The autoimmune hypothesis of narcolepsy and its unexplored clinical features

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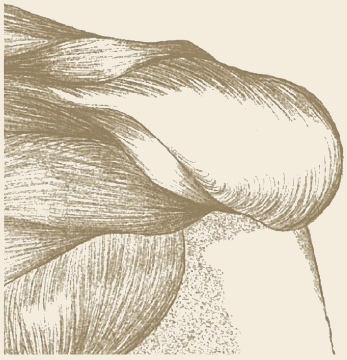

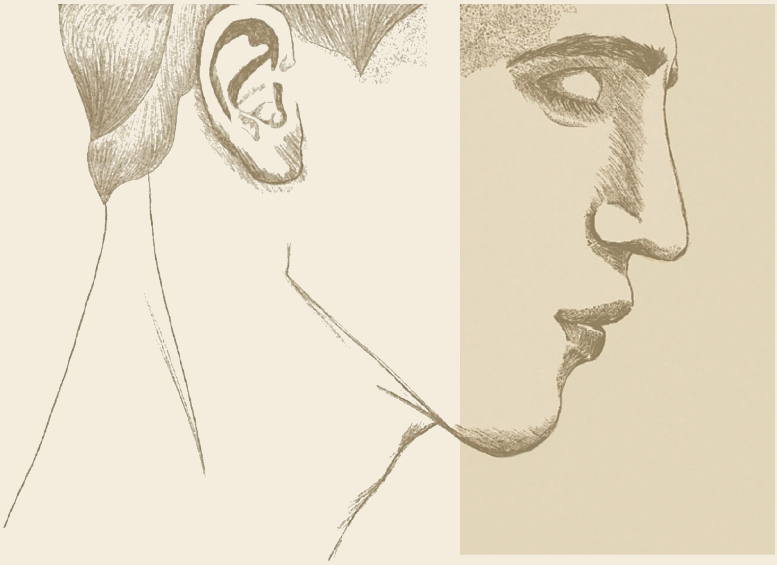

Summary, discussion and future perspectives

This thesis falls apart into two parts: part 1 focuses on the autoimmune hypothesis of narcolepsy, while the second part focuses on NT1 symptoms that are frequently neglected, even though they add significantly to the burden of the disorder.

Autoimmune hypothesis of NT1

The same figure that was shown in the introduction of this thesis is depicted here. Figure 1 displays the hypothesis that antigens from outside the body trigger an immune response in people susceptible for developing NT1 that leads to an autoimmune attack on the hypocretin-producing neurons in the hypothalamus. In the Chapters of this thesis, several of the presumed immune mechanisms involved are investigated: **Chapter 1** describes a potential new trigger for the autoimmune response; **Chapter 2** focuses on HLA-DQB1 associations in NT1 before and after the H1N1 influenza pandemic; **Chapter 3** describes the possible role of cross-reactive CD4+ T cells in the autoimmune response leading to NT1; and **Chapter 4** evaluates all immune cells that are depicted in this figure to identify enriched populations in NT1.

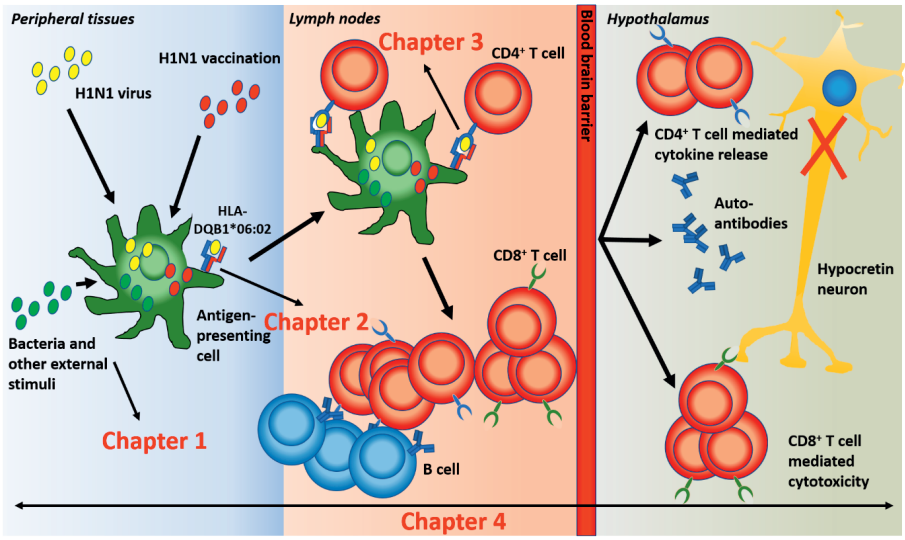

Figure 1. A depiction of the autoimmune hypothesis of narcolepsy and the mechanisms that are investigated in the different Chapters of this thesis.

Chapter 1 describes a NT1 case in which an enteroviral infection may constitute the environmental trigger for the hypocretin-1 deficiency that develops. This has not been described before and highlights the possibility that a variety of infections may trigger the immune system to elicit an autoimmune response targeting the hypocretin-producing neurons. Thereby, this case report stresses the importance of future research focusing on the question how a variety of environmental triggers can lead to a similar outcome: the destruction of hypocretin-producing neurons.

- The short interval between hypocretin-1 measurement and symptom onset in this case suggests that the decrease in hypocretin-1 concentration from normal to undetectable levels may be a process lasting only a few weeks or even less
- Enteroviral infection is a new potential environmental trigger for the autoimmune response leading to NT1

In **Chapter 2,** we describe HLA-DQB1 associations in a cohort of NT1 patients who developed symptoms before or after the H1N1 influenza pandemic. With this approach, we address the postulation of Scandinavian researchers that post-H1N1 NT1 is a separate disease entity. Over the last few years, many studies (Juvodden et al., 2019b, Nordstrand et al., 2019a, Nordstrand et al., 2018, Bomfim et al., 2017, Juvodden et al., 2018, Sarkanen et al., 2016), particularly in Scandinavia, have focused on post-H1N1 only, thereby excluding so called sporadic NT1 patients. We argue, based on the fact that HLA-DQB1 associations have not changed since the H1N1 influenza pandemic, that there is no convincing evidence to justify a distinction between post-H1N1 and sporadic NT1.

- No differences were found in HLA-associations in pre- vs post-H1N1 NT1 patients
- Positive association of pre- and post-H1N1 NT1 with HLA-DQ7 was confirmed
- Negative associations of pre- and post-H1N1 NT1 with HLA-DQ2, -DQB1*05:01, -*06:03 were confirmed
- These results argue against sporadic and post-H1N1 NT1 being separate entities

Chapter 3 describes our study on CD4+ T cells that cross-react with H1N1 influenza and hypocretin peptides. In this study we show that HLA-DQB1*06:02-restricted CD4+ T cell responses against H1N1 influenza peptides are more frequent in NT1 patients than in controls. However, we did not identify any hypocretin peptide-specific CD4+ T cell responses and, thus, no cross-reactivity between H1N1 and hypocretin. This argues against a causal role for these H1N1 peptides in the autoimmune pathogenesis of NT1.

In contrast to our findings, several studies do report the presence of CD4+ and CD8+ T cells that recognize hypocretin (Cogswell et al., 2019, Latorre et al., 2018, Pedersen et al., 2019, Jiang et al., 2019) or show cross-reactivity to hypocretin and H1N1 influenza peptides (Luo et al., 2018). One of the reasons for this discrepancy may be the different techniques that were used. In Chapter 3, we focus specifically on reactivity to H1N1 influenza and hypocretin peptide fragments that were chosen based on their structural resemblance as assessed by crystallography. All other studies used broad peptide pools or peptide libraries, thereby decreasing specificity, but largely increasing the possibility of finding a response.

Moreover, we validated the supposed specificity of expanded cells in T cell cultures with the H1N1 influenza and hypocretin peptides. We assessed proliferation of T cell clones generated from these T cell cultures, upon a challenge with a hypocretin or H1N1 influenza peptide fragment. This essential step was not performed in two of the aforementioned studies (Luo et al., 2018, Cogswell et al., 2019), thereby rendering conclusions about the specificity of T cell cultures in those latter studies uncertain.

The authors of the first study (Luo et al., 2018) responded to our study in the Journal of Neuroimmunology (Mignot et al., 2019). Firstly, they stress the importance of performing experiments with C-amidated peptides, since they only demonstrated cross-reactivity between hypocretin and H1N1-haemagglutinin peptides when hypocretin peptides were C-amidated. We have unfortunately not been able to repeat our experiments with these peptides to ascertain whether this difference indeed explains the differences between the outcomes of both studies. Secondly, Mignot et al. state that the conclusion that we could not find evidence for molecular mimicry with the peptides tested, was premature, because our sample size would not allow for detecting cross-reactivity to begin with, as we only measured 33 T cell clones. We do not agree with this comment. In our publication, we tested peripheral blood of 81 NT1

patients and 19 HLA-matched healthy controls. In none of our proliferation experiments with T cell lines stimulated with both peptides, we found any proliferative response to the two hypocretin peptides used. Additionally, we isolated H1N1-$HA_{275–287}$-specific T cell clones (157 T cell clones from 12 patients, shown in Table 3.3 in **Chapter 3**) in which we have not been able to detect any reactivity to both hypocretin peptides.

Thirdly, Mignot et al. state that the depth of our TCR sequencing is too low to be able to find any sequences common for NT1. We agree with them on this point, but this was not the primary focus of our project, we elaborate on this limitation of our study in the Discussion section of **Chapter 3**.

Important unsolved issues in studies showing autoreactivity to hypocretin are the identification of hypocretin-specific CD4+ and CD8+ T cells in healthy controls (Jiang et al., 2019, Pedersen et al., 2019) and the finding of HLA-DR-restricted instead of HLA-DQB1*06:02-restricted hypocretin-specific T cells (Latorre et al., 2018), as would have been expected based on the autoimmune hypothesis.

To conclude, the discussed studies add complementary information to the autoimmune hypothesis of narcolepsy. It seems more and more probable that the H1N1 influenza pandemic has a direct link with the development of narcolepsy type 1, but direct evidence of T cells being implicated in the autoimmune destruction of hypocretin-producing neurons is still missing.

- Hypocretin and H1N1-hemagglutinin peptides that are bound to HLA-DQ6 show structural homology
- H1N1-hemagglutinin-specific $CD4^+$ T cells are detected in NT1 patients and controls
- Cross-reactivity between H1N1-hemagglutinin and hypocretin peptides is not observed
- H1N1-hemagglutinin-specific $CD4^+$ T cell reactivity is HLA-DQ6-restricted
- There is no biased expression in the H1N1-hemagglutinin-specific T cell receptor repertoire

The fact that direct evidence for the autoimmune hypothesis of NT1 is lacking, led us to believe that pursuing a different approach to identifying the mechanisms responsible for the destruction of the hypocretin-producing neurons is needed. In **Chapter 4**, we describe our mass cytometry experiments that allow us to compare the immune cell composition in the peripheral blood of NT1 patients with that of healthy HLA-matched controls. This method allows for an in-depth analysis of differences in immune cell composition between both groups. In this way, it is possible to assess the immune system in an unbiased fashion. Our results show several memory $CD4^+$ and $CD8^+$ T cell populations expressing activation markers and regulatory $CD4^+$ T cells indicative of an activated phenotype that are more frequent in NT1 patients with recent disease onset compared with HLA-matched controls. These differences seem to support the autoimmune hypothesis of narcolepsy. However, since differences are mainly found in small populations, they should be regarded with caution and at present can only serve as starting point for identifying novel mechanisms involved in the autoimmune response leading to the destruction of hypocretin-producing neurons. Furthermore, experiments aiming on replicating our findings are very important, since interindividual variability is high and we were only able to replicate a minority of the differences between NT1 patients and healthy controls that were reported previously. Performing experiments on immune cell reactivity to antigens of interest (such as hypocretin, H1N1 or other, still unknown antigens) with the identified NT1-specific immune cell clusters seems a promising strategy, which allows for diminishing the background noise that other immune cells might have caused in experimental research on the autoimmune response leading to NT1 executed until now.

- NT1 patients with recent disease onset have increased frequencies of several memory $CD4^+$ and $CD8^+$ T cell populations compared to HLA-matched controls
- Differences in immune cell composition are small between NT1 patients with recent onset compared to those later after onset
- Identified $CD4^+$ and $CD8^+$ T cell populations seem plausible candidates for identifying mechanisms involved in the autoimmune response leading to the destruction of hypocretin-producing neurons

Future perspectives

The remaining question that this thesis does not touch upon, however, is whether we are searching for an autoimmune response to the right antigen. Thus far, hypocretin has always been regarded as the sole candidate antigen targeted in the autoimmune response in the development of NT1. This seems logical, since hypocretin is not produced in other neurons in the hypothalamus and hypocretin deficiency is strongly linked to the phenotype of the disorder (Lin et al., 1999, Nishino et al., 2000a, Peyron et al., 2000). However, whether other proteins or peptides exist that are relatively specific for hypocretin-producing neurons is largely unknown. Several studies have suggested genes that are relatively specific for hypocretin-producing neurons. However, this data is mostly derived from mouse studies (Liu et al., 2015, Romanov et al., 2017, Cvetkovic-Lopes et al., 2010, Dalal et al., 2013, Mickelsen et al., 2017, Seifinejad et al., 2019). Only one study uses posterior hypothalamus transcriptome data from human post-mortem brains (Honda et al 2010). Further information on the human expression levels of these genes in the hypocretin-producing neurons, or even in the hypothalamus, is lacking. The recent study in which hypocretin-specific CD8+ T cells were identified in healthy controls and NT1 patients was the first to compile data of these studies attempting to identify alternative candidate antigens and also perform experiments to assess reactivity to those antigens (Pedersen et al., 2019). Even though they did not find convincing evidence for autoreactivity, this approach seems promising. However, more reliable data on proteins being produced in hypocretin-producing neurons in humans is a prerequisite for designing experiments assessing immune responses to alternative candidate antigens other than hypocretin in NT1 patients. With the emergence of open access single cell RNA sequencing datasets and *in silico* data analysis methods becoming more and more advanced, identifying these alternative candidate antigens becomes feasible.

Frequently neglected symptoms of NT1

The second part of this thesis focuses on clinical symptoms of NT1. The assumed strong causal and temporal association between the destruction of hypocretin-producing neurons and the appearance of symptoms of NT1 is reinforced in **Chapter 1**. In this Chapter, we describe a case that shows that the relation between the development of hypocretin deficiency and the appearance of NT1 symptoms is causal and can be a process of only weeks. The case is

unique in terms of the short interval between a normal hypocretin-1 value in the cerebrospinal fluid and the first clinical signs of the disease. A second lumbar puncture confirmed the diagnosis of NT1 showing a complete disappearance of hypocretin-1 from the cerebrospinal fluid. In this chapter, we describe the pentad of core symptoms of NT1, but we also highlight the weight gain that the patient experienced.
The weight increase that frequently emerges early in the disease, as in the patient described in Chapter 1 (Daniels, 1934, Wang et al., 2016, Nordstrand et al., 2019b), is one of the most debilitating symptoms. Especially in children who develop NT1 this symptom plays a central role in the burden of the disease (Ponziani et al., 2016).

In **Chapter 5**, we describe a retrospective study on NT1 patients who start using sodium oxybate. Weight loss has been described as a side effect of this medication in one of the hallmark clinical trials on the effect of sodium oxybate in NT1 (The U.S. Xyrem® Multicenter Study Group, 2003) and in our outpatient clinic, patients using sodium oxybate frequently report a decrease in weight when they start using this compound. We compared NT1 patients that started using modafinil with those who started using sodium oxybate and found that sodium oxybate was associated with a mean BMI decrease of 0.84 kg/m^2 in men and 2.56 kg/m^2 in women. NT1 patients that started using modafinil did not significantly lose weight.

Several explanations for both the weight increase when developing NT1 and the subsequent weight loss when initiating sodium oxybate treatment have been proposed. Inactivity (Middelkoop et al., 1995), medication use (Black SW, 2015) and different eating habits (Lammers et al., 1996) as a cause for obesity in NT1 have not been confirmed, even though several symptoms of eating disorders, such as food craving and binge eating were reported in a majority of NT1 patients (Fortuyn et al., 2008). One study reported a lower basal metabolic rate (BMR) close after disease onset in children with NT1, which restored to normal levels in the following months (Wang et al., 2016). Therefore, the effect of NT1 on the individual body mass index (BMI) set point is regarded as the most important cause of the observed obesity. Recent observations suggest that also food-related responses in the cortex are enhanced in NT1 and could potentially contribute to increased food intake leading to obesity, even though this effect on intake has not been shown (van Holst et al., 2018).

Our findings on the effect of sodium oxybate use on body weight are in line with other studies that suggest that NT1 patients lose weight when using sodium oxybate (Boscolo-Berto R, 2012, Husain et al., 2009). Explanations for this treatment effect are lacking. However, recent pre-clinical research suggests that energy-combusting brown adipose tissue may be causally involved in the development of obesity in NT1 (Madden et al., 2012, Sellayah et al., 2011).

- NT1 patients lose weight upon initiating sodium oxybate treatment
- Modafinil use does not lead to a weight decrease in these patients
- The effect of sodium oxybate on weight is more pronounced in women and patients with a high BMI

Chapter 6 contains our findings on daytime sleep-state misperception in NT1 and other sleep disorders. In this prospective study, we assessed the occurrence of, and factors influencing sleep state misperception in several sleep disorders with excessive daytime sleepiness. We looked at both classical and reverse sleep state misperception and found that sleep state misperception is common in people with NT1 and -2, idiopathic hypersomnia, obstructive sleep apnea and insufficient sleep syndrome. Classical sleep state misperception is more frequent in patients with longer sleep onset latencies who only reach non-REM sleep stage 1 during a nap.
Daytime sleep state misperception is a relevant symptom for NT1 patients, since it may influence the capacity to notice daytime naps. In this way, information on the prevalence of daytime sleep state misperception is valuable for both patient and sleep physician when assessing disease severity and medication effect. Based on our findings, this is not only the case for NT1, but also for patients with other, much more prevalent, sleep disorders with excessive daytime sleepiness, such as obstructive sleep apnea and insufficient sleep syndrome. Hence, information on daytime sleep state misperception could also provide valuable information in the assessment of disease severity and treatment effect in other sleep disorders (Liu et al., 2018).

- Daytime sleep state misperception was measured during the short naps of the multiple sleep latency test
- Daytime sleep state misperception is prevalent in NT1 and -2, and other sleep disorders

- A long sleep latency and the occurrence of only NREM stage 1 sleep both predict daytime sleep state misperception
- Patients and physicians should take the presence of daytime sleep state misperception into account when making treatment decisions

Future perspectives

Scientific data on the pentad of NT1 core symptoms is gathered in almost every clinical trial or cohort study that is performed in NT1. Even though the primary endpoint in most studies is either cataplexy frequency or excessive daytime sleepiness, the effect of interventions on hypnagogic hallucinations, sleep paralysis and disturbed night sleep is frequently reported as a secondary endpoint. However, the burden of the disease is only partly dictated by these symptoms. Other symptoms, or neglected expression of core symptoms, such as weight gain, disturbed vigilance, depressive symptoms and sleep state misperception, are adding to the social disability, educational difficulties and mental problems that NT1 patients experience in everyday life. Clinical trials and studies using information derived from prospective clinical databases, that describe the effect of interventions on these other symptoms, are much needed. It will greatly support NT1 patients' futures to be able to treat their symptoms that are not part of the core pentad, based on clinical evidence.

Based on **Chapter 5**, it is clear that weight gain can be influenced by medication, but also that future research should focus on the mechanisms that underlie weight gain in NT1. Investigating the role of brown adipose tissue in both weight gain and subsequent weight loss upon initiation of sodium oxybate treatment seems promising. Many preclinical studies suggest a role for the hypocretin system in brown adipose tissue activation (Madden et al., 2012, Sellayah et al., 2011), but also in the differentiation and development of this tissue (Rogers et al., 2012, Sellayah and Sikder, 2014). However, the first study to assess brown adipose tissue in NT1 patients did not find any differences when its activation was compared to that of healthy controls, but the sample size was small and the protocol did not allow for adequate maximal brown adipose tissue activation measurements (Enevoldsen et al., 2018). Prospective larger studies are needed to evaluate the role of brown adipose tissue in the weight gain accompanying the development of NT1.

The mechanism that leads to the weight loss that follows the initiation of sodium oxybate treatment in NT1 might also involve brown adipose tissue activation, since the main metabolite of sodium oxybate, succinate, was recently shown to strongly induce brown adipose tissue thermogenesis (Mills et al., 2018).

Chapter 6 on the other hand, shows that symptoms that are not easily recognized, such as sleep state misperception, might influence a patient's ability to assess daytime function adequately and might therefore influence treatment decisions. Being aware of the presence of these symptoms is important for sleep physicians and should always be taken into account when assessing treatment effect. Gathering information on daily functioning from relatives or other people that are close to the patient seems pivotal in adequately assessing the burden of the disease for that patient. This is also highly relevant when performing clinical research, since excessive daytime sleepiness as a primary endpoint is often measured by questionnaires. Developing tools that enable researchers to include symptoms such as sleep state misperception and disturbed vigilance in the outcome parameters of clinical trials could be a first step to acknowledging the diversity of symptoms that make up the burden of NT1.

Overall conclusion

Both basic and clinical research on NT1 have increased rapidly over the past two decades, sparked by the discovery of hypocretin in the late 1990s. However, many questions remain unanswered. A clear understanding on what mechanisms drive the presumed autoimmune response leading to the development of NT1 is pivotal to eventually develop a causal treatment for or prevention of the disorder. This thesis describes several factors that might play a role in the pathophysiological process. At the same time, interest in invalidating consequences beyond the core clinical symptoms slowly but steadily increases, as Chapter 5 and 6 show. For NT1 patients this is arguably even more important than small improvements in preventing daytime sleep and the occurrence of cataplexy.