the cellular machinery necessary (Yuste and Denk, 1995) for the detection of temporal changes in inputs, a fact that inspired the notion of spines as units of integration in the neuron.

Integration capacity

Dendritic spines are the main target of excitatory inputs in pyramidal neurons. Given that neurons are integrators of several inputs, the fact that spines are the gateway of inputs to neurons, it has been suggested that dendritic spines are "units of integration" in neurons. Spines are the smallest compartment in neurons that has the machinery to perform input-output computations between presynaptic neurons and the dendrite segment of the postsynaptic neuron. Spines have calcium channels and act as individual calcium compartments, and when action potentials arrive, spines are able to detect the temporal distribution of synaptic activity (Yuste and Denk, 1995). Multiple spines in a single neuron increase the capacity of the neuron to perform input-output integration, which corresponds to the *integration capacity* of the neuron. The fact that animals increase their capacity to learn when spine density increases, suggests that higher spine numbers are correlated with higher *processing capacity* (Moser et al., 1994). An important feature of neurons is their distribution in space.

Processing capacity

Moser et al. (1994) proposed that changes in excitability or spine density could be caused by the neural stimulation of a complex environment, and that the outcome of such increase would have an effect on processing, rather than storage of information alone. Previously, Globus et al. (1973) had shown that environments richer in sensory stimulation cause increases in spine density, but these results alone could not distinguish whether animals had to store larger amounts of information or, as Moser et al. (1994) proposed, had a higher processing demand. In psychology, response time tests are interpreted as a measure of processing (Jensen, 2006, pp. 1-10), where shorter reaction times could be an indicative

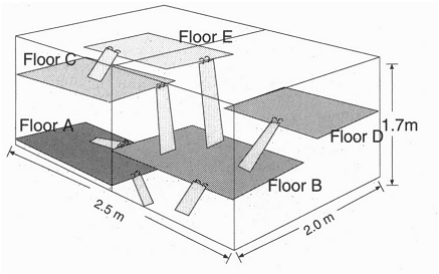

Figure 2.10: Rats were trained in complex environments that had a high demand on rats. Animals had to search for food and water, which was placed in different floors or levels. The cage had different objects that could stimulate exploration, for example leaves, wooden blocks and paper bags. Animals had several training sessions where they could be able to utilize their spatial navigation abilities in climbing and exploring different floors. After the training session, animals were tested in the Morris water maze (see fig. 2.11) Moser et al. (1994).

of faster processing. To test whether processing was affected by spine density changes, Moser et al. (1994) compared the response times to a spatial task (Morris water maze) of two different groups of rats with experience in different environments. Animals of the "enriched" group were exposed to a complex environment with different objects that could induce exploration (e.g. leaves, wooden blocks, paper bags), with different levels where the animals could train their spatial abilities. Food and water was hidden and placed in different levels to stimulate the animals to search for it. In fig. 2.10, a diagram of the training cage is shown. Animals that had been in spatial training sessions in the complex environment had shorter times while solving the Morris water maze and had higher spine densities in the basal dendrites in the hippocampus (i.e. a brain structure correlated with memory and spatial nagivation). The comparisons between animals and the typical response per group is shown in fig. 2.11. In the study by Moser et al. (1994) suggests that increased spine densities are correlated with the acquisition of a capacity to learn better (i.e. improved processing), and not necessarily information storage alone.

Spine density can be a measure of processing and integration capacities of the brain. The analysis of spine density and interpretation of the spine count will be discussed in section 2.5. Additionally, spines come in different come in different shapes, and because the shape of the spine is correlated with modifications in function, the classification of spines is common in cellular studies. However, spines undergo dynamic changes between spine types, which has brought to the question of whether spine classification is based on established spine types or temporal states.

Different types of spines

Dendritic spines come in different shapes. The functional properties of spines depend on their shape. Ion diffusion and compartmentalization change with the neck thickness, and surface area for synapse and receptor function change with the size of the spine head. Differences in functional properties have lead to the question of whether classification of spines in different classes is consistent with established spine subtypes with different

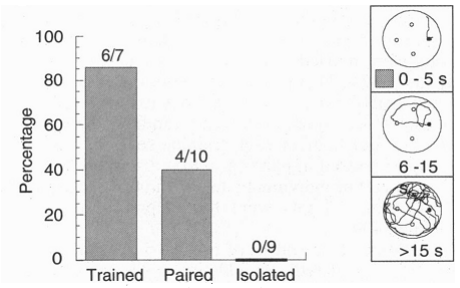

Figure 2.11: Rats of different groups (trained, paired and isolated) were tested in a response time spatial task. Animals of the trained group showed shorter response times, which suggests that the animals could achieve a faster processing Moser et al. (1994).

functional implications, or tentative stages in development or synaptic plasticity.

Spines are often classified as thin or mushroom-shape types. Their different shape modulate their ion diffusion and compartmentalization, and the space available for receptor trafficking and size of post-stynaptic density (PSD), and have different receptor types proportions. Each spine type has different temporal and cellular characteristics. Thin spines are transient and change in response to synaptic activity, have smaller PSDs with more NMDA receptors (NMDARs) than AMPA receptors (AMPARs). The transient and flexible thin spines are thought to provide the possibility of change and flexibility in cortical circuits, which have assigned them as candidate for "learning spines". In contrast, mushroom-shape spines are persistent (months), have large PSDs and have more AMPARs than NDMARs. The large mushroom spines form strong, long lasting synapses, and have the space to have a smooth endoplasmic reticulum, which can provide internal stores of calcium. Taken together, the characteristics of the mushroom spines have suggested that networks with mushroom spines have persistent activity, thus the mushroom spines have become the candidate for "memory spines" (Kasai et al., 2003).

Arellano et al. (2007) analysed morphology of dendritic spines in neocortical II/III layer pyramidal neurons. They found a high variability between putative spine types (e.g. thin, mushroom), which showed no clear evidence of bi- or multi-modality in the distribution of spine head volume to neck ratio. They conclude that there is a continuum of spine morphologies, thus classification into putative categories was not possible with their results because the fraction of spines in an "intermediate" type was the largest. Nevertheless, Arellano et al. (2007) found several correlations between spine head volume, PSD size, neck length and diameter, characteristics of spines that have functional implications in synaptic function (Nusser et al., 1998; Schikorski and Stevens, 1999; Yuste et al., 2000). Therefore, they suggest that the information about the distribution and types of spines in a neuron can be used to reconstruct a functional input map of a given neuron, which could represent the total input connectivity of the neuron, the first step in the study of processing in the neuron or "computational capacity".

Spines undergo dynamic morphological changes, which may indicate that spine types are developmental stages (Fischer et al., 1998), supporting the notion that categorization can only reflect transient states of spines, in the same line of the argument of the "contin-

uum of morphological types" (Arellano et al., 2007). Conversely, mushroom spines have been found to last for long periods (months) (Holtmaat et al., 2005), which suggests that spine types are persistent, and that spine types are a viable way to describe differences in spine diversity when knowledge about spine development and dynamics are considered (Bourne and Harris, 2007).

Neurons have appendages called dendrites that branch extensively and form large dendritic trees. Spines are distributed along the dendritic tree. There is a possibility that each spine can have independence (Shepherd, 1996), due to the electrical properties of dendrites. The way by which dendrites can give spines and inputs independence throughout the whole dendritic tree and the implications in integration and computational capacity will be discussed in the following section.

2.4.4 Spatial integration in dendrites

Dendrites are extended tree-like cellular processes that are able to receive inputs at different locations. When inputs are separated spatially, they are integrated by *spatial summation*. This process determines the transmission effectiveness and amplitude of PSPs, and depends on localization of the input, dendritic tree geometry, and electrical properties of the membrane, given by the distribution and diversity of ion channels (Etherington et al., 2001).

PSPs attenuate as they travel through the membrane because there are ion channels constitutively opened, allowing charge to *leak* out of the cell (Etherington et al., 2001). These channels are of the same kind of the opened channels that passively contribute to the maintenance of the membrane potential (Koester and Siegelbaum, 2013). The membrane, acting as a capacitor, and the *leakage* of charge act together as an *electrical filter*, which attenuates PSPs as they travel, making their spread slower on the dendritic tree. Even though the amplitude of PSPs becomes reduced, their slower transmission compensates by increasing their duration and so increasing the opportunity for temporal summation. The prolongation of EPSP causes *blurring* that reduces the resolution or independence of EPSPs.

Voltage gated channels are important to *sharpen up* (i.e. normalize) the signal transmitted through dendrites, or in other words, giving independence to different inputs that otherwise would be filtered (i.e. blurred) by the dendrites and summed regardless of their temporal difference (Williams and Stuart, 2000). One example is hyperpolarization-activated mixed cation channels, which are also known as hyperpolarization cyclic nucleotid-activated channels (HCN) (Shah, 2014). The HCN channels are distributed in a non-uniform manner, with linearly increasing densities towards the dendrites, generating a localization-independence for EPSP, making distally generated EPSP similar in amplitude and speed as somatically generated EPSPs. When HCN channels are inhibited, this independence of EPSP is no longer available, and distally generated EPSPs become blurred, having a pronounced temporal summation (i.e. the individual EPSPs seem closer, and have less resolution between them). Thus, HCN channels contribute to the sharpening of temporal summation by allowing EPSPs to have independence or a higher temporal contrast (Williams and Stuart, 2000).

Dendrites can branch extensively, and as they are anatomically complex, they become able to subserve more complex tasks. When synaptic contacts are placed directly on the

axon, as in the case of interneurons, such contacts have a relatively simple relay function, where few integration is required. Conversely, when neurons have a complex anatomy with extensively branched dendritic trees, such neurons are often involved in highly complex information processes (Etherington et al., 2001).

Dendrites not only act as pipes that lead all the inputs to the axon, but provide the space to expand the surface area for the establishment of different densities of synapses and their independent processing. Spatial segregation of inputs reduces nonlinear summation of EPSPs, because when inputs are distributed over a wide area, the local depolarization caused by an entrant inputs have enough space to diffuse without affecting neighbouring electrochemical driving force and reducing the amplitude of neighbouring EPSPs. Therefore, spatial distribution of inputs provides the spatial capacity to operate each different input independently, even if synapses are activated at the same time. Furthermore, the available dendritic space allows the placement of synapses at different distributions (i.e. higher or lower densities) and in patterns (i.e. combination of inhibitory and excitatory inputs at different proportions), a diversity that provides the possibility of localized forms of integration (Etherington et al., 2001).

One of the purposes for dendritic tree geometry to have evolved into complex branching patterns may be to satisfy the need of independent processing and localized integration. Having diverse mechanisms of integration on complex cellular architectures, neurons are powerful integrative units, with the capacity of computing thousands of inputs distributed in space and time, to produce an integrated signal sent to other neurons, which may act as relays for more processing, or be final effectors of coordinated bodily functions (e.g. glands, organs, muscles) (Etherington et al., 2001).

2.4.5 Cortical layers

The cortex is the brain structure concerned with cognition and consists of a layered neuronal sheet that covers the cerebral hemispheres. Most of the neocortex has six layers, which are named with a number, from the outer surface, to the inner white matter. Particularly in humans, the cortex is larger than other areas, and forms foldings or grooves (sulci) that separate elevated regions (gyri), in a way that the cortical area is maximized in the volume of the brain. In neuroscience, the relationship among neurons, layers and cortical areas is described as cycles of input and output, with diverse complexity in connectivity and activity patterns. The neocortex is the area of the cortex that is closest to the brain surface. The neocortex is connected to the thalamus, other cortical areas of both brain hemispheres and subcortical structures, from which it receives inputs and sends outputs to. The connectivity patterns described as input-output relationships are orderly arranged in different cortical layers, yielding a different connectivity patterns per layer type.

The mammal neocortex consists of a 2 mm thick sheet of cells, divided conventionally in six layers, even though some areas can present more layers. Figure 2.12 shows how different neurons are located in different layers and form connections with other neurons in the same or a different layer. In the cortex, neurons can be defined as local interneurons or principal (projection) neurons. Local interneurons have axons that remain within the same area (Kandel, 2013a, 348). Projection neurons typically have pyramid shaped bodies and are mainly located in layers III, V and VI. The layer III and V have pyramidal neurons

that can be identified by their triangular shape, and can be an experimental target for the study of cellular architecture and connectivity.

Neurons in the layer III project locally to neurons of the same cortical area and to other cortical areas, mediating the main corticocortical connectivity. Therefore layer III pyramidal neurons can be a sample of corticocortical connections within and between different cortical regions (Kandel, 2013a, 348). All layers in the neocortex have connections with principal neurons of the layer V (Burkhalter, 1989), and pyramidal neurons of the layer V give rise to the main output pathways to other cortical regions and subcortical structures (Kandel, 2013a, 348), therefore layer V pyramidal neurons can be a sample of output connections to other cortical regions and subcortical structures. A summary of the characteristics of the different layers is found in table 2.2.

2.4.6 The hierarchical organization in the brain

Clinical observations have suggested that neurons form relays of information processing, and that as information is integrated and sent to different populations of neurons, such information becomes more complex (Kandel, 2013a, 341-343). Benson proposed a hierarchical organization (Benson, 1993) that classifies different regions of the cortex by their role in a train of integration. Benson's classification was inspired by the proposal of Mesulam et al. (1998) of a gradient of connectivity in the synaptic relays of sensory systems. Benson's classification defines four types of cortex: primary, unimodal, heteromodal and supramodal cortices.

Primary cortex is the kind of cortical region that receives and sends information about stimuli (Benson, 1993). Receptor organs send their inputs to the thalamus and this structure relays the raw information to primary sensory areas. Neurons in primary areas encode single features of sensory stimuli, and as a population they can represent complex information. The first areas concerned with cortical processing are primary cortical areas, while the final processing step is the primary motor cortex (Kandel, 2013a, 341-343).

Unimodal association cortex is the region of the cortex that surrounds the primary cortical regions. The unimodal association cortex processes the information of a single primary cortical region or of a single sensory modality (Benson, 1993). At higher-orders of processing, neurons become responsive to highly integrated information, for example instead of responding to a point in the visual field, neurons can detect complete faces (Kandel, 2013a, 341-343).

Next to the unimodal cortex lies the *heteromodal association cortex*. United perceptual symbols are generated with the integration of information of different modalities. Most cross-modal (i.e. heteromodal) percepts require serial processing in the prefrontal cortex to maintain the correct sequence of information. In this level of hierarchy, perceptual symbols and memories are formed, such as the name of an object encoded in the sound of a word. Lesions in heteromodal association cortex leads to relatively limited dysfunction (e.g. aphasia, agnosia, acalculia), which supports the notion that high level cognitive abilities (e.g. language, imagery, computation) are performed in heteromodal association cortices (Benson, 1993).

Supramodal association cortex is the kind of cortex that monitors the activity of other networks, selects preferred responses and directs non-reflex responses (Benson, 1993). Evidence of supramodality in the brain comes from studies in congenital-blindness, where

Table 2.2: Cortical layers with their name and relevant characteristics like the type of cells found in the layer and the connectivity patterns (Kandel, 2013a, 348).

Cortical layer	Name	Characteristics
I	Molecular layer	Occupied by the dendrites of cells located in deeper layers and axons that travel through this layer to make connections in other areas of the cortex.
II	External granular layer	Contains small spherical neurons and next to layer III projects locally to different layers and cortical regions.
III	External pyramidal layer	The neurons located deeper in layer III are typically larger than those located more superficially. The axons of pyramidal neurons in layers II and III project locally to other neurons within the same cortical area as well as to other cortical areas, thereby mediating intracortical communication.
IV	Internal granular layer	Main recipient of sensory input from the thalamus and is most prominent in primary sensory areas.
V	Internal pyramidal layer	Mainly pyramidal neurons that give rise to the major output pathways of the cortex, projecting to other areas and to subcortical structures.
VI	Polymorphic layer	It blends into the wite matter that forms the deep limit of the coortex and carries axons to and from areas of cortex.

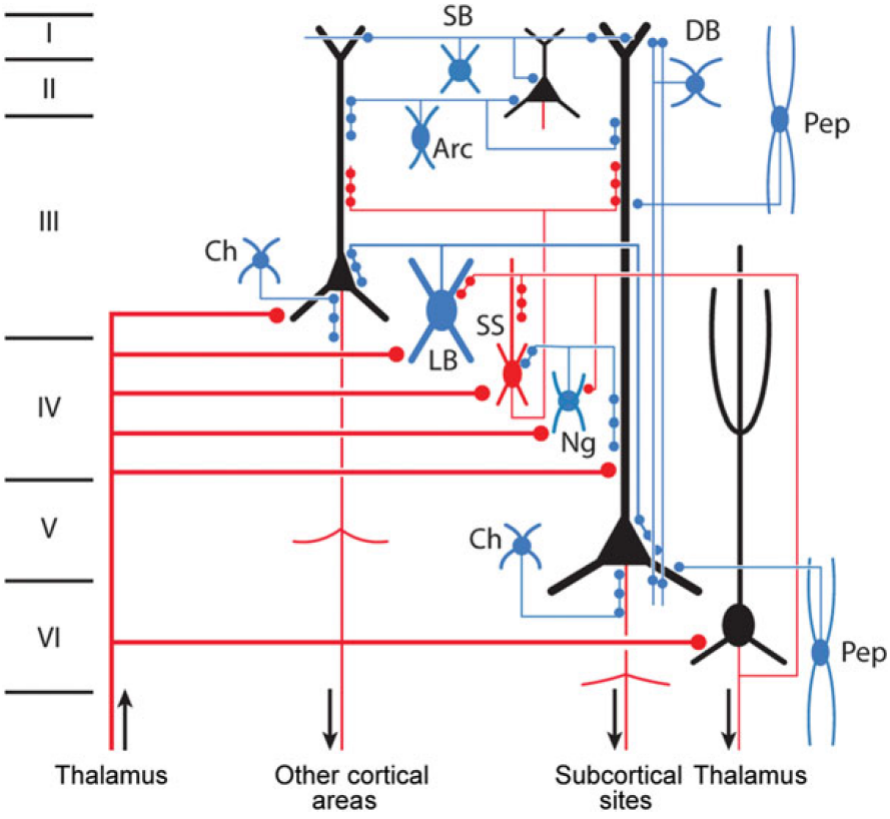

Figure 2.12: Diagram of the cortical layers and the known input-output relationships such as thalamocortical and corticocortical connections. Blue cells are inhibitory interneurons tagged with the names that they received when identified in monkey and cat: Neuron with arciform axon (Arc), Chandelier cell (Ch), double bouquet cell (DB), large basket cell (LB), neurogliaform cell (Ng), peptidergic neuron (Pep). Excitatory neurons are in red, and include principal pyramidal neurons of layers II-VI and spiny stellate cells (SS) from layer IV (Javier DeFelipe, 2010).

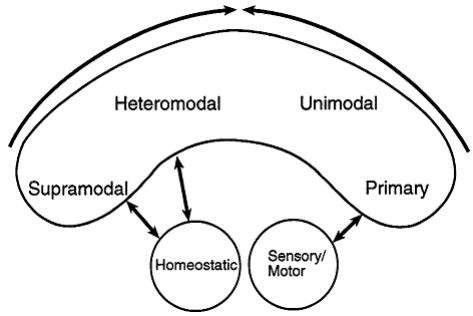

Figure 2.13: Diagram that illustrates the connectivity in the cortex according to Benson's hierarchical organization. Subcortical and sensory/motor connections are bimodal, shown by two-headed arrows, while the general directions between different hierarchy modal cortices are unidirectional, shown by one-headed arrows (Benson, 1993).

supramodal cortex was implicated in abstract features regardless of modality, for example the occipito-parietal stream of "where and how" process information about localization and form for both perception and mental imagery, also regardless of modality (Ricciardi et al., 2014).

Heteromodal and supramodal association cortices receive considerably more connections from subcortical motor and limbic structures, compared to primary cortices, which instead receive more sensory-motor immediate information. Relatively few immediate sensory/motor information reaches the heteromodal and association cortices. Cortico-cortical and subcortical-cortical connectivity according to Benson's hierarchical classification are summarized in fig. 2.13 (Benson, 1993).

Mesulam et al. (1998) proposed the existence of a different kind of cortex in the hierarchy, the *transmodal cortex*. Supramodal association cortices are not the same as transmodal cortices. Transmodal cortices are not the final destination of convergent inputs, but act as gateways (nexuses or functional hubs) that access specific information that is distributed across all the system. Multimodality in the brain, including Benson's classification and Mesulam's transmodality, has been described in fMRI studies Sepulcre et al. (2012). The default mode network (DMN) have two core nodes with high levels of connectivity that were described as "functional hubs" by (Andrews-Hanna et al., 2010b) and then have become to be known as "transmodal" regions by Braga et al. (2013).

2.5 Morphometric analysis of neurons

Morphology in neurons has functional implications with computational and integration capacity. The quantitative analysis of different morphological characteristics of neurons is called "Morphometry" (Milatovic et al., 2010), and in general terms consists of image analysis. Different methods exist to reveal the neuronal cytoarchitecture, followed by image sampling and a quantification that often involve the separation of the dendritic tree in segments with a length, and a spine count. Spine density is the result of the number

of spines per dendritic length. Morphometric analysis have been used to describe normal brains (Claiborne et al., 1990; Desmond and Levy, 1982; Blackstad et al., 2015) and to identify aberrant morphology in disease (Milatovic et al., 2010; Glantz and Lewis, 2000; Tang et al., 2014).

Morphometric analysis of neurons have two main applications, spine quantification and dendritic tree geometry. Spine density profiles can be an approximation of the input connectivity of a cortical region (Arellano et al., 2007), and whether disease (Glantz and Lewis, 2000) or experimental manipulations (Lieshoff and Bischof, 2003; Mychasiuk et al., 2013) affect such profiles, provide an empirical grounding for the study of neural cortical function in normal and diseased brains. The geometry of dendritic trees can be an estimate of the spatial integration capacity and to explore whether single neurons have extended available area for synaptic input reception and independent processing (Claiborne et al., 1990; Etherington et al., 2001). The products of morphometric analysis, spine density and dendritic geometry, together with the molecular biology of the neuron (ion channel and receptor composition), can be used to reconstruct a functional map (Arellano et al., 2007) and ultimately be used to approximate the electrical and computational properties of a given neuron (Sterratt et al., 2011, pp. 72-95).

In this section, aspects of morphometric analysis of neurons that are relevant to the current study are discussed. First, a comparison between two widely used staining methods for the visualization of neurons is discussed in section 2.5. The main quantification parameter of the current study is synaptic density, therefore in section 2.5, aspects of the interpretation of the synaptic number are discussed.

Comparison between Golgi stain and dye injection

Golgi stain is relatively simple, less expensive, and can be used in post-mortem samples of human brains in normal and diseased brains. The stain consists of an impregnation of brain tissue samples in chromate solutions, that after an incubation period, are exposed to silver nitrate, which reacts with the chromate and produces silver chromate crystals inside neurons. The result is the revealing of the neuronal features by silver deposits, which appear black, contrasted to a light background (Milatovic et al., 2010). Golgi stain can be adjusted and optimized for the needs of a project and the conditions of samples (Rosoklija et al., 2003). Experiments that use Golgi staining for morphometric analysis often use slices of $90\,\mu\mathrm{m}$ to $200\,\mu\mathrm{m}$ to achieve optimal resolution (Das et al., 2013; Tang et al., 2014). However in a volume in this range, distal dendrites cannot be followed. Blackstad et al. (2015) used consecutive slices to produce hand drawings of neurons with a camera lucida, and manually aligned, joined start and end cuts of dendrite segments of each slice. The drawings were then digitalized for reconstruction and morphometric analysis. Even though manual alignment of consecutive sections can be time consuming, it represents an alternative to use Golgi stain to reconstruct whole neurons.

Another alternative to be able to reconstruct whole neurons is dye injection. Dye injection consists of the injection of dyes into neurons to reveal morphological features (Claiborne et al., 1990) and can use fluorescence (Duan et al., 2002). This method can involve the use of the *Lucifer yellow* dye, a dye that when irradiated with blue light, forms a polymer that is electron-opaque (i.e. does not interfere with electron microscopy) (Meißlitzer-ruppitsch et al., 2009). Injection methods use sections of $400\,\mu\mathrm{m}$ that include a great extent

of the neuronal dendritic tree, and by performing injections in far apart neurons, there is no overlap as in Golgi stain preparation. Neurons can be traced with retrograde dyes to ensure better identification. Injection methods reveal only one selected neuron, thus layer identification requires additional staining procedures (Duan et al., 2002), as opposed to Golgi stain, which reveals several neurons that appear as bands of cortical layers and can be approximately distinguished (Rosoklija et al., 2014). Compared to Golgi stain, injection of dyes can have some difficulties. Animals must undergo surgery and have a survival time of 21 days to ensure retrograde transport for the identification of neurons. An example of a complete procedure for morphometric analysis of neurons using Lucifer yellow is explained in the article by Duan et al. (2002).

Interpretation of the synaptic number

The spine count can be interpreted in two ways: as number of excitatory synapses (input connectivity), or as processing/integration capacity. The first interpretation is as an estimate of connectivity. However the spine number does not include all cellular connectivity, since axon terminals (output connectivity) and inhibitory synapses are not included in the number. The second interpretation is as an approximation of processing and integration capacities, in accordance with two observations. Increases in spine density are correlated with increase in learning abilities (Moser et al., 1994,9; Globus et al., 1973), and individual spines have the ability to detect temporal changes in different inputs, acting as units of integration in a neuron (Shepherd, 1996; Yuste and Denk, 1995). Jacobs et al. (2001) studied different cortical regions arranged by integration hierarchy, as regions of low integration (primary cortices) and high integration (associative regions), and found that regions expected to have higher integration demands, had higher spine densities and more complex dendritic trees.

Both increased spine count and increased functional connectivity are correlated with increased processing. Studies in ageing have been useful to determine whether connectivity has implications with function. Using fMRI, the activity between the posterior cingulate cortex (PCC) and medial prefrontal cortex (mPFC) in subjects in advanced age was measured, excluding Alzheimer's disease effects. A comparison with young subjects showed that subjects with high functional connectivity regardless of age, had increased memory function. Therefore, increased functional connectivity is correlated with increased function. Given that increased spine density is also correlated with increased function (Moser et al., 1994,9; Globus et al., 1973), there is a possibility that cellular connectivity expressed as spine density could reflect similar results in functional connectivity. However, spine density count alone cannot tell whether a neuron with high spine count has efficient or noisy processing.

Several regions of the brain, including the prefrontal, temporal and parietal cortices undergo synaptic pruning during adolescence. A possible reason for the reduction in the synaptic levels is that an excess of synapses may decrease the signal-to-noise ratio. Synaptic pruning eliminates the excess of synapses, fine-tuning the connectivity into more specialized cortical networks that has a smaller number of highly efficient spines to do the same cognitive work (Blakemore, 2008). With age, cortical networks develop a signal-to-noise ratio higher with optimized numbers of spines. Following this argument, a small spine count cannot rule out the possibility of having highly efficient neural networks in par-

ticular brain regions that are implicated in highly complex intellectual functions (Laughlin and Sejnowski, 2003). Conversely, a computational model for synaptic pruning demonstrated that if synapses are Hebbian, the system benefits of higher spine density to ensure correct optimization of cortical networks by synaptic pruning, compared to networks with low densities before pruning (Chechik et al., 1998). Synaptic prunning is a developmental stage different from ongoing adaptation of neurons (synaptic plasticity), but the fact that brain networks with higher spine density have a more optimized synaptic prunning, suggests that a neuron with higher spine densities may have more flexibility.

The quantification of spine density is a sample of the excitatory input connectivity, and can be an estimate of the processing and integration capacities in the brain. A small spine count does not necessarily means less complexity, given that sparse networks can be highly efficient, but a high spine count could be an indicative of network flexibility. Spine count is a measure that can describe some aspects of neural architecture, such as integration and processing capacities, connectivity and flexibility, but that has to be interpreted taking in consideration the existence of different patterns of connectivity with different functional capacities.

2.6 Whole brain mapping

The brain is organized in a hierarchy of regions that are relatively independent, but that require connectivity to operate as a whole. Different regions have different anatomical patterns that can be observed in the cytoarchitecture and myeloarchitecture of the tissue. Korbinian Brodmann is one of the neurobiologists who pioneered in the study of cytoarchitecture in the brain, next to Cécile and Oskar Vogt. Brodmann studied the distribution of cell bodies in the cortex different patterns like layers and cortical columns, and the diversity of cell types, they attempted to parcellate the cerebral cortex in regions. Brodmann classified and defined brain areas according to differences in patterns of cytoarchitecture, and demonstrated that the human brain has an homologue structure in different mammals. Brodmann areas (BA) have an identification number and are often used in medicine and research to identify different functional brain systems. Even though, individual differences between subjects of the same species is a major problem of the cytoarchitectonic Brodmann's map, functional brain imaging did not invalidate it. Brain activity in different task conditions could be linked to the cytoarchitectonic characteristics of the regions activated (Zilles and Amunts, 2010).

A brain map or atlas can be the means to integrate different information. Different aspects of brain function and structure have started to be applied to describe the brain in terms of differences across regions. Receptor fingerprint is a term proposed by Zilles and Amunts (2009), who quantified and described the receptor population in brain slices, while keeping the anatomical organization of the sample. Zilles and Amunts (2009) suggest that by knowing the function of different receptors in a region and their distribution pattern, the possible functional implications of a whole region can be estimated. The Allen brain atlas is a database that integrates whole-brain genetic expression profiles in humans, rats and mice, in a spatial manner with high detail (Sunkin et al., 2013). An example of the utility of brain atlases like Allen Brain, is the study by Richiardi et al. (2015), who conducted a meta-analysis of genetic expression in the DMN and found increased expression of ion

channels implicated in synchronization. Different morphological and functional characteristics of the brain can be unified in brain atlases, and provide the possibility to explore brain function distributed in space.

3D atlases are an emerging tool that integrate spatial and functional information. Histological atlases in the human and the rat often consist of a series of slices with spatial indications, such as the Paxino's rat atlas. Even though navigation in such atlases is simplified by the use of coordinates, the separation of the brain in slices can become problematic. The banana problem is often used to illustrate the complications of dividing an object in 2D representations (Malandain et al., 2004). Suppose that a structure with the form of a banana is cut in slices is provided to a person who has never seen a banana before. There is a possibility that the round slices would be reconstructed in a straight cylinder form, instead of the curved shape of a banana. In the same way, brain structures have forms with slight variations that could be misunderstood.

The Paxino's atlas consists of a series of plates with drawings of anatomical patterns observed and interpreted from histological slices in the coronal plane. External or cranial landmarks are used in atlases like this to provide spatial reference in stereotaxic brain surgery. For example, one landmark is the distance between the point lambda and bregma. Brain areas can be approximately identified using stereotaxic atlases, and coordinates to be used should be adapted to the rat strain used due to variations in brain size and structural locations. However, such landmarks are not visible in brain imaging or cannot be measured when the skull is not available (Papp et al., 2014). Waxholm space coordinates are different from stereotaxic coordinates because the landmarks used are internal, not external. The origin point in Waxholm space is the anterior commisure, which can be identified directly in brain imaging or stained slices, without requiring the skull (Hawrylycz et al., 2011).

Brain atlases of different functional and morphological features, at different levels of organization (i.e. single neuron maps, cortical regions, whole brain) are a tool that can aid the study of the varying patterns in the brain. Given that the DMN is a brain-wide network that interacts with multiple systems, atlases of different characteristics could be an useful tool for the study of DMN function.

Chapter 3

Aims of the project

The default mode network (DMN) is a complex brain cortical network that underlies important aspects of everyday mental function, namely self-generated thought and mind wandering. One of the main purposes of research in the DMN is that explanation of spontaneous brain activities elicited in the DMN could improve the understanding of human thought processes like mind wandering, that have implications for the study of consciousness, philosophy of mind (Metzinger, 2013; Irving, 2015), and the nature of several mental diseases (Broyd et al., 2009). Mind wandering (Baars, 2010) and the DMN (Spreng and Grady, 2010) are correlated with thoughts about the self and social interaction, which has lead to the hypothesis that mind wandering has an important adaptive role for social behaviour (Andrews-Hanna et al., 2014), and that the DMN is part of the "social brain" (Mars et al., 2012). Smallwood et al. (2012) proposed the hypothesis that because social environments are complex and continuously changing, they posit high adaptation demands on the brain, and if the DMN is indeed a network implicated in social behaviour (Mars et al., 2012), cortical networks of the DMN may have high capacities for synaptic plasticity, processing as well as integration. Additionally, two regions of the DMN, the posterior parietal cortex (PCC) and anterior medial prefrontal cortex (aMPFC) have come to be known as regions in the brain specifically implicated with self-knowledge (Moran et al., 2013) and as functional hubs, regions in the brain with high connectivity, mediating information processing within the DMN (Andrews-Hanna et al., 2010b) and between several networks in the whole brain (Braga et al., 2013).

Even though fMRI connectivity studies of the DMN have been able to describe its functional patterns to a high degree (Andrews-Hanna et al., 2014), fMRI cannot describe explicitly local neural architecture (Grillner et al., 2005). There is a lack of studies of the DMN involving the neural architecture or the synaptic levels. Studies of functional connectivity (fMRI) describe the interdependency of different brain areas (Friston, 2011), while knowledge about spine density can determine the structural basis for this connectivity (Jacobs et al., 2001). The facts that processing demands can increase the spine density in basal dendrites of cortical circuits (Globus et al., 1973; Moser et al., 1997), and that individual spines can integrate inputs and be regarded as units of integration (Yuste and

Denk, 1995), suggest that the measure of spine density can be an approximation of processing and integration capacities, in addition to input connectivity. Spine count is an approximation to the input connectivity, and processing as well as integration capacity of the network. This would then represent a demonstration of the structural basis of current theories of connectivity and function in the DMN.

The first objective of the project is to describe DMN connectivity at the synaptic level, as this has not been elucidated yet. To do so, my aim is to quantify dendritic spine density in basal dendrites of layer V pyramidal neurons, in different regions of the DMN, compared to primary cortices and a task-activated network, the lateral cortical network (LCN) (Schwarz et al., 2013). Jacobs et al. (2001) has pointed out a problem with spine quantification in images: spines in the front and the back of the dendrite cannot be counted and therefore spine densities are underestimated. To solve this problem, I have chosen to use 3D modelling of dendrite segments to be able to identify spines in all angles of the dendrite segment with a standard computational tool, Imaris filament tracer, which has already been used in previous spine density analyses with Golgi stain (Tang et al., 2014).

Layer V pyramidal cells were selected as sample because they have connections with all layers in the neocortex (Burkhalter, 1989) and give rise to the main output of the brain to cortical and subcortical regions (Kandel, 2013a, 348). Therefore, layer V pyramidal neurons can be a sample of cortico-cortical and cortical-subcortical connectivity, and given that they are a major projection neuron, possibly have higher processing and integration demands. A measure of the spine number could be an estimate of how much processing as well as integration capacity layer V pyramidal neurons have to produce an output that communicates between different regions in the brain.

Basal dendrites of pyramidal neurons were selected for analysis. Basal dendrites have less spines than apical dendrites in the visual system (Larkman, 1991), but have shown to change in spine density with learning in the hippocampus, which is not observed in apical dendrites (Moser et al., 1994).

The general hypothesis is that the DMN high connectivity observed in fMRI has a structural basis that can be demonstrated in terms of spine density. Andrews-Hanna et al. (2007) showed that functional connectivity is correlated with function. Connectivity and memory function decrease with age, but subjects that had high functional connectivity despite age, had increased memory function. Therefore, degree of functional connectivity is correlated with function. Input connectivity to a neuron, measured by spine density, may be an estimate of integration capacity of the cortical region, while functional connectivity may be reflected in the levels of spine density as well, even though output connectivity is not being measured.

A secondary goal of the project is the integration of the DMN in an atlas of the rat brain. Three-dimensional atlases are an emerging tool that aid the localization and understanding of the anatomy of the bran. Localization of brain networks can aid in understanding possible relationships amongst them, and three-dimensional atlases provide an useful tool in this regard. The DMN is a network distributed across much of the brain, and its localization in the rat has been described by fMRI (Lu et al., 2012), which makes it possible to include in an atlas. This project aims to include the DMN in a commonly used 3D rat brain atlas (Papp et al., 2014) and to use the atlas to represent findings in regional spine density.

3.1 The DMN compared to primary cortices

The fourteen cortical regions of the rat brain considered in the quantification were classified according to Benson's hierarchical organization (Benson, 1993). In this classification, *primary cortex* is the first processing step of sensory stimulus, or the final output of motor action, *unimodal cortex* integrates information of a single modality to produce mental representations of a single modality, *heteromodal cortex* integrates mental representations of different modalities and memories to produce or update multimodal internal representations, and *supramodal cortex* deals with abstract representations independent of modality (e.g. where and how). Additionaly, a fifth class suggested by Mesulam et al. (1998), the *transmodal cortex*, a region that is rich in connectivity and acts as functional hub, selecting information from all networks distributed in the whole brain. According to both classification schemas, cortical regions were designated as low integrative (primary and unimodal) and high integrative (heteromodal, supramodal, transmodal), in a similar fashion as Jacobs et al. (2001) and Braga et al. (2013) did in their studies. According to the hypothesis by Smallwood et al. (2012), if the DMN is indeed a network implicated in social behaviour, given that social environments are complex and change constantly, the DMN may be subject to high adaptation, processing and integration demands. Therefore, assuming a hierarchical organization in the brain, the first hypothesis is that neural cortical circuits in the DMN may have more connectivity (i.e. higher spine density) than primary cortices due to high integration demands.

Research Question 1: Is dendritic spine density higher in the DMN compared to primary cortices?

Hypothesis 1: The DMN may have significantly higher spine density than the primary cortices of the modalities sensory (S1), visual (V1) and motor (M1).

3.2 The DMN compared to the LCN

The lateral cortical network (LCN) is a task-activated network that is not affected by changes in learning paradigms, as opposed to the DMN (Li et al., 2015). The activity in the DMN can be modulated with changes in learning paradigms and extinction, which suggests that the DMN may require high spine density to be able to change. Neuronal networks with higher spine density can have a more optimal synaptic pruning (Chechik et al., 1998). Even though synaptic pruning is a developmental stage that is different from ongoing synaptic plasticity, but the fact that higher spine density ensures a more optimal refinement of neuronal networks, suggests that a high spine density could provide more flexibility to a network.

Research Question 2: Is dendritic spine density different in the default mode network (DMN) compared to the lateral cortical network (LCN)?

Hypothesis 2: The DMN may have high spine density in order to have more flexibility to adapt to changes in the environment.

3.3 Multiple comparisons between regions of interest

The DMN is an heterogeneous network that has regions of different hierarchies arranged in subsystems (Andrews-Hanna et al., 2010b). All the cortical regions analysed were classified in different groups according to Benson's hierarchical classification. Therefore, one may expect to find differences between regions. Multiple comparisons between different DMN regions may show differences within the default mode network. Two regions of the human DMN, the posterior cingulate cortex (PCC) and the anterior medial prefrontal cortex (aMPFC), have been described as functional hubs or regions with high connectivity in the human (Andrews-Hanna et al., 2010b), (Braga et al., 2013). Three candidate regions were chosen as possible hubs in the rat: Detected in fMRI, the retrosplenial and the prefrontal cortices (Lu et al., 2012), and the cingulate cortex as a homologue in cytoarchitecture (Vogt and Paxinos, 2014). The homologue functional hubs in the rat brain have high functional connectivity that may be structurally supported by cortical neural networks with high spine density.

Research Question 3: Is dendritic spine density different between regions of the DMN?

Hypothesis 3: The rat DMN is constituted by different kinds of cortices, from primary to associative cortices (Lu et al., 2012). High integration regions (e.g. temporal association, cingulate, retrosplenial and prefrontal cortices) may have higher spine density than low integration regions within the network (e.g. auditory cortex).

Research Question 4: Is dendritic spine density significantly higher in the functional hubs of the DMN?

Hypothesis 4: The homologue functional hubs in the rat DMN may have higher spine density than other regions within the DMN, specially the low integration regions.

Chapter 4

Methodology

Rats were selected as experimental animal due to the accessibility of whole brains and the possibility to process tissue immediately after sacrifice. The rat has homologue networks to the DMN and provides a sample with fewer variations and is easy to process with Golgi stain. The sample consisted of 5 rats. All subjects were female, 100 days old and of the same strain (Dark Agouti). All rats lived at the animal facility in a cage, without any additional treatment. After sacrifice with guillotine under anaesthesia, rat brains were extracted and immersed in impregnation solutions A and B of the FD Neurotechnologies Rapid Golgi stain kit. After incubation of 3 weeks, the whole brain was embedded in agarose blocks, sliced in the vibratome at a thickness of $100\,\mu\mathrm{m}$, and immediately mounted on superfrost glass slides, lying flat. The slice thickness was selected after several trials to obtain the best resolution results. All the cortex was sliced, maintaining the order of slicing identified per glass slide. If a brain slice was damaged and unable to provide information, its corresponding position was registered on the glass slide. The brain slices were stained lying flat with solutions D and E of the FD Neurotechnologies Rapid Golgi stain kit, in aqueous solution. After washing with MilliQ water, slices were mounted with glycerol gelatin and cover slipped. The staining protocol was a modified version of the manufacturer instructions. The modifications are explained in A.

A set of ROIs were identified in the brains, using the Paxinos stereotaxic rat brain atlas, and stereotaxic coordinates were converted to Waxholm space. The coordinates of the ROIs were tailored for the size of each experimental rat brain.

Imaging was performed with a Leica Microscope (DM5500B) with motorized axes control and the imaging software Leica Application Suite X (LAS X). Cortical layers were approximately identified by counting the layers. Nissl staining to reveal cortical layers could not be used to avoid excessive background staining that could interfere with the three-dimensional reconstruction of dendrite segments. LV pyramidal neurons were identified by their characteristic triangular shape and dendrite segments that belonged to the cell were imaged. Only basal dendrites were imaged since it is easier to follow basal dendrites with the soma as starting point. Dendritic segments of $60\,\mu\mathrm{m}$ to $80\,\mu\mathrm{m}$ were imaged in z-stacks with $0.1\,\mu\mathrm{m}$ step size. Resolution was selected as recommended by

Rodriguez et al. (2008) in the documentation of their software for spine detection. Images were pre-processed with color inversion (black neurons become white) and deconvolution, both processes performed by the microscope software LAS X. The z-stacks were processed by Imaris to produce 3D reconstructions and to quantify spine density along the dendrite. 3D reconstructions have the benefit of including spines at different angles of the dendrite. Imaris was selected because of the possibility of having technical support and guidance, as well as the user-friendly interface. The resulting data was represented on a three-dimensional rat brain atlas with the corresponding ROI coordinates in Waxholm space. The ROI were drawn in ITK-SNAP, using as a base the rat brain map by Papp et al. (2014) and the adapted coordinates from the Paxinos rat brain atlas (Paxinos and Watson, 2009). The drawing process is described with detail in appendix C.

4.1 Identification of regions of interest

Several cortical regions of interest (ROI) were selected for the analysis, including regions that belong to the DMN, primary cortices and the lateral cortical network (LCN). All regions were classified by Benson's hierarchy, as proposed in (Benson, 1993). Primary regions (S1, V1, M1), were grouped as "primary cortex´´, while the other regions were grouped by network (DMN and LCN). All regions were designated as low integration region for primary and unimodal cortices and high integration region for heteromodal, supramodal as suggested by Jacobs et al. (2001). The hippocampal formation is part of the DMN in humans Andrews-Hanna et al. (2014) and rats (Lu et al., 2012), but the hippocampus is complex in terms of molecular biology and anatomy, and the cytoarchitecture is different from the neocortex (i.e. there are no layer V pyramidal neurons). In order to reduce variability, the hippocampus was not considered in this study.

The brain areas selected for the DMN were the regions identified by (Sierakowiak et al., 2015) and Lu et al. (2012), and the selected regions of the LCN were the ones identified by Schwarz et al. (2013). The Paxinos rat brain atlas (Paxinos and Watson, 2009) was used to confirm the stereotaxic coordinates of all regions of interest, and internal landmarks described by (Sergejeva et al., 2015) were used for rat size adjustment and prediction of coordinates per rat brain . The process is described in detail in the appendix B. See table 4.3 for an overview of regions of interest.

4.2 Imaging of dendrite segments

For each region of interest, 5 LV pyramidal neurons were selected, and for each neuron, a dendrite segment of $60\,\mu\mathrm{m}$ to $80\,\mu\mathrm{m}$ was imaged. The full description of the criteria can be found in the appendix D.

Images of the selected dendrites were obtained with light microscope (Leica) with motorized control, to obtain z-stacks with a step of $0.1\,\mu\mathrm{m}$, with 100X oil immersion objective. Aperture was set to 32 and light intensity as high as possible to eliminate the background, but not too high to avoid blurring the spines. After acquisition, images were preprocessed with color inversion and deconvolution using the same software (LAS X). Details on image preprocessing methods are described in the appendix D.

Table 4.1: Methodology summarized in a list of steps. Each step corresponds to one of the main sample processes in the methodology and contain several details that are described in the text or in the corresponding appendix. Steps 1 to 4 are widely explained in A, including the modifications done to the FD neurotechnologies kit protocol; steps 5 to 8 are described in 4.1 and in detail in B and C; steps 9 to 11 are described in 4.2 and 4.3, and in detail in D. The last step is described in 4.4.

#	Step	Description
1	Sampling	Anesthesia, sacrifice and extraction of whole rat brains.
2	Golgi stain impregnation	Impregnation of the brains in solution A (FD NeutoTechnologies).
3	Embedding, slicing	Embedding in agarose and slicing of the whole brain in vibratome
4	Staining and Mounting	Immediately after slicing, each slice is stained lying flat and then mounted on glass slides with glycerol gelatine.
5	ROI coordinate prediction	Prediction of coordinates using internal landmarks (Sergejeva et al., 2015).
6	ROI identification	Identification using the Paxinos rat brain atlas (Paxinos and Watson, 2009)
8	ROI drawing	Drawing of ROI in a three-dimensional atlas using ITK-SNAP.
9	Imaging	Identification of LV pyramidal neurons, imaging of dendrite segments in z-stacks, color inversion and deblurring by deconvolution.
10	3D reconstruction	In Imaris, semiautomatic reconstruction of dendrite segments with spines, and manual removal of false positives.
11	Spine quantification	Registration of spine count and dendrite length per reconstructed dendrite segment. Quantification of spine density.
12	Statistical analysis	Statistical description and testing of the sample spine density means.

Table 4.2: List of materials, equipment and software used in the experiments.

Purpose	**Tool**
Golgi stain	Rat specimens
	Razor blade
	FD NeutoTechnologies FD Rapid golgiStain kit, PK-401
	$50\,\mathrm{mL}$ plastic tubes with lid
	Disposable pasteur pippetes, plastic and glass
	Coverslips
	Tissue paper
	Aluminum foil
	Superglue (water resistant, Roit)
	MilliQ water
	Brush with natural hair
Embedding	Agarose (Sigma, Type 1, Catalog # A6013 or A0169, no other)
	$NaIO_4$ (Sigma); protect from light, use in chemical fume hood
	Phosphate buffer (pH 7.4)
	Borax solution 0.5 M $Na_4B_4O_7 * 10H_2O$, Sigma,
	Boric Acid Solution 0.05 M H_3BO_3, Sigma
	$NaBH_4$ (Sigma, Catalog # 452882) use in chemical fume hood
	Stir plate and stir magnets
	Molds (VWR)
	Superfrost microscope glass slides
	MilliQ water
Equipment	Vibratome
	Light Microscope Leica, objective 100X
	Leica Motor control for X, Y and Z axes
	Wacom Pen Tablet for digital drawing
Software	Imaris version 8.1 with Filament Tracer
	ITK-SNAP version 3.0
	Leica Application Suite X
	IBM SPSS Statistics 21 for statistical tests
	Origin Pro 2015 for chart drawing

Table 4.3: Regions of interest grouped by network or Benson hierarchy and designated as low integration region for primary and unimodal cortices and high integration region for heteromodal, supramodal as suggested by Jacobs et al. (2001). Clasification includes integration class and if applicable related network (i.e. Network/Integrative class). All the regions included in this study are listed in the table, including the abbreviation used, the region category by which each region was grouped, the landmark used per region for coordinate adjustment per rat, and the Waxholm space coordinate range where the region is found according to the Paxinos rat atlas (Paxinos and Watson, 2009). Coordinates were converted from stereotaxic coordinates of the Paxinos rat brain atlas to Waxholm space (Johnson et al., 2010) using the anterior commisure as internal landmark of the origin.

ROI	Classification	Landmark	Coordinate range (mm)
Frontal association cortex (FrA)	DMN/High	FM1	6.4 – 5.7
Medial orbital cortex (MOrb)	DMN/High	FM1	5.7 – 4.1
Prelimbic cortex (PrL)	DMN/High	FM1	4.5 – 2.6
Cingulate cortex (Cing)	DMN/High	FM1	3.3 – -1.28
Retrosplenial cortex (Retro)	DMN/High	HM1	-1.67 – -6.7
Auditory cortex (Aud)	DMN/Low	IP	-2.8 – -6.7
Temporal association cortex (TeA)	DMN/High	IP	-4.8 – -7.14
Posterior parietal cortex (PtP)	DMN/High	IP	-4.2 – -5.6
Primary sensory cortex (S1)	Primary cortex/Low	FM1	3.0 – -0.9
Primary motor cortex (M1)	Primary cortex/Low	FM1	4.5 – 0.3
Primary visual cortex (V1)	Primary cortex/Low	IP	-4.4 – -7.1
Anterior secondary motor cortex (aM2)	LCN/High	FM1	5.2 – 2.5
Jaw region of the primary sensory cortex (S1J)	LCN/Low	FM1	3.3 – 2.5
Insular cortex (Ins)	LCN/High	FM1	4.4 – 2.6

4.3 3D reconstruction and spine density quantification in Imaris

In Imaris, z-stacks were used to build a maximum intensity projection that resembles the 3D structure of the dendrite segment. Then, using 3D editing tools, the dendrite of interest was isolated from the image. With the filament tracer module, the main dendrite was traced with the semi-manual tool Autodepth, and dendritic spines were traced with automatic detection. False positives were removed manually. Only spines that were visibly present in the original images where kept to avoid overestimation of spine density. Details on the 3D reconstruction and quantification are in the appendix D. Dendrite segment length, surface area and spine number were obtained from the 3D reconstructions. Spine density per area was quantified from the extracted data.

4.4 Statistical methods

At least 350 neurons should be imaged in total. The power of the test should be calculated for the sample size. Outliers should be detected using labelling rule with g = 2.2 as suggested by Hoaglin and Iglewicz (1987). In this case, more than two independent groups are compared, and each neuron was considered independent even within the same brain. Thus, ANOVA should be performed with the adequate post-hoc tests (Milatovic et al., 2010). Assumptions for ANOVA should be tested, including normality (Levene's test), and equal variances (Shaphiro-Wilk's test). Whether the sample has equal variances is a deciding factor for the choice of post-hoc tests.

Chapter 5

Results

In this study, rat brain slices were processed with Golgi stain to reveal the architecture of neurons, including dendrites and spines, and then the spine density of dendrite segments was quantified. Figure 5.1 shows a photography of one stained slice with magnification of 4X with the structure of several neurons revealed with black silver nitrate crystals on a white background. Spine densities in different regions of the Default Mode Network (DMN) was compared to the lateral cortical network (LCN) and the primary sensory, visual and motor cortices. A summary of the spine densities of all regions with statistical descriptors are found in table 5.1. The DMN is the network involved in mind wandering. Activity in the DMN is anticorrelated with the LCN in rats, and is thought to have processing and integration demands higher than primary cortices. A total of 14 regions were selected and classified as DMN, LCN or primary cortex, and the slice used for each sampling point was registered to calculate the coordinate in Waxholm space (fig. 5.2). All the cortical regions were identified in the Paxinos Waxholm atlas (Paxinos and Watson, 2009) and then with the stereotaxic coordinates provided by the atlas, calculated their Waxholm space coordinates and integrated to the 3D rat brain atlas by Papp et al. (2014). The regions together in different angles are shown in fig. 5.3 and classified by group in fig. 5.4.

All statistical tests were performed with IBM SPSS statistics 21. Spine densities per region were compared between rats and no significant differences were detected, therefore each measurement was assumed to be independent and the data were pooled across rats. Welch-ANOVA and Games-Howell tests were selected because the sample had normality and different variances. Using outlier labelling rule with g = 2.2 as suggested by (Hoaglin and Iglewicz, 1987), no outliers were detected. The calculated power of the test for a sample size of 200 was 0.9993, which indicates that statistical tests had a high probability to correctly detect differences.

Regions were compared both individually and grouped, using the mean spine density for comparing single regions, and the mean of all the individual mean spine densities for each region in the group when comparing groups. This way, three different comparison schemes were used: *region-to-region*, *region-to-group*, and *group-to-group*. In the region-to-group comparisons, the group average was adjusted. The adjustments consisted in the

exclusion of specific individual regions for the calculation of the group average. The regions included in the region-to-group comparisons are shown in table 5.2.

Figure 5.5 shows a group-to-group comparison. Error bars with standard error of the mean (SEM) were used in the figures because this kind of bars are shorter and offer a display that allows a clearer visual comparison. Values reported in the text are mean with standard deviation (mean$\pm SD$). No significant differences between the DMN, LCN and primary cortices could be detected. However, in absolute values, the DMN was higher than the LCN, and the primary cortices had the lowest values (fig. 5.5).

Region-to-region comparisons are shown in figure 5.6, where significant differences were detected between some individual regions. The cingulate cortex had the highest spine density (1.64 ± 0.55 spine/$1\,\mu\mathrm{m}$). The cingulate cortex had significant differences with three regions. Temporal association cortex (TeA), a region within the DMN, had significantly less density ((1.07 ± 0.29 spine/$1\,\mu\mathrm{m}$), p = 0.020) of all. Regions of the primary sensory cortex also had significantly lower density, both when samples were from the jaw region (S1J, part of DAN, 1.03 ± 0.21 spine/$1\,\mu\mathrm{m}$, p = 0.008), or the primary cortex in general (S1, 1.05 ± 0.43 spine/$1\,\mu\mathrm{m}$, p = 0.044). The rest of the region-to-region comparisons did not lead to significant differences, but showed a tendency of DMN regions to have higher density as they become closer to the cingulate cortex, being the closest the prelimbic cortex (PrL, 1.44 ± 0.40 spine/$1\,\mu\mathrm{m}$) and the retrosplenial cortex (Retro, 1.41 ± 0.37 spine/$1\,\mu\mathrm{m}$). The 3D rat brain atlas with the included relevant regions was used to map the mean spine density per region with a gradient of color from highest (red) to lowest (dark blue), fig. 5.9.

The cingulate cortex had the highest spine density and region-to-region comparisons lead to significant differences. Then, the cingulate cortex was included in a region-to-group comparison. The comparison in fig. 5.7 showed significant differences (DMN, LCN and primary). However, as seen in figure 5.8, there are no significant differences when the group mean was adjusted (DMN', Primary', LCN') by excluding the regions that already had shown significance (TeA, S1, S1J). Table 5.2 shows the regions included for region-to-group comparisons.

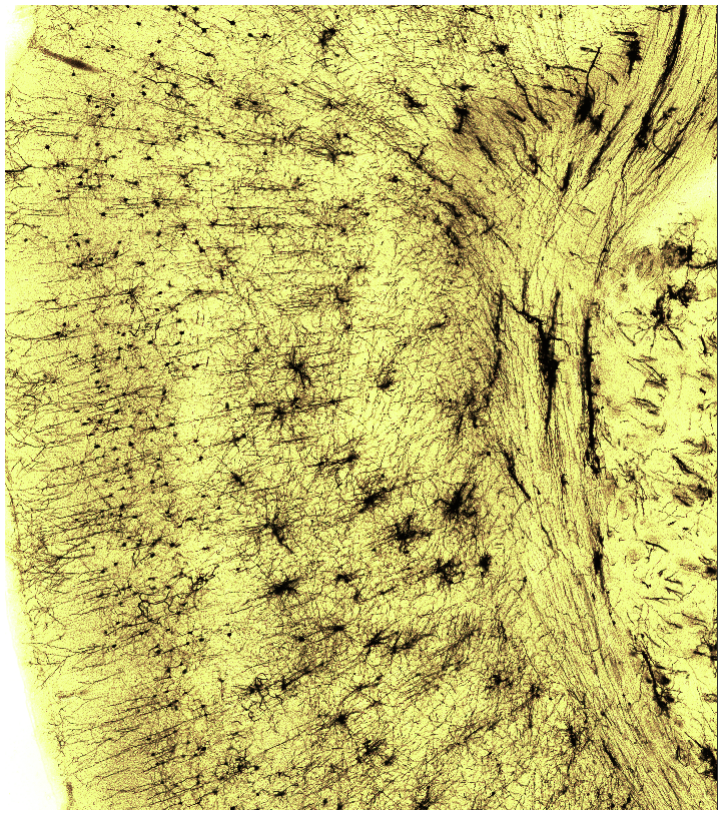

Figure 5.1: Photography of a brain slice processed with Golgi stain in magnification of 4X.

Table 5.1: Spine density in all the regions of interest analysed. The class of each region corresponds to the default mode network (DMN), primary cortices and the lateral cortical network (LCN). The statistical descriptors are given, including the standard deviation and 95% confidence intervals.

ROI	Class	Average	SD	95% CI
Frontal association cortex (FrA)	DMN	1.08	0.38	0.81 - 1.36
Medial orbital cortex (MOrb)	DMN	1.31	0.25	1.10 - 1.52
Prelimbic cortex (PrL)	DMN	1.44	0.40	1.20 - 1.68
Cingulate cortex (Cing)	DMN	1.64	0.55	1.38 - 1.90
Retrosplenial cortex (Retro)	DMN	1.41	0.37	1.17 - 1.65
Auditory cortex (Aud)	DMN	1.14	0.31	0.98 - 1.30
Temporal association cortex (TeA)	DMN	1.14	0.29	0.93 - 1.21
Posterior parietal cortex (PtP)	DMN	1.07	0.43	1.03 - 1.45
Primary sensory cortex (S1)	Primary	1.24	0.43	0.83 - 1.27
Primary motor cortex (M1)	Primary	1.05	0.32	0.94 - 1.38
Primary visual cortex (V1)	Primary	1.16	0.31	1.16 - 1.52
Anterior secondary motor cortex (aM2)	LCN	1.34	0.46	1.16 - 1.17
Jaw region of the primary sensory cortex (S1J)	LCN	1.47	0.21	0.92 - 1.14
Insular cortex (Ins)	LCN	1.03	0.29	1.18 - 1.54
Total		1.26	0.41	1.20 - 1.32

Table 5.2: Overview of comparisons and regions included. Three comparison schemes were made: Region-to-region, group-to-group and region-to-group. In the region-to-group comparisons, the group average was adjusted, denoted by a apostrophe added to the name of the group (DMN', Primary', LCN'). The adjustments consisted in the exclusion of specific individual regions for the calculation of the group average. The regions included in the region-to-group comparisons are shown in table

Comparison scheme	Group	Regions
region-to-region	–	*All regions included*
group-to-group	DMN	FrA
		MOrb
		PrL
		Cing
		Retro
		Aud
		TeA
		PtP
	Primary	S1
		M1
		V1
	LCN	aM2
		S1J
		Ins
region-to-group	–	Cing
	DMN'	FrA
		MOrb
		PrL
		Cing
		Retro
		Aud
		PtP
	Primary'	M1
		V1
	LCN'	aM2
		Ins

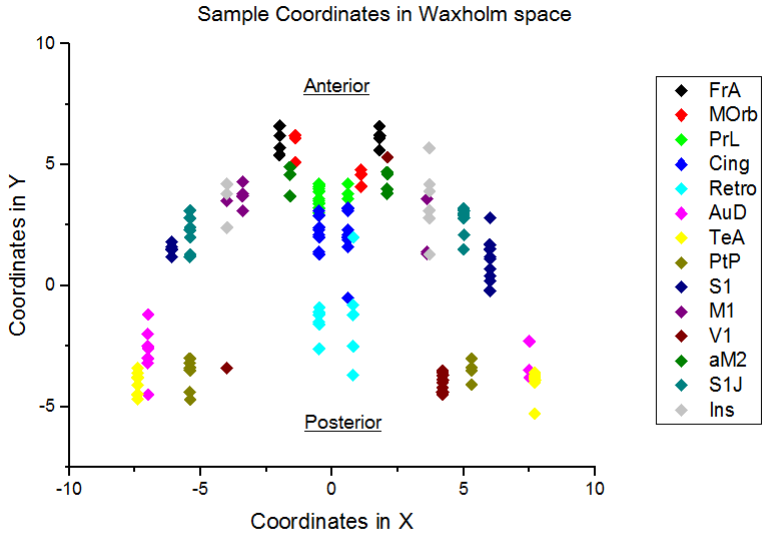

Figure 5.2: Coordinates in Waxholm space of each sampling point. The rat brains were sliced completely and the position of each slice, relative from other slices, was registered. The slice position of each sampling point was registered and then the Waxholm space coordinate of each sampling point was registered. In this graph, the calculated coordinates of all sampling points is plotted together. Only one neuron was sampled per point.

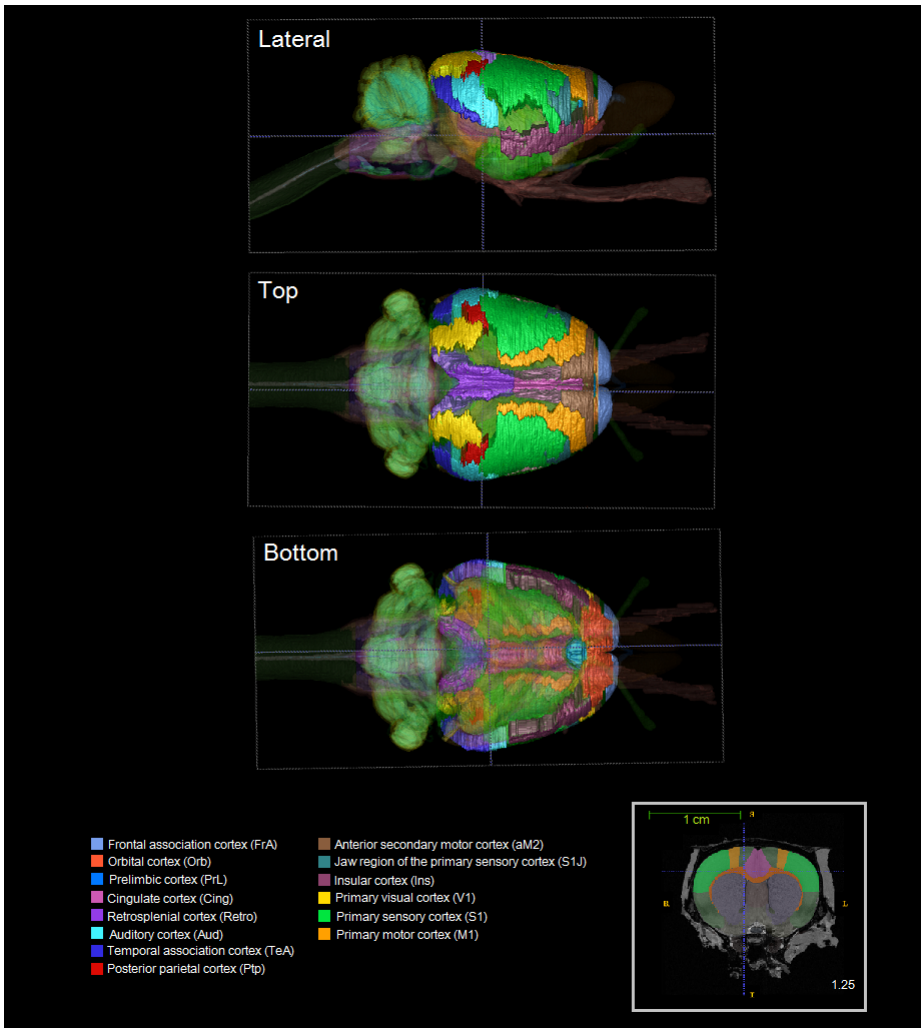

Figure 5.3: Localization of all cortical regions of interest in a 3D rat brain atlas. Each cortical region was identified in the Paxino's rat brain atlas (Paxinos and Watson, 2009) and with the provided stereotaxic coordinates, the Waxholm space coordinates were calculated to identify the corresponding localization of the regions in the 3D rat brain atlas by Papp et al. (2014). Then, each region was 3D modelled and integrated in the 3D rat brain atlas. In this figure, each region is labelled with a color. The slice in the right bottom corner shows an approximate scale of the map and the number in the bottom right corner of the slice is the Waxholm coordinate in millimetres.

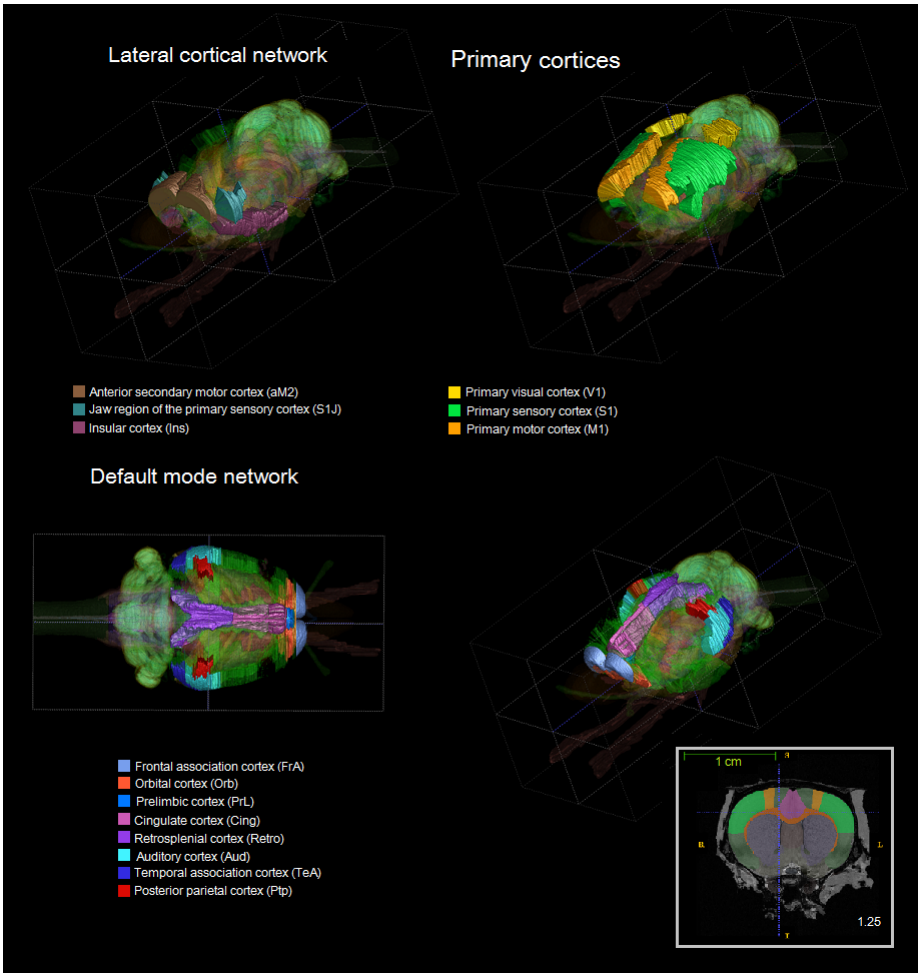

Figure 5.4: Localization of the DMN, LCN and sampled primary cortices in the rat brain. The slice in the right bottom corner shows an approximate scale of the map and the number in the bottom right corner of the slice is the Waxholm coordinate in millimetres.

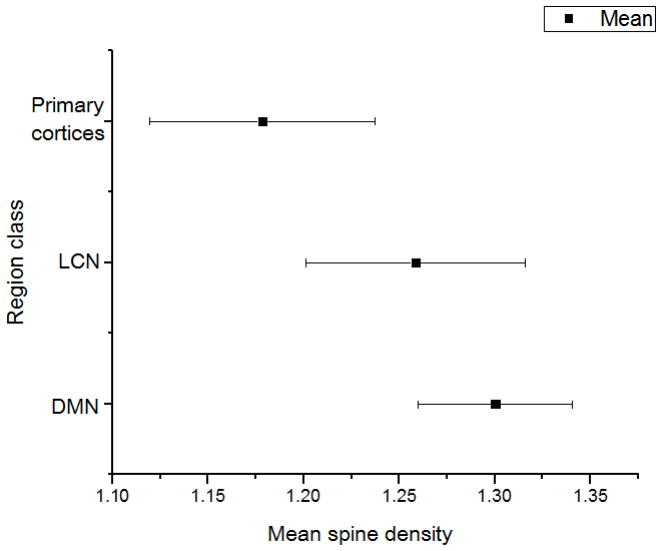

Figure 5.5: Group-to-group comparison of the mean spine density. Default mode network (DMN), lateral cortical network (LCN) and primary cortices. Error bars show standard error. No significant differences could be detected.

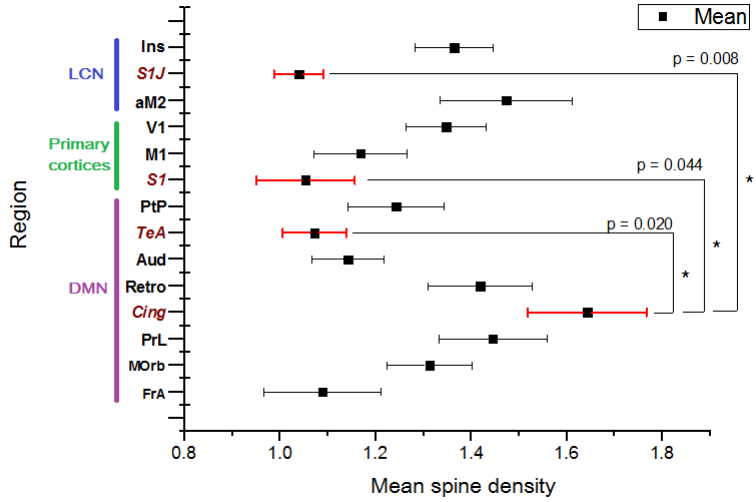

Figure 5.6: The mean spine density of each region of interest. Multiple comparisons detected significantly higher spine density in the cingulate cortex, compared to the temporal association cortex (TeA), primary sensory cortex (S1) and the jaw region of the primary sensory cortex (S1J). The cingulate cortex had the highest spine density. Error bars stand for the standard error of the mean.

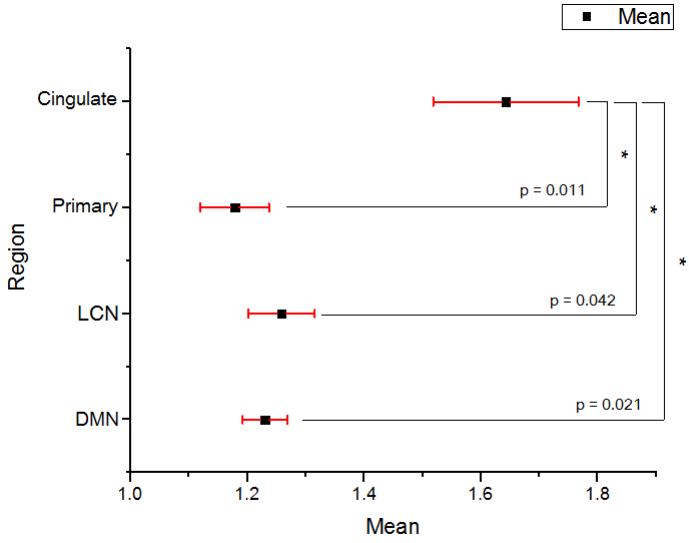

Figure 5.7: Given that the average spine density in the cingulate cortex was the highest of all regions, the cingulate cortex was compared in a scheme of region-to-group. The DMN average was adjusted by excluding the cingulate cortex. The spine density in the cingulate cortex showed significantly higher levels when compared to all groups. LCN: Lateral cortical network, DMN: Default mode network, Primary cortices: V1, S1, M1. Error bars stand for the standard error of the mean.

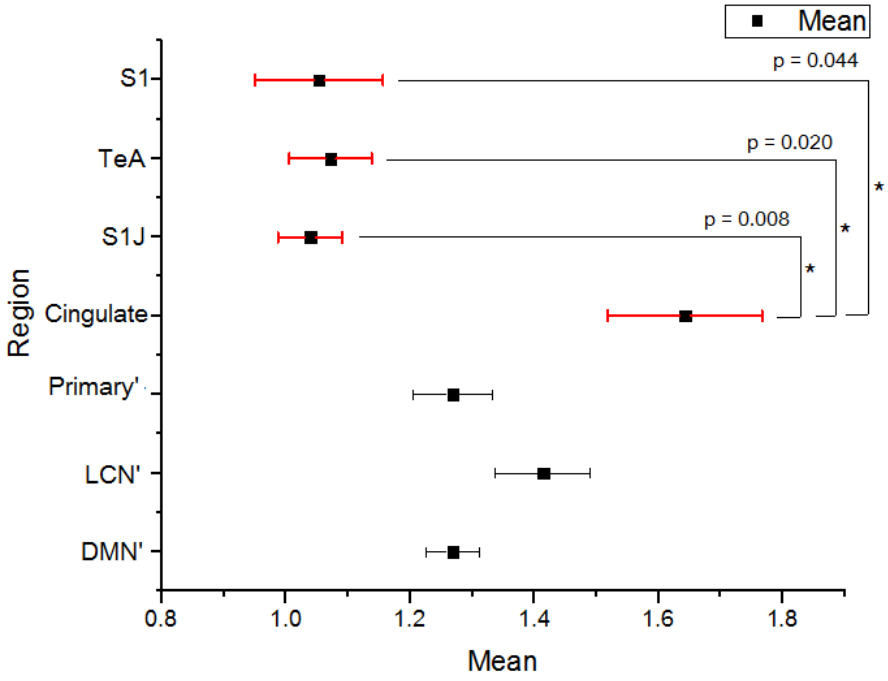

Figure 5.8: Comparisons between the cingulate cortex and the region classes DMN, LCN, primary cortices, and single regions TeA, S1, S1J. Regions that previously lead to significant differences in multiple comparisons (TeA, S1, S1J) are included in this figure, but were not part of the calculation of the mean spine density of groups (DMN', LCN', Primary'). The regions used for the average adjustment are listed in table 5.2

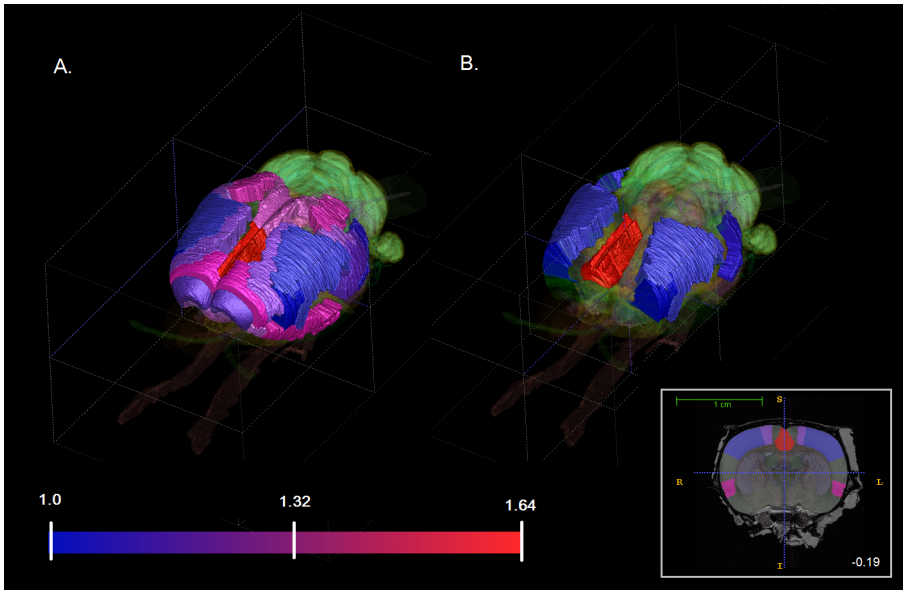

Figure 5.9: The 3D rat brain atlas with the integrated regions of interest showing a map of spine density gradient. A. Spine density brain map of all brain regions. B. Spine density brain map of the brain regions that presented significant differences. The color gradient represents the spine density, from lower levels in blue, to higher levels in red. The cingulate cortex has the highest level in the color gradient, while S1, S1J and TeA are the regions with the lowest densities. The slice in the right bottom corner shows an approximate scale of the map and the number in the bottom right corner of the slice is the Waxholm coordinate in millimetres.

Chapter 6

Discussion

Golgi stain of whole rat brains combined with 3D modelling of dendrite segments enabled the quantification of spine density in regions of the default mode network (DMN), primary cortices (S1, V1 and M1) and the lateral cortical network (LCN). The DMN is implicated in internal cognition and social behaviour, and because social environments are complex and continuously changing, it has been hypothesized that the DMN is exposed to high adaptation and integration demands (Smallwood et al., 2012), thus it would be expected to have high processing and integration capacities. Spine density increases with processing demands in basal dendrites of neurons in cortical circuits (Globus et al., 1973; Moser et al., 1997). The DMN also has regions with high functional connectivity, called *functional hubs* (Andrews-Hanna et al., 2010b) and functional connectivity is correlated with increased memory function in the DMN (Andrews-Hanna et al., 2007). All layers in the neocortex have connections with principal neurons of the layer V (Burkhalter, 1989), and the layer V pyramidal neurons give rise to the main output of the brain (Kandel, 2013a, 348). A measure of the spine number is an estimate of how much integration capacity layer V pyramidal neurons have to produce an output that communicates between different regions in the brain. In this project, I have investigated a hypothesis proposing that a network expected to have high processing demands and high integration capacity may have high spine density.

Multiple comparisons between regions were carried out to find out whether the DMN regions can be distinguished by their spine densities. The results point to the cingulate cortex, which had the highest spine density of all regions and significantly higher than important regions like S1, S1J and TeA.

6.1 Differences between the DMN, primary cortices and the LCN

The DMN was compared to regions of the rat brain that presumably have different functions and activation patterns. Two candidate regions were chosen: primary cortices and a

task-activated network, the lateral cortical network (LCN). Primary cortical regions were assumed to have low integration capacities, assuming Benson's hierarchical organization (Benson, 1993). In the rat, the LCN is a task-activated network, anticorrelated with the DMN (Schwarz et al., 2013). The LCN is unaffected by resting states and shows no task-dependent modulation (Li et al., 2015). Selecting the cingulate cortex as a representative region of the DMN, the results supported the hypothesis that high integration regions would have high spine density: the cingulate cortex had significantly higher spine density than S1 (primary cortices group), and than the S1J (part of LCN). In accordance with Benson's hierarchy organization, layer V pyramidal neurons in the cingulate cortex had a higher spine density, meaning that these neurons have high processing and integration capacities.

Rats that live in an enriched environment have been found to increase in spine density in some studies (Globus et al., 1973), and to improve learning capacities (Moser et al., 1997), thus an enriched environment may have higher processing demands that could develop the DMN, specially if the environment is continuously changing. The rats included in this study lived in a cage without any additional treatment in a fairly simple and constant environment. Spine density quantification has been applied to the study of synaptic plasticity. Mychasiuk et al. (2013) quantified spines before and after a sensory stimulation in rat pups, and found a difference in spine density, which they termed anatomical plasticity. Differences in the number of spines between different treatments could be a structural demonstration of synaptic plasticity. Morphometric analysis of rats exposed to different demanding situations, possibly could detect more differences between cortical regions and shed light into the adaptability of the neural cortical circuits of the DMN.

The layer III pyramidal neurons are another candidate for the estimation of integration capacity and connectivity. Pyramidal neurons in layer III have connections with neurons in the same region(intercortical connections) and other cortical regions (corticocortical connections), therefore a future study of layer III pyramidal neurons with morphometric analysis could be a measure of the connectivity *within* regions (Kandel, 2013a, 348). The measure of spine density in diverse layers could be useful to detect differences in connectivity between and within cortical regions.

The lack of significant differences with the remaining regions of the DMN when compared to the other regions (i.e. primary and LCN), motivated me to compare the average spine density per region group against the cingulate cortex and the regions with significance individually (fig. 5.8). This comparison did not lead to more significant differences, but showed an unexpected trend. The average spine density of the remaining regions of the DMN were close to the averaged primary cortices, and slightly smaller than the averaged LCN. This tendency indicated that the DMN without the cingulate cortex could not be significantly distinguished from primary cortices and the LCN.

6.2 Differences between regions of the DMN

The DMN consist of several local cortical regions of different hierarchy (Benson, 1993; Andrews-Hanna et al., 2010b; Lu et al., 2012), thus it can be expected to have differences in connectivity levels. In line with the hypothesis that high integration regions (i.e. heteromodal, supramodal and transmodal cortices) may have higher densities due to high

processing demands, the cingulate showed the highest spine density even within the DMN. However, the temporal association cortex (TeA) is another high integration region of the DMN that had significantly less spines than the cingulate cortex. The significant difference between two high integration regions indicates that regions of the DMN have varying spine densities, regardless of cortical hierarchy. Nevertheless, the cingulate cortex may have more connectivity than the TeA.

In the rat network described by Lu et al. (2012), the TeA has weak correlations within the network compared to the cingulate (fig. 2.4). In humans, the posterior cingulate cortex (PCC) is a transmodal association cortex (Braga et al., 2013) and is one of the functional hubs of the DMN (Andrews-Hanna et al., 2010b), while the TeA is an heteromodal association cortex. The human PCC has high metabolic demands, dense wide spread connectivity and signal echoes from multiple brain networks. As a cortical hub, the PCC integrates the information processed in distinct functional brain networks about a constantly changing environment, yielding flexibility to the individual for adaptation (Leech et al., 2012). Additionally, the cingulate cortex is implicated in the initiation of goal-directed, motivation and affective behaviours (Devinsky et al., 1995), in self-knowledge and social behaviour (Moran et al., 2013), two functions that have been proposed for the DMN and mind wandering (Baars, 2010), and that require processing by limbic system (Devinsky et al., 1995) and have implications for depression (Sheline et al., 2009). The temporal association cortex is an heteromodal association cortex that integrates multisensory information and has a topographic organization of neurons that are responsive to features of different sensory modalities (Dahl et al., 2009). The TeA has connections with the primary visual cortex (V1), secondary visual (V2) and auditory (Aud2) cortices, and subcortical regions such as the caudate-putamen and the amygdala. The connectivity of the TeA has been suggested to be a pathway of visual/auditory integration that converges in the limbic system through the perirhinal cortex and the amygdala (Vaudano et al., 1991).

The cingulate cortex had significantly higher spine density than the TeA. This result is consistent with the fact that the human PCC is a transmodal cortical region (i.e. a brain-wide functional hub) that requires high connectivity, and processing as well as integration capacity. The relatively low spine density in the TeA suggests that despite the complexity of multisensory processing, a transmodal region might require higher spine density to ensure flexibility. A computational model for synaptic pruning demonstrated that if synapses are Hebbian, the system benefits of higher spine density to ensure correct optimization of cortical networks by synaptic pruning, compared to networks with low spine density (Chechik et al., 1998). Synaptic pruning is a developmental stage of the brain, which is different from ongoing adaptation and synaptic plasticity, but the fact that brain networks with higher spine density undergo more optimized synaptic pruning suggests that a network with higher spine density may be better suited for optimized changes in cortical circuits due to adaptation needs, such as the PCC as a functional hub.

When the mean spine densities were plotted together ordered by proximity (fig. 5.6), a pattern of gradual change in spine density was observed, with the cingulate cortex as point of highest spine density, and neighbouring regions decreasing in density as they become further apart (fig. 5.9). Thus, the temporal association cortex is far apart from the cingulate cortex. This gradient suggests that the cingulate cortex may have an influence in the density of neighbouring regions, and is an observation consistent with previous descriptions

of the cingulate cortex as functional hub of the DMN. Nevertheless, this observation had no statistical significance and requires further investigation.

One may expect that as a functional hub, the cingulate cortex in rats would have a high connectivity, however, in the rat DMN network identified by Lu et al. (2012), hub-like connectivity patterns were described for a group of regions collectively denominated "PFC" by the authors, including the whole cingulate cortex (see section 2.2.5). It is not clear whether only the cingulate cortex had a role of functional hub, or it was a shared function amongst several regions. The cytoarchitecture of the rat cingulate cortex shows direct resemblance with the human cingulate, but unlike humans, it does not have the posterior part (i.e. PCC in humans) (Vogt and Paxinos, 2014). Future work would greatly benefit from elucidating a more specific homology between the human and the rat DMN in a more detailed way.

Most resting state connectivity studies of the DMN in rats are performed under anaesthesia (Lu et al., 2012), and there is a lack of agreement on the exact definition of resting state: whether it means to be awake, waiting in a fMRI scanner as in human studies (Raichle et al., 2001), or it means to be under anaesthesia, as in studies with experimental animals (Kelly et al., 2012). In-vivo studies of intrinsic activity in the DMN could be an useful tool to aid in the functional parcellation of the rat DMN. Implanted carbon paste electrodes (CPEs) can record low-frequency fluctuations of tissue oxygenation (Li et al., 2015) and have proven to be an useful tool to measure DMN patterns of activity compared to task-activated networks like the LCN. The use of CPEs could open the possibility of behavioural tasks. Even though mind wandering is studied only in humans because of the strong dependence on self report for thought sampling (Smallwood and Schooler, 2015), rats with several CPEs implanted could be studied in different behavioural tasks with conditions known to induce mind wandering in humans, such as future planning (Morsella et al., 2010), and then observe whether the DMN has different patterns of activity.

6.3 Staining quality of the Golgi method

Two main benefits of Golgi staining are the relatively fast and simple preparation of the samples, and that there are no correlations between staining and cellular features, rendering a randomization factor required for statistical analysis. Given that the staining is suited for postmortem samples, animals do not require to undergo surgery with recovery periods, making the whole experimental protocol relatively simpler than dye injection (see section section 2.5). Other benefits consist in the production of sample archives that can be analysed long after their preparation and the possibility to use postmortem samples of human brains.

In this project, it was assumed that Golgi stain works throughout single neurons and that neurons are randomly stained, which aids to control confounding variables. From the pool of randomly selected neurons within each chosen cortical area, dendritic segments were selected only if they showed a high quality of staining, independently of the number of visible spines. However, the staining did not work homogeneously across all slices. Only some slices were stained and some regions had few cells that had high enough staining quality. There were few neurons to choose from, thus distance between neurons could not be controlled. In some cases, selected neurons were close, being $100\,\mu\mathrm{m}$ to $200\,\mu\mathrm{m}$

apart, or further away, being $300\,\mu m$ to $400\,\mu m$ apart.

In the study by (Claiborne et al., 1990), neurons of the same class that are close to each other are similar in anatomy. This finding indicates that close neurons might have similar spine density. Nevertheless, neurons that are close in a radius of $1\,mm$ have a low probability to be connected to each other (Laughlin and Sejnowski, 2003) and that might result in less influence between close neurons. To control confounding variables, future experiments would benefit from having a more homogeneous stain to being able to select neurons at equal distances from each other.

Golgi stain is a technique that requires long durations of incubation. Even though the exact mechanism of neuron selection for staining is not known, trials are recommended for achieving an optimization of staining conditions. In this project, despite the overall high quality of staining, it may possibly be improved. Future research could benefit from using Golgi staining solutions prepared in the laboratory and a toluene-based mounting medium. Serial sectioning and staining requires large volumes of solutions, while kits have limited amounts of impregnation solution. The kit producers recommend to add at least $5\,mL$ of impregnation solution per cubic centimetre (i.e. per hemisphere), which has to be replaced once after the first day of impregnation, which will add up at least $20\,mL$ per brain. Keeping hemispheres together reduces processing times of slices by half, but staining of whole brains with the FD Neurotechnologies rapid Golgi stian kit is not recommended by the manufacturer of the kit, because it can cause uneven stain across all sections. Home-made Golgi stain solutions allow for the production of large volumes and a flexible optimization of the method. While the FD Neurotechnologies rapid Golgi stain is best suited for fresh tissue, home-made solutions can be adapted for different samples, for example by changing concentrations and monitoring the pH for achieving the best impregnation results for the specific kind of samples (Angulo et al., 1994).

Keeping hemispheres together reduces processing times of slices by half, but staining of whole brains with the FD Neurotechnologies rapid Golgi stian kit is not recommended by the manufacturer of the kit, and yielded uneven staining across all sections. There is documented successful staining of whole rat brains using Golgi-Cox staining (Gibb and Kolb, 1998), added to optimization of Golgi staining (Angulo et al., 1994), could be useful for future research.

6.4 Future applications of morphometric analysis of neurons

Measurement of spine density can be used to study changes in morphology in brain diseases such as schizophrenia. Currently, there are contradictory results in studies that use different methods. The connectivity profile in DMN studies describe schizophrenic brains with hyperconnectivity (Broyd et al., 2009), while dendritic spine counts have found decreased densities in neurons of prefrontal cortices (Glantz and Lewis, 2000). Quantification of dendritic spines in diseased brains could help to elucidate contradictory results such as the increased connectivity observed in fMRI studies of schizophrenic brains, and the low spine densities observed in Golgi stain preparations of the prefrontal cortex of post-mortem samples of schizophrenic patients.

Neuron Studio is a computer program developed by Rodriguez et al. (2008), which using iterative algorithms, divides the 3D volume of a z-stack, analyses the signal and is able to reconstruct neurons, dendrites and classify spines without manual intervention, reducing human error. The algorithms used in Neuron studio split and merge clusters of signal to detect and construct 3D shapes that represent the neuron. In multiple iterations (repetitions) of the algorithm, Neuron studio analyses the dataset with user-defined thresholds. Neuron studio matched 85% of a human consensus classification when compared with expert operators, which indicates that the software can perform quantifications close to those made by human operators. Additionally, Neuron Studio is open source, but requires specialized knowledge to operate (programming skills in C++), and is preferred for fluorescence. It remains to be tested whether Neuron Studio can be used with Golgi stain.

6.4.1 Reconstruction of dendrites

The advantage of cell morphology studies is that quantification of dendritic branching (i.e. integration capacity) and synaptic count (i.e. connectivity) can be performed together because the samples and the methods are the same.

The quantification of the complexity of dendritic tree geometry can be an estimate of integration capacity because of the importante of spatial integration for input resolution. However, other cellular features influence integration, including ion channel diversity and distribution across the membrane (Sterratt et al., 2011, pp. 96-132), presence of inhibitory interneurons and modulatory systems, connectivity patterns among different neuronal types (Javier DeFelipe, 2010, pp. 5-14), and the contribution of astrocytes (Lee et al., 2014). The mapping of cellular properties of the DMN requires an integral approach.

Distant segments of basal dendrites have shown to have higher spine densities than segments close to the soma (Jacobs et al., 2001). In this project, only segments close to the soma were considered due to ease of quantification and time concerns. Golgi stain in consecutive slices can be used to reconstruct whole neurons, regardless of the slice thickness (Blackstad et al., 2015), however the process is time consuming. An alternative method is dye injection (Duan et al., 2002) but animals must undergo surgery and recovery time of approximately 21 days. Stress influences the spine density in the mPFC of rats (Michelsen et al., 2007), and posttraumatic stress disorder has been correlated with altered DMN connectivity patterns (Bluhm et al., 2009). Whether uncontrolled exposure to stress of experimental animals for dye injection alter the spine density is a possibility, and future work with stress in the DMN could elucidate this possibility.

A meta-analysis of genetic expression differences in the DMN, using the database Allen Brain (Richiardi et al., 2015) showed that the regions in the DMN are enriched in ionic channels. Ion channels are implicated in the generation of membrane potentials, which are a requirement for integration in dendrites (see section 2.4.4). An accurate 3D reconstruction (Arellano et al., 2007) using Golgi stain or dye injection can be used to model the electrical and computational (Sterratt et al., 2011, p. 72-95) properties of a given neuron.

6.5 Conclusion

This is a study that approaches connectivity in the DMN at the synaptic level. Overall, the DMN shows a trend of higher spine density compared to other important cortical systems, like the LCN and primary cortices, even though without significant difference. When broken down to individual regions, it is evident that the cingulate cortex stands out as the one region with the highest spine density, also within the DMN itself. A high spine density may be an indicator of high cellular connectivity, which possibly is responsible of giving the cingulate cortex high integration and computational capacities. The cingulate cortex has been previously described as one of two functional hubs in the human DMN. As a functional hub, a high spine density is consistent with high activity demands. Whether other regions of the DMN and the cingulate cortex itself can be distinguished by more significant differences remain to be tested more robustly, but the current results indicate that future research of the biology of mind wandering and the DMN may benefit from focusing on the cingulate cortex.

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Appendix A

Staining and preparation of microscope slides

In this section technical details of experimental procedure are explained and justified, added to the description of the protocols used for tissue sampling and embedding, Golgi stain and mounting on microscope slides. Adjustments to the FD Neurotechnologies rapid Golgi stain kit protocol are described and justified in appendix A.7.

A.1 Tissue sampling and impregnation

At least 48 hours prior to the experiment (Milatovic et al., 2010), the impregnation solution has to be prepared as a mix of solutions A and B (FD Neurotechnologies Rapid Golgi stain kit) in equal proportions (1:1). For anaesthesia of rats, use $2\,\mathrm{mL}$ of isoflurane per rat in an incubation box and then sacrifice with guillotine. Extract the whole brain and store it in impregnation solution. Then, store the brains in the dark for 2 weeks. Solution should be replaced the next day for new solution (in a mix of A and B in proportion of 1:1). Solutions A and B contain $HgCl_2$, K_2CrO_4 and $K_2Cr_2O_7$ (Milatovic et al., 2010). These substances are toxic to the skin and fatal if swallowed, thus tissue and solutions must be handled with gloves and under a fume hood.

After two weeks of storage in impregnation solution(A and B 1:1), move brains to solution C (FD Neurotechnologies Rapid Golgi stain kit). The next day, solution C must be replaced for new solution C. After 72 hours of storage in solution C, the rat brains are ready for slicing and staining. Samples should be processed as soon as possible because there is loss of quality for staining after a month of storage. More details can be found in the instructions for the kit (FD Neurotechnologies).

A.2 Embedding of whole rat brains

Impregnated brains should be embedded in agarose to avoid tissue loss during slicing and to keep anatomical organization. In this project, the remaining spinal cord and cerebellum were separated from the forebrain prior to embedding because otherwise the blocks would be too big for the vibratome. The hemispheres were not separated.

Two main protocols are needed for brain embedding in agarose, the preparation of agarose and fixing the agarose block to the brain.

A.2.1 Preparation of oxidized agarose

Prepare a solution of 4.5% agarose in 10 mM $NaIO_4$, use phosphate buffer (pH 7.4). Stir in the fume hood for 2 hours (up to 3 hours) at room temperature, protected from light. Filter the solution with filter paper (with pores no smaller than $0.2\,\mu\mathrm{m}$). Wash three times the agarose with phosphate buffer ($50\,\mathrm{mL}$ per wash), and then resuspend the agarose in $50\,\mathrm{mL}$ of phosphate buffer. The agarose is then ready to use, but can be stored in the fridge for up to two weeks, protected from light.

A.2.2 Embedding and fixing the agarose block to the brain sample

To embed the brain, the agarose has to be brought to a boil, and then cool to 60-65 °C, and then pour in the mold with the brain. Allow to cool down for some hours (2 to 3, or overnight). Then the block of agar can be extracted from the mold. To fix the block to the brain, the block should be added to a solution of $NaBH_4$.

To prepare this solution, first prepare borate buffer by mixing $19\,\mathrm{g\,L^{-1}}$ of borax and $3\,\mathrm{g\,L^{-1}}$ of boric acid in MilliQ water. Then adjust the pH to 9.0 to 9.5 with 1N NaOH. This buffer can be kept at room temperature for long periods. Heat $100\,\mathrm{mL}$ of the borate buffer at $40\,°\mathrm{C}$, add $0.2\,\mathrm{g}$ of $NaBH_4$ in the fume hood, and stir for $15\,\mathrm{min}$ to $30\,\mathrm{min}$, while protecting it from the light. There will be formation of CO_2, thus the bottle should be left overnight with the cap (not tightened). Next day, the cap can be tightened. The solution should not be used immediately after preparation (the same day as preparation), because the gas formation can break the agar block. The $NaBH_4$ solution can be stored for up to one week.

A.3 Vibratome slicing

The block of agarose with brain has to be cut prior gluing on the plate, so the size and angle fits a coronal sectioning, at $90°$ from the midline of the rat brain. The surfaces of the vibratome plate and the agar block (the side that will be facing the plate) should be dried with tissue paper. Add superglue to the plate of the vibratome, and then wait some seconds for the glue to dry before adding the block in the correct angle. It is recommended to put the block in with the posterior part in the bottom and the olfactory bulb in the top. Once the dried side of the block is put on top of the glue, let dry for some seconds. Given that the impregnated brain is hard, the vibratome blades should checked and the angle adjusted to avoid the production of slicing artifacts.

The box of the vibratome has to be filled with phosphate buffer and cover the tissue block completely to avoid drying and cracking, and uneven cutting due to vibration artifacts. Set speed to 9 and frequency to 5, which works best for impregnated tissue because it is hard. Slices should be $100\,\mu m$. Slice the brain, and retrieve each slice as it is produced using the brushes. Keeping the agar frame is optional and depends on the amount of subregions because the tissue might require the frame to keep them together, but in some cases the frame is unnecessary and is better to discard it. Avoid unnecessary manipulation of non-mounted brain slices because they can break easily.

A.4 Mounting on microscope slides

After slicing in vibratome, brain slices should be immediately mounted in a glass slide with a drop of solution C previously put on the glass surface (FD Neurotechnologies Rapid Golgi Stain kit). Superfrost glass slides do not require previous treatments to enhance sticking of the slices onto the glass surface. The whole brain slice should be mounted with or without the agar frame, while being sure that the position with respect to the left and right hemispheres is the same as the brain embedded in the block being cut. Several slices can be mounted per glass slide, depending on the space available (three to four, if the size allows it). The order of mounting on the slice is selected to be the first slice at the top, and following slices at the bottom. The bottom of the slice is the rough edge where the identification number of the glass slide is annotated. During mounting, glass slides with brain slices should remain flat at all moments. Protect from light as much as possible.

When the slices have been placed on them with drops of solution C, the glass slides can be stored in a humidity box for some hours, but should be monitored to avoid drying, protected from light. The slices are not completely stuck to the glass, and should be manipulated carefully.

Before staining, each glass slide with brain slices should be dried using tissue paper with gentle touches. A sheet of a single layer (as thin as possible) can be lied on top of several slides to absorb solution. Small paper squares are easier to manipulate. Slices do not stick to thin paper, making it easier to dry, unless there is residues of dura or pial membrane, which tend to cling to the paper. It is recommendable to have close a petri dish with buffer to submerge the paper if the slice sticks to it, and then use a brush to retrieve the slice to mount again. Avoid using too large papers because the larger they are, the more difficult it is to recover slices.

A.5 Staining

Lying flat, add two drops of MilliQ water per slice. Leave for $4\,\mathrm{min}$ protected from light. Remove the MilliQ water by drying with single layer tissue paper. Add another drop of MilliQ water and repeat the procedure (wait for $4\,\mathrm{min}$ and dry). Next, add a drop of staining solution D and E (diluted in a proportion of 1:1:2, D, E and MilliQ water respectively, freshly prepared, FD Neurotechnologies Rapid Golgi Stain kit). Leave protected from light for $10\,\mathrm{min}$. Remove the staining solution with tissue paper, and wash with MilliQ water for $4\,\mathrm{min}$, twice. Remove the water drop and allow to dry for $4\,\mathrm{min}$ at room tem-

perature. Supervise the drying step continuously because time can vary. The last step of drying is very important. Drying should not be prolonged, but if not done, the slices will not stick to the glass slide and can move out and break during cover slipping.

A.6 Coverslipping

Heat gycerol gelatin (mounting medium) at $40\,^{\circ}\mathrm{C}$. Apply only one drop of liquid glycerol gelatine on the mounted dry slice. Let cool down for one to two seconds. If not, the mounting medium is too liquid and can move the slices out. Coverslip by gently putting the glass cover on top, starting from only one side of the glass, avoiding bubble formation. Press gently the mounted section to extract the excess of mounting medium. Do not press too much because the tissue can be broken. If the mounting medium has hardened before, pressing can be done on the hot plate.

Slices mounted on glass slides and cover slipped must be left on a hot plate at $30\,^{\circ}\mathrm{C}$ for 24 hours before being able to look at them in the microscope. Clean the surface of the glass slides gently with water and a cloth, and then store at room temperature, protected from light.

A.7 Adjustment of the Golgi stain protocol

The staining protocol provided by the rapid Golgi stain kit (FD Neurotechnologies) had to be adapted to satisfy three main needs of the project. First, staining and slicing of the whole brain should avoid tissue loss while preserving anatomical organization. Second, slices should be thick enough to be able to follow and analyse dendrite segments with a length of $120\,\mu\mathrm{m}$ to $160\,\mu\mathrm{m}$, without causing resolution loss due to excess of tissue; and third, the whole pipeline process should be simple and fast, due to the large number of regions of interest per brain.

A.7.1 Slice thickness

For spine quantification using Golgi stain, the recommended thickness of the slices varies. Thickness of $200\,\mu\mathrm{m}$ is often used (Mychasiuk et al., 2013), (Tang et al., 2014), while some studies use thinner slices ($90\,\mu\mathrm{m}$) (Glantz and Lewis, 2000). However, at $200\,\mu\mathrm{m}$ thickness, cells appear with more background staining and there is loss of resolution. Furthermore, if slices are too thick, the microscope cannot focus in depth. For spine quantification with Imaris, resolution and a clear background are essential to reduce error. Conversely, thin slices do not have enough space to contain the large structure in three dimensions of dendritic trees, which appear cut. Slices of different thickness were tested for visualization in the microscope. Slices of $100\,\mu\mathrm{m}$ were found to have a better resolution than slices of $200\,\mu\mathrm{m}$ for the purpose of imaging of dendrite segments (See details of neuron and dendrite selection in D).

The rapid golgi staining procedure requires several solution changes for dehydration of the tissue that make up to one hour of sample processing without mounting. Furthermore, manipulation of the samples while floating can break the tissue. Therefore, instead

of staining floating slices, having slices attached to gelatinized slides is desirable to optimize the procedure times and to protect the integrity of the tissue anatomical organization. According to (Das et al., 2013), slices should be $200\,\mu\mathrm{m}$ to have enough electrical charge to be attracted to the chromium in the gelatinized slides. While slices of $200\,\mu\mathrm{m}$ keep attached to the slides, slices of $100\,\mu\mathrm{m}$ did not stick to the gelatinized slides and fell off during the rapid Golgi staining procedure.

On one hand, $200\,\mu\mathrm{m}$ could stick successfully to the slides, but had poor resolution. On the other hand, $100\,\mu\mathrm{m}$ slides had better resolution, but detached from the slides, when submerged vertically on the solutions for staining. Resolution is a factor of major importance for the quality of spine quantification. Thus, slices were stained while lying flat in superfrost slides.

A.7.2 Adjustment of the staining procedure

According to the FD Neurotechnologies Golgi stain kit instructions, the procedure needs dehydration and an organic-based mounting media such as Permount. However, this mounting media was not available, thus glycerol gelatin was used instead. Glycerol gelatin is a water-based mounting media, so the dehydration steps were skipped. An additional reason that supports the water-based staining procedure is the need of embedding the whole brains in agarose, a procedure that produces slices with a frame of agarose that protects the anatomical organization of the samples. While dehydration and organic-based mounting media interfered with the agarose frame, the use of glycerol gelatine completely integrates the agarose frame in the mounting media. Staining was performed on the mounted slices while lying flat on superfrost slides. Gelatinization of slides was not necessary.

Appendix B

Coordinate localization of regions of interest

Waxholm space is a standardized coordinate space that uses internal landmarks to define the localization of points of interest. In this project, the coordinates of regions of interest (ROI) were assigned to Waxholm space in order to fit our results to a rat atlas in the Waxholm space (Papp et al., 2014). The coordinates of the ROI set have to be tailored per experimental animal due to size differences. The Paxinos atlas is the reference atlas for the localization of brain areas used in this project. However, the rats involved in the quantification were of a different strain (Dark Agouti) than the used for the atlas (Wistar). Thus, an adjustment of the atlas is required to identify the regions of interest in the experimental rat brains. In this section, the procedures for strain adjustment of the atlas and prediction of experimental coordinates per rat brain are described.

B.1 Identification of internal anatomical landmarks

In this project, four internal landmarks were used, (see table B.1) (Sergejeva et al., 2015), and to measure and adjust the proportional differences between the rat strains, the Scalable Brain Atlas template was used as a guide (Papp et al., 2014). Landmarks were chosen according to their proximity to the ROIs because proximal landmarks may be able to reflect local anatomical variations. Once we had the difference, we could estimate the coordinates of each ROI per rat brain with adjustments to the strain.

To estimate the experimental coordinates of the ROIs in each brain, different landmarks (Sergejeva et al., 2015) have to be identified in stained brain slices, and the distance between them measured. Given that the rat brains were sliced in a coronal plane, only one plane of coordinates is necessary, which is the equivalent to the coronal plane (Y plane in the new version of the rat brain Atlas). First, search all the landmarks in the slices, according to the instructions by (Sergejeva et al., 2015), and if the landmark is available, mark the slice in which the landmark is found. The rat brain atlas in Waxholm space (Papp

Table B.1: Internal landmarks used for the adjustment of the rat brain atlas and the prediction of ROI coordinates in the experimental brains. Landmarks consist of brain structures that can be identified by their morphology in histological slices. A whole brain must be sliced with regular slice thickness so that the distance between slices and landmarks is known. In the last column, the Waxholm space (WHS) coordinates correspond to the location of the landmarks as a distance from the anterior commisure (AC), which is the origin in the Waxholm space coordinate system. In this table, the coordinates for the landmarks are for the rat brain atlas by Papp et al. (2014).

Landmark	Full name	WHS coordinate
FM1	Frontal Middle 1	2.188
HM1	Hippocampus middle 1	-4.453
IP	Interpeduncular nucleus middle	-6.25
AC	Anterior commissure	0

et al., 2014) should be used as a reference for the anatomical appearance of the slices with landmarks.

B.2 Origin in the experimental brains

The most important landmark is the anterior commisure (AC) because it represents the origin (zero) in Waxholm space. Therefore, the AC should be identified in all samples. The number of slices between the AC and a point of interest is equivalent to the distance in micrometres given that each slice is a step of $100\,\mu\mathrm{m}$. The distance in millimetres from the AC to the point of interest is the Waxholm space (WHS) coordinate of the point of interest. In this way, the WHS coordinate of each landmark (FM1, HMI, IP) should be calculated per brain. The experimental WHS coordinate of the landmarks is compared to the WHS coordinate in the atlas provided by (Sergejeva et al., 2015). The conversion is shown in eq. (B.1), where W_{poi} is the WHS coordinate of a point of interest in millimetres, and S_{AC} is the number of slices before the anterior commisure.

$$W_{poi} = S_{AC} * 100\,\mu\mathrm{m}(\frac{1\,\mathrm{mm}}{1000\,\mu\mathrm{m}}) \tag{B.1}$$

B.3 Prediction of coordinates in experimental brains

The prediction of ROI experimental coordinates requires two steps. First, the WHS atlas has to be scaled down because the experimental animals are smaller than the atlas rat brain. Second, per brain, the first and last slices of the set are anatomically compared to the rat brain atlas. Then, the coordinates of the equivalent slice in the atlas are registered. The coordinates of the first and last slices can be expressed in WHS millimetres, and when added the total represents the length of the intermediate volume in the atlas. This measurement is the atlas expected brain length. To obtain the experimental length, the WHS coordinate of the first and last slices has to be measured and calculated in the same way as

the landmark experimental WHS coordinates, by counting the number of slices between the first slice and the AC. The experimental length is equivalent to the sum of the WHS coordinates in millimetres of the first and last slices. This relationship is described in the relation B.2, where C_j is the adjusted WHS coordinates, C_a is the atlas WHS coordinates, v is the NIfTI voxel size (0.0390625) in millimetre over voxel ($mm/voxel$), L_e is the experimental rat brain length, and L_a is the atlas expected brain length.

For each ROI identified in the atlas, the ROI WHS coordinate can be adjusted. Using the atlas and experimental length the rat brain atlas can be scaled down to be able to proportionally compare the atlas to the smaller brains of the sample.

$$\frac{C_j}{C_a}v = \frac{L_e}{L_a} \tag{B.2}$$

The Waxholm space origin in the experimental rat brains has to be adjusted as well. Instead of using zero (WHS mm), the origin can be adjusted using the NIfTI voxel coordinate (this coordinate is the slice number in ITK-SNAP for each plane of the Waxholm space brain atlas).

The prediction of the experimental coordinates has to take into consideration the local anatomical variability of the region of interest. Three landmarks were used to calculate the position of ROIs in experimental brains, FM1, HMI and IP (Sergejeva et al., 2015). assuming that the relationship of proportionality is true for local ROIs and close landmarks eq. (B.3), where W_{el} is the WHS coordinate of the landmark in the experimental brain, v is the NIfTI voxel size (0.0390625) in millimetre over voxel ($mm/voxel$), W_{al} the WHS coordinate for the landmark in the adjusted atlas, E_{ROI} is the predicted WHS coordinate for the ROI in the experimental brain, and A_{ROI} the WHS coordinate for the ROI in the adjusted atlas.

$$\frac{W_{el}}{W_{al}}v = \frac{E_{ROI}}{A_{ROI}} \tag{B.3}$$

Appendix C

Drawing of the regions of interest in a 3D rat brain model using ITK-SNAP

In this project, we used the 3D model of the Sprague Dawley rat brain, as a base to draw the DMN and other regions of interest (ROI). With a 3D model we could represent our data as a 3D heatmap in the standardized Waxholm space. Given that we already had the equivalent stereotaxic coordinates for the Paxinos atlas (strain adjustment explained in section 4.1), we could use the Paxinos atlas coordinates to draw the 3D model of the DMN and represent the values found in the corresponding areas of the DA rat brain.

We used ITK-SNAP 2.20(Yushkevich et al., 2006) to visualize and edit the 3D rat brain model in Waxholm Space of the Sprague-Dawley rat (Papp et al., 2014). Two NIfTI files (.nii) and one label (.label) file conform the rat brain atlas, and are available in the documentation of the model (Papp et al., 2014). To work with these files in ITK-SNAP, first open the rat brain MRI image file (T2.nii), then open the segmentation file (atlas.nii) and finally under the tab (segmentation), select ”import label descriptions” to open the label file (.label) C.1, C.2. The same procedure is required to open the file including the DMN drawing, which should be selected instead of the atlas.nii file. First the MRI image file, and then the DMN segmentation file.

In ITK-SNAP 2.20, the *label editor* is in the tool bar at the left (shown in figure C.2). Using the Label Editor, we create a new label to draw on a new volume that will correspond to a region of interest. To adapt the visuals of the working environment, using the label editor we can select only the volumes we are interested in, for example the neocortex label. In ITK-SNAP, the areas selected (with a thick in a box) are hidden, therefore unselected areas will be visible (figure C.3). Alternatively, we can change the opacity of non-working labels to low levels.

Before drawing, we need to identify the slice in which the ROI is found. For that, first we find the structure in the Paxinos rat brain atlas, which has coronal slices and stereotaxic

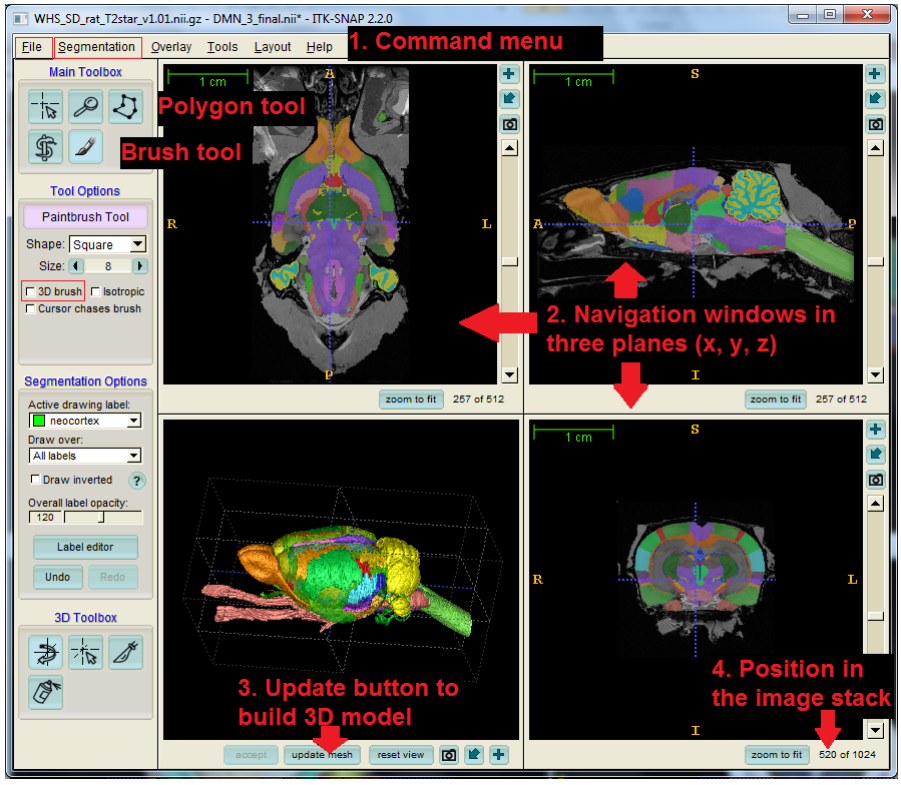

Figure C.1: Common appereance of an ITK-SNAP window (Yushkevich et al., 2006), with the 3D rat brain model in Waxholm Space of the Sprague-Dawley rat (Papp et al., 2014). Using red arrows and boxes, the image shows the localization of useful commands and information required to draw 3D volumes in a given coordinate. 1. Command menu: under the tab *file*, MRI images (.nii) can be opened, and under *segmentation* 3D volumes (.nii) and label files (.label) can be added to the workplace; 2. Navigation in three planes (x, y, x), ITK-SNAP allows the user to navigate and draw in three planes, updating each plane as the user draws; 3. Update button initializes the 3D building of the model after drawing in each slice at different planes; 4. The position in the image stack in ITK-SNAP is in NIfTI voxels, and can be navigated by moving the side bar. This position must be changed to draw in different slices. Finally, the localization of the polygon tool and the brush tool with the 3D functionality are shown.

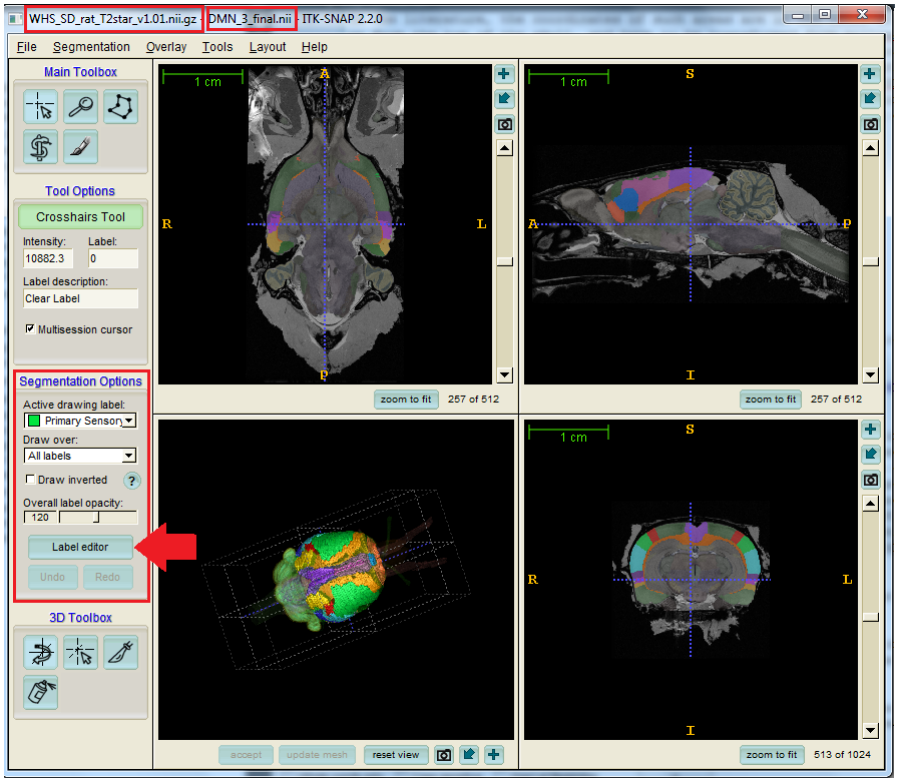

Figure C.2: Localization of the Label Editor in ITK-SNAP and the names of the NIfTI files highlighted, first the MRI image, and then the segmentation image

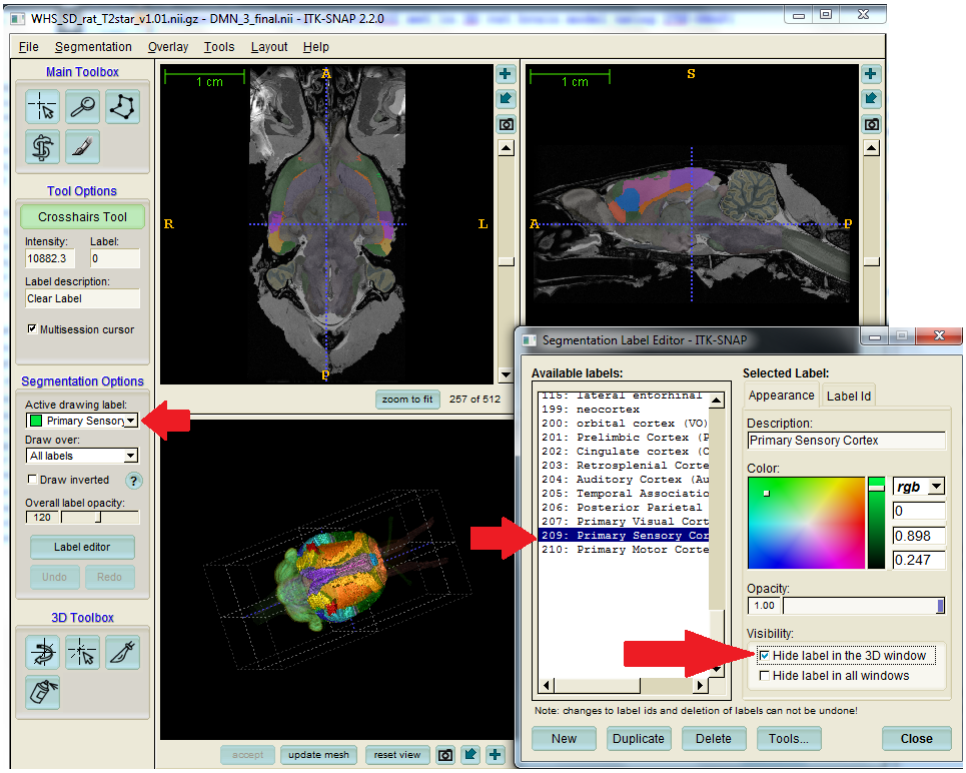

Figure C.3: Label editor window showing how to hide labels

coordinates in the order of millimetres from Bregma. Then, we need to convert from stereotaxic coordinates to Waxholm space (WHS) voxels and to NIfTI voxels. The NIfTI voxel represents the z-slice in which ITK-SNAP shows the current MRI image (see figure C.1). We calculated the NiFTI voxels or the ITK-SNAP position in the coronal plane (y) only. The origin in stereotaxic coordinates (Bregma point B) is offset by two voxels compared to the origin in Waxholm space (the anterior commisure). Therefore, given a point of interest (POI) in the stereotaxic coordinate system should be added two voxels in mm. Then, to obtain the NIfTI voxel position (N_{POI}), the adjusted POI coordinate has to be divided by a conversion factor of millimetres to voxels, and finally adding the NIfTI voxel coordinate of the Waxholm space origin (N_{ORI}), the anterior commisure, in the y plane (voxel no. 623). The relation C.1 shows how to calculate the voxel position N_{POI} (NifTI voxels) of a point of interest. POI is the distance between the point of interest (POI) to bregma in millimetres (0.0781250 mm), B is the WHS coordinate of the bregma in millimetres, v is the NIfTI voxel size (0.0390625) in millimetre over voxel ($mm/voxel$), and N_{ORI} is the voxel in which the WHS origin is located, the anterior commisure. More details of the conversion from stereotaxic to Waxholm space coordinates can be found in (Papp et al., 2014).

$$N_{POI} = \frac{POI + B}{v} + N_{ORI} \tag{C.1}$$

For example, a given POI of $2.3\,\mathrm{mm}$ would be equivalent to the NIfTI voxel 683 in the ITK-SNAP navigator, according to the following calculation:

$$N_{POI} = \frac{2.3\,\mathrm{mm} + 0.078\,125\,0\,\mathrm{mm}}{0.0390625\frac{mm}{voxel}} + 623voxel = 683voxel;$$

To draw we can use the paintbrush mode for fine details and complex shapes. The brush can be set to volumetric (3D voxel brush) and be used to cross over one or two slices. The polygon tool allows to select points surrounding a shape and fill it with voxels, which is recommendable to draw over for large areas. In this project we used a Wacom Bamboo Pen Tablet for digital drawing instead of a normal computer mouse to ease the drawing task. To segment the ROI, we draw in each slice where the ROI is found, by moving slice to slice, drawing by free hand or adjusting polygons, and looking at the different planes C.4. We hit the *update mesh* button to build the 3D model to see the progress.

In ITK-SNAP, voxels of new volumes replace voxels of underlying volumes. For example, if we draw a new volume over the label of the neocortex, the voxels that belong to the neocortex will be deleted. In segmentation options, the "Active Drawing Label" tab should contain the new volume we want to draw, and under "Draw over" we select the label that should be replaced, for example the neocortex label. Alternatively, we can select all labels, but be careful to draw only in the desired regions C.2.

To save the changes in a new file, first we use the command *Export Label* to save changes in the label file, and then under the tab of "segmentation", we save as a new image. In this way we can draw the DMN on top of the volume that represents the cortex in the atlas, but save in a new file.

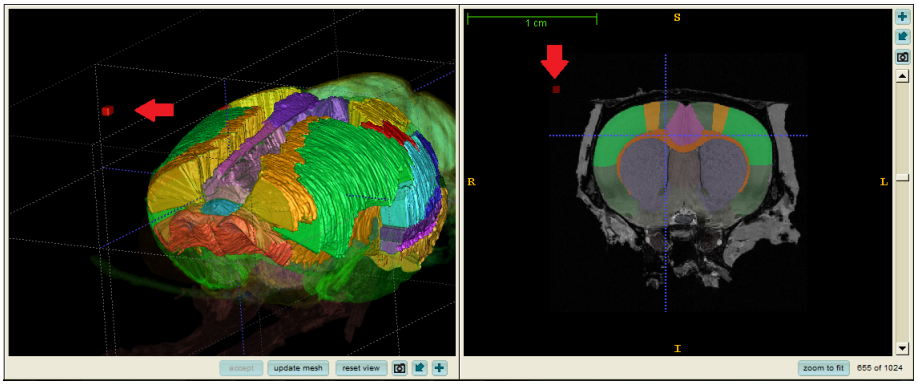

Figure C.4: We can build 3D volumes in ITK-SNAP by drawing on the desired ROIs on the three different planes. In the image, a red square was drawn using the 3D-brush in three slices of the coronal (x) plane (right), and then build in the 3D model (left)

C.1 Selection of a color gradient to represent data in the brain map

The brain map contains volumes that represent each region of study. Each region had an average spine density, and all the means had a range from 1.0 to 1.64. Thus, a gradient of 64 steps was generated, so that a tone could be assigned per region. The color gradient was generated using an online tool (perbank.dk). In ITK-SNAP, colors can be selected according to the color gradient.

Figure C.5: Selection of color gradient for the spine density map of the rat brain. The color gradient had 64 steps and was generated using as base colors blue and red. The selection of steps was based on the range of the mean spine density of all regions.

Appendix D

3D reconstruction of dendrite segments and spine density quantification in Imaris

In this appendix, the criteria for selection of neurons and the technical details on the reconstruction of dendrite segments is covered. A video of a fast-speed reconstruction can be found in https://youtu.be/0a_unxUxwiU. The video is also attached as supplementary material.

D.1 Criteria for the selection of neurons and dendrite segments

When using Golgi stain for quantification of spine density, different studies use different criteria for selection of neurons. In this study, we used a combination of the criteria used by (Roitman et al., 2002), (Glantz and Lewis, 2000) and (Tang et al., 2014).

Selection of neurons and dendrite segments should be in accordance with the following criteria:

- Cell bodies should be darkly and completely stained.
- The location of the dendrite segment should be in the middle of the tissue (z-axis thickness).
- Cell soma should have the characteristic pyramidal cell body shape and be in layer V.
- Sample segments of basal dendrites per neuron should have a length of $60\,\mu m$ to $80\,\mu m$ and be at $60\,\mu m$ to $80\,\mu m$ of distance from the soma, as in fig. D.1 and fig. D.2. Spine density and spine size change linearly with distance from the soma

Figure D.1: Diagram that represents the cell soma and the two dendrite segments that are identified and measured. From the soma, after a first segment of 60 µm to 80 µm, a second segment of 60 µm to 80 µm is imaged.

(Benavides-Piccione et al., 2002), thus the distance between start point of the segment and the soma should be kept as constant as possible for all segments.

- The selected dendrite segment should be fully impregnated and preferably in the same plane as the brain slice.
- Dendritic segments should not have interruptions and be able to follow clearly.
- Soma or dendrites should not be obscured by background opaque artefacts larger than 5 µm

Acquired images must be preprocessed before using Imaris. Preprocessing consists of color inversion and deblurring by deconvolution as shown in figure D.3.

D.2 Stack processing

In Imaris, convert the imaging files in .LIF format to .IMS format. Open the .IMS file in Imaris, and a maximum intensity projection will be generated automatically. This projection allows to see the imaged dendrite in a pseudo-3D model. The reconstruction is done with this projection.

To be able to operate with smaller files with shorter processing times, two processing steps should be accomplished: resampling and cropping of the region of interest. To resample the file, under the menu ”Edit”, select the option ”Resample 3D”. Always select the box ”fixed ratio x/y/z” before changing any value in x, y and z. Reduce the value in x to half, no more to avoid quality loss. Then, rotate the maximum intensity projection until the dendrite is vertical. In the menu ”Image processing", choose the option ”Free rotate”. Finally, under the menu ”Edit”, select the option ”Crop 3D”, to crop the projection in all axes. Crop the file to include only the dendrite of interest in the file. If the dendrite cannot be cropped in a cubic shape, create a surface in manual mode. Draw the 3D figure using different planes until the dendrite is included inside of it. When the surface is done, under the tab ”Edit”, convert the surface into a ”Mask”. A new channel is created that includes only the dendrite inside the surface. See fig. D.4 for an example of cropping a file in Imaris.

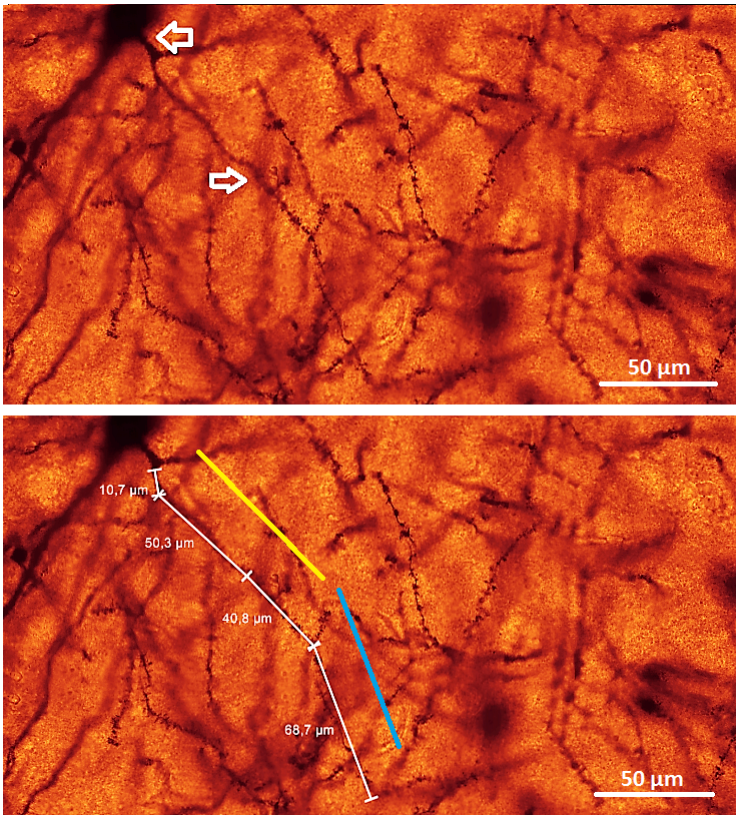

Figure D.2: Example of dendrite segments selection. Segments of basilar dendrite should be in total 120 µm to 160 µm. Yellow and blue bars represent the two segments measured, that should be of the same length. The yellow segment is measured, but not imaged, while the blue segment is measured and imaged in a z-stack.

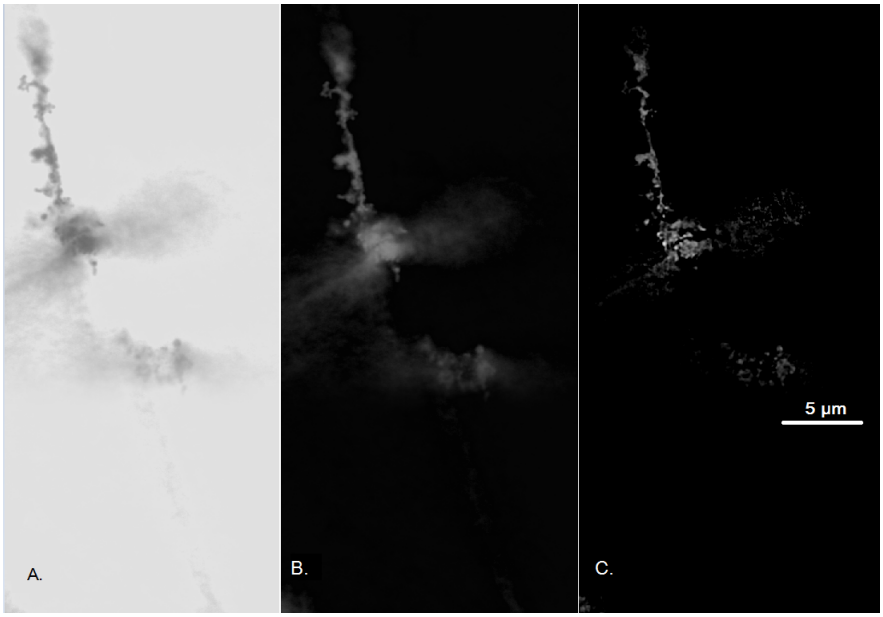

Figure D.3: Image preprocessing of the z-stacks consisted of color inversion and deconvolution. Imaris detects white pixels as positive signal, therefore images of neurons stained with Golgi method must be inverted in color. Finally, images are deblurred using deconvolution algorithms. A. Original image, B. Image with inverted colors, C. Image with inversion and deblurring by deconvolution.

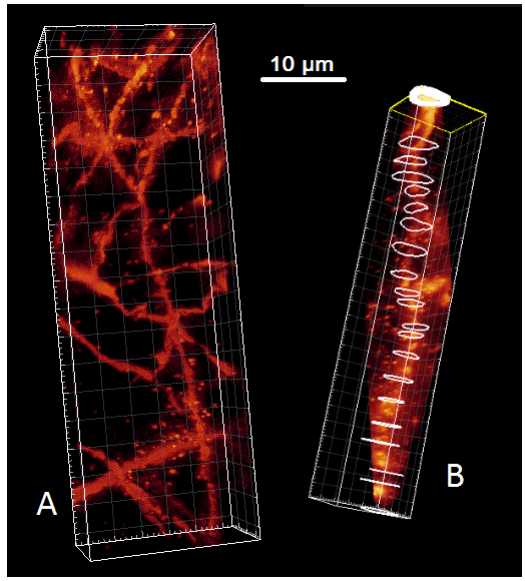

Figure D.4: Example of 3D cropping. A. Whole z-stack in a maximum intensity projection, B. Dendrite segment after rotating and cropping inside a surface. In B. the surface is drawn in different levels of the x/y plane.

D.3 3D reconstruction of dendrite segments

Create a filament by clicking in the leaf icon in the window ”volume properties”. Skip automatic creation and use the Autodepth tool to draw a line with a mouse or a pen-tablet. Holding the shift key, draw a line where the dendrite segment is located. If Autodepth does not work properly, rotate the image and draw on the side, see fig. D.5.

Drawing errors can be edited under the tab ”filament edit”. Select the whole filament by holding Ctrl key and clicking on the segment. Then use the tool ”smooth” several times to soften the main line. Then use the tool ”center” to adjust the line onto the point of maximum signal intensity. The adequate value of contrast threshold for the sample is calculated during automatic reconstruction of spines and is suggested by Imaris. To adjust the thickness of the dendrite filament, select the ”cone mode” under display options, and then under ”edit”, select ”diameter”. Select the masked channel and a contrast threshold of 0.89. Adjust the maximum and minimum desired values for diameter until the 3D model fits the original image of the dendrite. An example of diameter adjustment is shown in fig. D.6.

D.4 Automatic detection of dendritic spines

To be able to quantify spines automatically, a ”false” spine has to be drawn manually in the model. Then, under the tab ”create”, select ”rebuild spine”. Then, an automatic process of three steps will follow. The resulting 3D model provides several parameters, such as spine

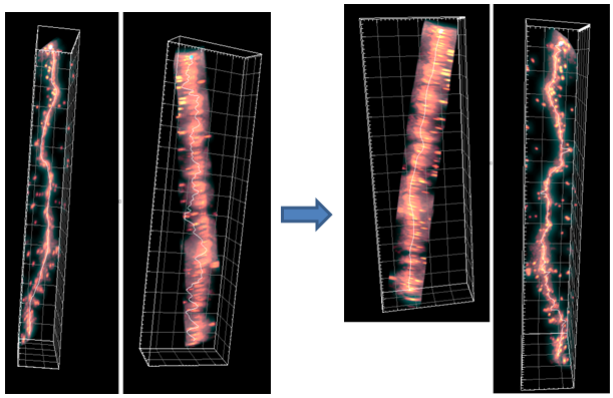

Figure D.5: If using Autodepth does not produce a straight line, rotate the dendrite and draw the line on the side as shown in the image.

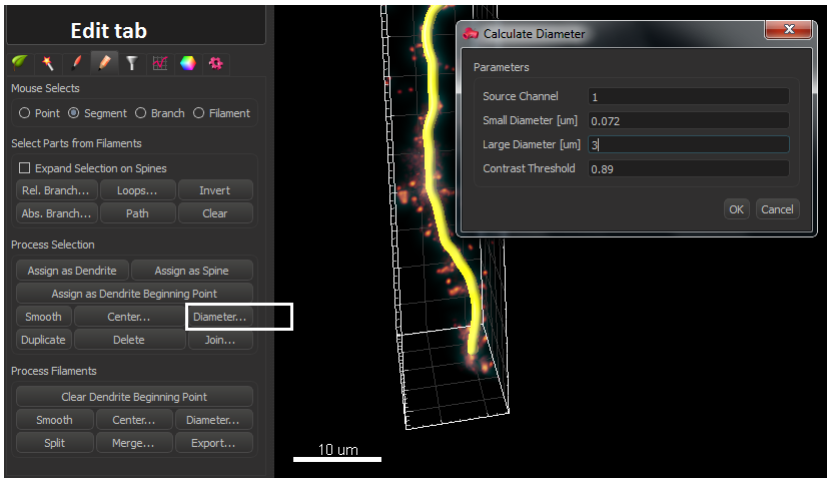

Figure D.6: Calculation of the diameter of the dendritic segment on the maximum intensity projection.

number, surface area and dendritic length.

- Measure in the slice view the minimum spine head diameter and the maximum spine length per sample. Specify the obtained values in the first step. Do not allow spine branches.
- Imaris suggests an automatic seed point threshold. This threshold can be adjusted if necessary. To adjust, select a threshold of seed points that reduces false positives but not visibly true spines. False positives can be filtered and deleted in later steps.
- Select a threshold that includes the diameter of all spines. Finish the detection process.

D.5 Filtering of false positives

Automatic spine detection in Imaris generates 3D spines with the signal in the z-stack, but with several false positives or non detected spines. Figure D.7 shows different cases of false positives and non detected spines. Due to low resolution, there is an extension of the signal in the Z axis, which causes Imaris to detect double spines where there is only one true spine, as shown in fig. D.10.

Filtering by selection and is required to adjust the quantification. To delete false positives by steps, use a filter for position in the Y axis, as shown in fig. D.8. Then, the model can be rotated and compared to the original maximum intensity projection to determine whether spines are true or false, as shown in fig. D.9. Only true positives are deselected, and the rest are left selected and deleted.

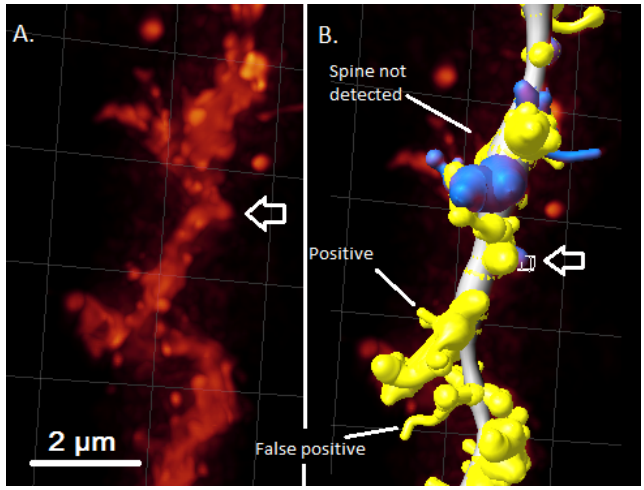

Figure D.7: Manual false positive filtering. Three different views of a dendritic segment. A. In the maximum intensity projection, the white arrow points to a true spine (positive). B. The 3D model with selected spines in yellow, and unselected in blue. Selected spines are manually evaluated and deleted if they are false positives. The white arrow shows a blue spine that corresponds to the positive in the first panel.

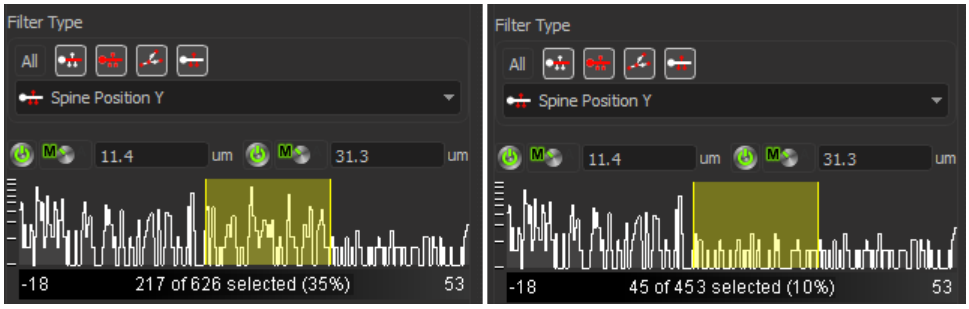

Figure D.8: In the filter tab, different filters can be used to select spines. A filter for Y-axis position is selected to manually filter spines by steps on the dendrite length. The figure shows the state of the spine number before and after filtering false positives in a group of spines at the middle of the dendrite segment.

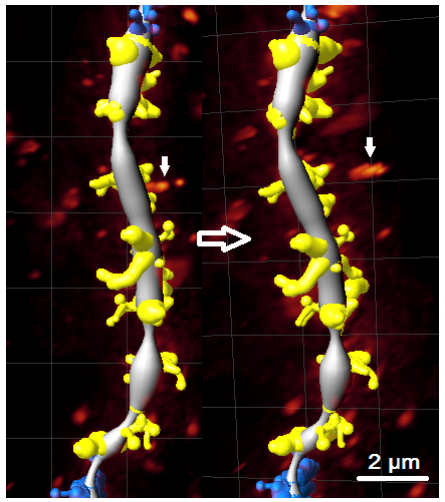

Figure D.9: Rotation of the 3D reconstruction of a dendrite to ensure a correct filtering. The 3D model and the maximum intensity projection can be rotated to reveal apparent spines or true spines. In the figure, the model is rotated to reveal an artefact that resembles a spine.

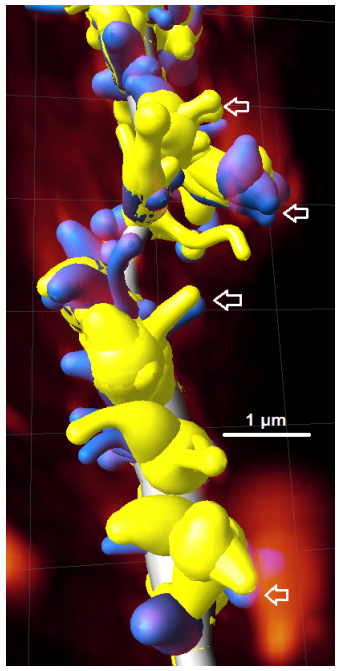

Figure D.10: Production of double spines in the dendrite model. There is an extention of the signal in the Z-axis due to low resolution. Imaris detects double spines where the true spines are located. White arrows show double spines that must be deleted to avoid overestimation of spine number.