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elucidating underlying mechanisms

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ANTINOCICEPTIVE AND ANALGESIC EFFECTS OF BOTULINUM NEUROTOXIN TYPE A: ELUCIDATING UNDERLYING MECHANISMS

BY
LARISSA BITTENCOURT DA SILVA

DISSERTATION SUBMITTED 2014

AALBORG UNIVERSITY
DENMARK

ANTINOCICEPTIVE AND ANALGESIC EFFECTS OF BOTULINUM NEUROTOXIN TYPE A: ELUCIDATING UNDERLYING MECHANISMS

Ph.D. Thesis

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Preface

The present thesis is based on the work done at the Laboratory of Experimental and Clinical Pharmacology, Centre for Sensory-Motor Interaction (SMI); Laboratory for Cancer Biology (LCB), Biomedicine, Aalborg University, Denmark; Department of Neurology, Aalborg University Hospital and Orthopedic Surgery Research Unit, Aalborg Hospital Science and Innovation Centre, Aalborg University Hospital, from January 2011 to August 2014. This thesis is based on three performed studies that are referred as Study I, II and III. These studies also yielded three original full-length papers that are either published or submitted to relevant international peer-reviewed journals. In addition, four conference abstracts, presented as posters in pain scientific meetings complement the scientific work conducted in this Ph.D. project.

- Paper I: *da Silva LB, Kulas D, Karshenas A, Cairns BE, Bach FW, Arendt-Nielsen L, Gazerani P.* **Time course analysis of the effects of botulinum neurotoxin type A on pain and vasomotor responses evoked by glutamate injection into human temporalis muscles.** *Toxins***.** 2014;6(2):592-607.
- Paper II: *da Silva LB, Karshenas A, Bach FW, Rasmussen S, Arendt-Nielsen L, Gazerani P.* **Blockade of glutamate release by botulinum neurotoxin type A in humans: a dermal microdialysis study.** *Pain Research & Management.* 2014;19(3):126-132.
- Paper III: *da Silva LB, Poulsen JN, Arendt-Nielsen L, Gazerani P.* **Botulinum neurotoxin type A modulates calcium-dependent vesicular release of glutamate from trigeminal satellite glial cells.** *Accepted to the Journal of Cellular and Molecular Medicine.* 2014.
- Abstract I: *da Silva LB, Kulas D, Karshenas A, Cairns BE, Bach FW, Arendt-Nielsen L, Gazerani P.* **Time-course of analgesic effects of botulinum neurotoxin type A (BoNTA) on human experimental model of pain induced by injection of glutamate into temporalis muscle.** *Scandinavian Journal of Pain.* 2012;3(3):187, No. T7.
- Abstract II: *da Silva LB, Kulas D, Karshenas A, Cairns BE, Bach FW, Arendt-Nielsen L, Gazerani P.* **Pain, sensitization, and vasomotor responses following intramuscular administration of glutamate into temporalis muscle in healthy men and women.** *In Abstracts of the 14th World Congress on Pain, International Association for the Study of Pain, IASP, 27-31 August 2012, Milan, Italy. International Association for the Study of Pain, IASP.* 2012. p. No. PF 426.

- Abstract III: *da Silva LB, Karshenas A, Rasmussen S, Bach FW, Arendt-Nielsen L, Gazerani P.* **Blockade of glutamate release by botulinum neurotoxin type A in a human experimental pain model: a dermal microdialysis study.** *In Abstract Book of the 8th Congress of the European Federation of IASP Chapters (EFIC),* 9-12 October 2013, Florence, Italy. *European Pain Federation EFIC.* 2013. p. No. 444.
- Abstract IV: *da Silva LB, Poulsen JN, Arendt-Nielsen L, Gazerani P.* **Effect of botulinum neurotoxin type A on vesicular release of glutamate from trigeminal satellite glial cells.** In 15th World Congress on Pain, 6-11 October 2014, Buenos Aires, Argentina. International Association for the Study of Pain/IASP Press. 2014. p. No. PF036.

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List of abbreviations

AUC	Area under the curve
BAPTA	(1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BoNTA	Botulinum neurotoxin type A
CGRP	Calcitonin gene-related peptide
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyography
FLPI	Full-field laser perfusion imager
GCP	Good clinical practice
HPLC	High performance liquid chromatography
IASP	International association for the study of pain
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LDF	Laser Doppler flowmetry
LDI	Laser Doppler imager
MPQ	McGill pain questionnaire
NMDA	N-methyl-D-aspartate receptor
PPT	Pressure pain threshold
SGCs	Satellite glial cells
SNARE	Soluble NSF attachment protein receptor
SNAP-23	Synaptosomal-associated protein 23 kDa
SNAP-25	Synaptosomal-associated protein 25 kDa
TG	Trigeminal ganglia
TMD	Temporomandibular disorder
TRPV1	Transient receptor potential vanilloid 1
VAS	Visual analogue scale

Summary

Despite advances in pain treatment, many patients do not achieve optimal pain relief or experience significant side effects from current medications. Botulinum neurotoxin type A (BoNTA) is a compound that has long been used for patients seeking muscle relaxation due to neuromuscular disorders and excess of muscle tension. In these patients, an additional pain relief effect was observed after BoNTA treatment, which led to further investigation on its potential analgesic efficacy. In 2010, BoNTA was approved for prophylaxis of chronic migraine; however, its mechanism of action is not fully known yet. Besides, BoNTA has become an option for many painful disorders in off-label studies. Elucidating its underlying mechanisms would enhance the possibility of further understanding its action and potentially its approval for other painful conditions. Moreover, BoNTA can be used as a pharmacological tool to study responsiveness of both neuronal and non-neuronal cells to different stimuli and substance release. Therefore, the purpose of this Ph.D. was to investigate potential BoNTA analgesic and antinociceptive mechanisms in 3 distinct studies applying different approaches. The first study investigated the time-course of pain relief from BoNTA in a human experimental pain model using psychophysical approaches and tissue imaging techniques. Healthy subjects were pre-treated with BoNTA in the temporalis muscle and saline as a control in the contralateral side and were followed in 4 sessions over 2 months. In each session, subjects received an artificially induced pain stimuli evoked by intramuscular injection of glutamate. BoNTA was able to exert its effect as early as 3 hours after its treatment, and this effect remained for 4 weeks, peaking at week 1. The second study involved a direct approach to measure glutamate, a potential substance released under painful condition, by means of a technique called microdialysis. Healthy volunteers received a subcutaneous injection of BoNTA in one forearm and saline in the other, and a week later, they were challenged by application of an experimental pain model of topical capsaicin combined with mild heat. This study revealed that BoNTA was able to reduce glutamate release in response to capsaicin pain model. Besides, BoNTA decreased pain and blood perfusion in the treated area when compared to the control side. Most studies have been focused on neuronal effect of BoNTA. Since the role of non-neuronal cells in pain is being revealed gradually, the third study was designed as a basic back-translational study on cells, to investigate effects of BoNTA on satellite glial cells (SGCs) of the peripheral nervous system from the trigeminal ganglia (TG) of rats. Isolated cells were cultivated for 7 days and were challenged by ionomycin to release glutamate after 24h of pre-incubation with BoNTA. In addition, *in vitro* and *in vivo* staining techniques were used to detect the presence of SNAP-25 and 23, the docking proteins that BoNTA cleaves to exert its effect and possibly inhibit the vesicular release of several substances that play a role in pain. This study revealed that SNAP-25 and 23 are present in isolated cells as well as in sections of the TG. BoNTA, at a concentration

of 100pM, could block ionomycin-evoked glutamate release that is most likely by cleaving SNAPs. This study not only showed vesicular release of glutamate from SGCs, but also confirmed that BoNTA can exert inhibitory effects on non-neuronal cells of the peripheral nervous system. Results from these three studies advanced our understanding of the potential analgesic/antinociceptive mechanisms of BoNTA that eventually can yield beneficial evidence for further and extended application of this agent in several painful conditions.

1. Dansk sammenfatning

Antinociceptive og analgetiske effekter af Botulinum neurotoksin type A: forklaring af de underliggende mekanismer

På trods af fremskridt inden for smerteforskningen oplever mange patienter, at de ikke får optimal smertelindring eller får betydelige bivirkninger fra den medicin, der anvendes til behandling af deres lidelse. Botulinum neurotoksin type A (BoNTA) er et stof, som i flere år har været anvendt som muskelafslappende middel på patienter, der lider af neuromuskulære sygdomme samt muskelspændinger. Hos disse patienter ses smertelindring efter behandling med BoNTA. Dette har ført til yderligere studier i stoffets analgetiske virkning. I 2010 blev BoNTA godkendt til brug for kronisk migræne; dog kendes mekanismen bag virkningen ikke fuldt ud. BoNTA anvendes derudover som mulig behandling på smertefulde lidelser i off-label studier. Hvis de underliggende mekanismer blev belyst, ville det give en yderligere forståelse af stoffets virkning og dermed potentielt godkendelse til behandling af andre smertefulde lidelser. BoNTA kan anvendes som et farmakologisk værktøj til at undersøge følsomheden i både neuronale og ikke-neuronale celler i forhold til forskellige stimuli og frigivelse af stoffer. Formålet med denne ph.d.-afhandling var derfor at undersøge BoNTAs analgesiske og antinociceptive mekanismer i tre særskilte studier med forskellige fremgangsmåder. Det første studie undersøgte tidsforløbet for virkningen af BoNTA i en human eksperimentel model, der anvendte psykofysiske tilgange og vævsbilledteknikker. Raske forsøgspersoner blev forbehandlet med BoNTA i m. temporalis samt saltvandsopløsning som kontrol i den kontralaterale side, og forsøgspersonerne blev herefter fulgt i fire forsøgssessioner over to måneder. I hver session påførtes kunstige smertestimuli vha. intramuskulær injektion af glutamat. BoNTA kunne vise sin virkning så tidligt som tre timer efter smertestimulationen, og denne virkning fortsatte i fire uger, med maksimal virkning i den første uge. Det andet studie bestod af en fremgangsmåde til at måle glutamat; et stof, som frigives under smerte, ved hjælp af den såkaldte mikrodialyse-teknik. Raske forsøgspersoner fik en subkutan injektion med BoNTA i underarmen og saltvandsopløsning i den anden underarm. Efter en uge påførtes en smertemodel med topisk capsaicin i kombination med let varme. Studiet afslørede, at BoNTA kunne reducere glutamatfrigivelsen som reaktion på capsaicin-modellen. Derforuden formindskede BoNTA smerten og blodgennemstrømningen i det behandlede område sammenlignet med kontrolområdet. De fleste tidligere studier har fokuseret på den neuronale effekt af BoNTA. Da ikke-neuronale cellers rolle i smerte gradvist afdækkes, var det tredje studie designet som et bagudrettet translationelt basisstudie i celler til undersøgelse af BoNTAs virkning på satellit-gliaceller (SGC) i det perifere nervesystem fra den trigeminale ganglion (TG) hos rotter. Isolerede celler blev dyrket i 7 dage og blev påvirket ved hjælp af ionomycin til at frigive glutamat efter 24 timers præinkubation med BoNTA.

2. Introduction

Pain is a debilitating condition that affects millions of people worldwide every year and chronic pain still remains as one of the major under-estimated and under-treated disorders (1). In 2006, a study estimated that 19% of Europeans suffered from moderate to severe pain and, among those, most did not visit a pain specialist, and 40% alleged their treatment was not adequate (2). In Denmark, a fairly recent study estimated that 16% of the general population suffered from chronic pain. As in most countries, in Denmark, chronic pain is also undertreated, and more than 80% of chronic pain patients had never seen a pain management specialist (3).

Pain is also a burden for societies from an economical point of view, impairing the productivity at work due to sick leaves, low functioning, and demanding enormous amounts of financial aid from governments (4). In a recent report by Global Industry Analysts, it was estimated that the global pain management market will reach US$60 billion by 2015. Besides, pain also relentlessly distresses patients' social and personal life, reducing the quality of life of individual sufferers and their families significantly. Patients' response to one treatment is not always in a similar way and adding comorbidities to a great variability can start the long path to find the optimal management plan (2,4).

It is estimated that currently analgesic medications provide 50% of pain relief for about 30% of pain patients' population (5). Undoubtedly, many patients still suffer from suboptimal treatments and side effects from current medications. Pain research, after many decades, still faces several obstacles. It is imperative the need of more research to pursue better treatment options, reliable and direct tools to assess pain, and determine if patients will positively respond to the current treatments. In order to achieve this, it is important to better understand pain pathways to target those efficiently. Another issue in this research field is the translational aspects, which are not always predictable and effective (6). This means that even drugs that are proved effective in pre-clinical phases may not always be effective when they move to human clinical trials. Analgesic drug development is therefore very challenging and not always successful (5,7).

BoNTA has been showing promising results for pain treatment and more research is needed to show the toxin efficacy in different pain conditions to elucidate its mechanism, optimal dosing, and safe application to further expand its approval for other painful conditions. One of the main advantages of this compound is its long-lasting effect (8) and, in the last years, researchers have shown that BoNTA might be a suitable treatment option for several pain patients with different painful conditions (9-11). However, besides chronic migraine, its use for all other pain conditions still remains off-label.

2.1. Human experimental pain models

Studying a potential analgesic compound or investigating underlying mechanism in pain patients is challenging. In addition to some ethical limitations, most certainly, it would yield a great variability in outcomes due to variable nature of pain in this targeted population; hence, making it unreliable to interpret or replicate the obtained data. Besides, since in many cases pain mechanisms are not easily understood, the complexity would be even larger in comparison with other medical fields (12,13). Experimental pain trials can therefore offer potential possibilities to overcome some of these challenges (14). One of the main advantages of experimental pain models is that one can partly focus on a particular involved mechanism and better assess the outcomes excluding confounding factors. However, only particular pain induction and assessment methods can be used in human volunteers or patients due to ethical limitations or safety reasons. Even though, when an experimental pain model is validated and well-established, it can be used for screening of potential analgesic compounds in early phases of drug development in a reliable and reproducible manner (15,16).

Experimental pain models can be used as a tool to help researchers to deeper investigate detailed aspects of a specific nociceptive input. Even though these models might not exactly mimic clinical situations, they still aid providing better insights for clinical studies and are broadly used to peripheral and central aspects of pain research. All available pain models share two common features: they utilize a standardized method to activate the nociceptive system, creating a painful stimulus and, once that is reached, a variety of measurements are used to assess the outcomes, that can be based on psychophysical, electrophysiological, or imaging techniques (17). These models can also be used to investigate hyperalgesia, that is an increased pain sensation caused by a normal noxious stimulus or allodynia, which is a painful experience caused by a stimulus that is usually not painful (14). These phenomena are often seen in chronic pain patients; thus experimental models that can mimic these aspects are highly relevant.

Several human experimental pain models have been tested and established and are now available and broadly used to induce an artificial state of pain. Pain induction methods vary from induction by aid of thermal, electrical, mechanical to chemical stimuli isolated or combined. The ideal pain stimulation would be the one that evokes a distinctive pain, exciting only certain nociceptive fibers, with as minimal tissue damage as possible and with a relationship between the stimulus and pain intensity. Besides, it should have a reliable intra and inter-session reproducibility (13-15,18).

In order to enhance the feasibility of an experimental model, it is imperative the need of a planned study design bearing in mind the available options for the pain model as well as for the measurement techniques. To better choose the optimal pain model, the researcher has to consider

the characteristics of a model and the purposes of an experiment, making sure the assessment techniques are relevant and available (14,15). For instance, in this Ph.D. project, two experimental models of pain were applied: injection of glutamate and topical capsaicin combined with heat stimulation. Both these models have been used safely in the humans with acceptable and reliable features required for studying pain pathways and analgesic drug mechanisms for this project.

2.2. Glutamate

Glutamate is a well-known excitatory neurotransmitter with an important mediatory and modulatory role in nociception and sensitization and is thoroughly investigated in the central nervous system (19). However, many evidences show that glutamate is also an important component involved in peripheral nociception (20,21). In animals, elevated levels of interstitial glutamate were found in peripheral tissues after nerve stimulation, capsaicin, and formalin induced pain model (22,23). In humans, increased levels in peripheral glutamate release have been observed due to chronic and inflammatory conditions (24-27). There is evidence that non-neuronal cells can also release glutamate, like astrocytes (28) and Schwann cells (29), contributing to maintaining the pain state and possible central and peripheral sensitization, respectively. It has also been shown that activation of peripheral glutamate receptors contributes to nociception and peripheral sensitization (30-32) and that the number of glutamate receptors is increased in the periphery during inflammation (33). It is evident that glutamate and its receptors are involved in pain pathways and it is believed that the modulation of both glutamate and its receptors could be used as a potential option to treat persistent pain (both neuropathic and inflammatory pain) (22,34).

Since glutamate is involved in pain modulation, it has been considered and used as a potential substance in experimental pain model. Glutamate injection has been used to evoke experimental pain in animals and humans (30,35-39). Injection of glutamate in the peripheral tissues activates peripheral glutamate receptors and has been used as a model to induce pain, sensitization, and neurogenic vasodilation. For example, glutamate injection in the masseter muscle or in the temporomandibular joint has been used as a TMD (temporomandibular disorder) pain model both in animals and humans (35,37,40).

It has been broadly shown that the injection of glutamate into human muscles evokes pain and mechanical sensitization and that is mediated, at least in part, through activation of peripheral glutamate receptors (30,37,38,41,42). When ketamine, an NMDA receptor (N-methyl-D-aspartate receptor) antagonist was injected, it could block the glutamate induced pain and revealed that glutamate receptors are involved in the peripheral effect of glutamate to induce pain (43). Glutamate-induced neurogenic vasodilation was also found to be mediated in part by the vasodilatory neuropeptides calcitonin gene-related peptide (CGRP) and, to a lesser extent, by

substance P. In study I of this Ph.D. project, it was hypothesized that if glutamate release is contributing to peripheral pain and sensitization, blockade of its release would result in reduced pain and sensitivity in the peripheral tissues. Therefore, an injection of glutamate in the temporalis muscle was used as an experimental pain model in presence and absence of BoNTA, which is a compound that can block the release of neurotransmitters, such as glutamate via SNARE dependent mechanisms.

2.3. Capsaicin

Capsaicin is the active compound from chili pepper that gives the hot and pungent sensation. This extract is also largely used as a pain model that causes a burning pain and a distinct flare reaction. Capsaicin-evoked pain, sensitization, and vasomotor reactions may partially mimic the mechanism underlying both inflammatory and neuropathic pain. When injected or applied topically, it activates the transient receptor potential vanilloid 1 (TRPV1) located on C-fibers of peripheral nociceptive sensory neurons (44,45) and provokes release of some substances, e.g. glutamate and/or CGRP. While the capsaicin injection produces a faster and stronger pain response for a short period of time, the topical application takes a longer time to get a similar response, but provides a more stable plateau. The effect seen following the topical formulations of capsaicin is highly influenced by skin stratum cornea depth, the skin vascular network, and temperature. In comparison between the routes of administration of capsaicin, the injection remains more reliable than the topical application, since it is not easy to determine exactly the concentration of capsaicin that is being absorbed and metabolized by the body to reach the expected response (44).

Interestingly, capsaicin is also used in a topical formulation to treat pain, including neuropathic pain (46), painful HIV neuropathy (47), and postherpetic neuralgia (48). The first usage is usually done after local anesthesia, because patients will experience the burning pain and sensitization caused by capsaicin. In the second treatment, they face a reduced sensitivity period and after repeated topical capsaicin application, they enter a desensitization phase. It is believed that the mechanism of action for this desensitization is through the depletion of substance P from the sensory nerve endings after repeated capsaicin application (49). Low concentration creams and lotions have been used for a few decades; however, recently, a high concentration capsaicin patch 8% (Qutenza®) was approved for pain treatment of painful peripheral neuropathies. The patch application is usually for 30 minutes and pain relief is due to last for 12 weeks (50,51). The phenomenon of first application causing burning pain with capsaicin patch was therefore used as the basis for the experimental pain model in the study II described in this Ph.D. thesis. Hence, it was combined with mild heat to increase the response. The heat/capsaicin sensitization model was established in 1999 by Petersen KL and colleagues (52), and it is based on the synergistic effect

from capsaicin and the mild stimuli from heat, which together will be greater than both of them applied separately. This model was proved to be more stable than capsaicin alone or heat alone. Since there was no record of using the patch with mild heat in literature, a pilot study was done before study II, where the efficacy of the model was approved. For the described study, it was hypothesized that if glutamate is released following the application of capsaicin plus mild heat, BoNTA can potentially block it. Therefore, release of glutamate in this model was investigated in presence and absence of BoNTA.

4.4. BoNTA

Botulinum neurotoxin type A is one of the seven serotypes of the neurotoxin produced by the gram-positive anaerobic bacterium *Clostridium botulinum* (53). For the last decades, BoNTA has been used in the treatment of disorders characterized by pathologically increased muscle tone, such as hemifacial spasm, dystonia, spasticity, strabismus, and cerebral palsy (54-57). In addition, it was also revealed that when the toxin was injected in the post-ganglionic cholinergic fibers it induced chemical denervation in the area, becoming useful for the treatment of hyperhidrosis and reduction of salivary secretions and drooling (58-60). Early observations in dystonia also demonstrated an analgesic effect of BoNTA (55,61), which led to further investigation on its efficacy for painful conditions. Currently, chronic migraine is the only pain condition that BoNTA treatment is regulated and approved for (62-64). However, BoNTA has proved beneficial and has been used off-labeled as an alternative treatment for several other painful conditions, like neuropathic pain (9,65,66), low back pain (10), myofascial pain syndrome (67,68), fibromyalgia (11) among others.

4.4.1. Mechanism of action

BoNTA's mechanism of action in muscle relaxation is well established, and it has proved useful for conditions with muscle hyperactivity. When injected intramuscularly, BoNTA inhibits the exocytotic release of acetylcholine at the neuromuscular junction leading to muscle relaxation. Following the injection, the toxin molecule is transported to the cytosol of the pre-synaptic neuron and there, the light chain selectively cleaves the SNAP-25, one of the proteins of the SNARE complex. This complex is responsible for the docking of vesicles containing acetylcholine and several other substances and neurotransmitters, e.g. glutamate and CGRP. When SNAP-25 is cleaved, the whole complex is not functional and these storing vesicles cannot attach and fuse to the membrane to release the acetylcholine that would further promote the muscle contraction (69-72). Figure 1 shows a detailed schematic of the mechanism of action of BoNTA in the synaptic cleft.

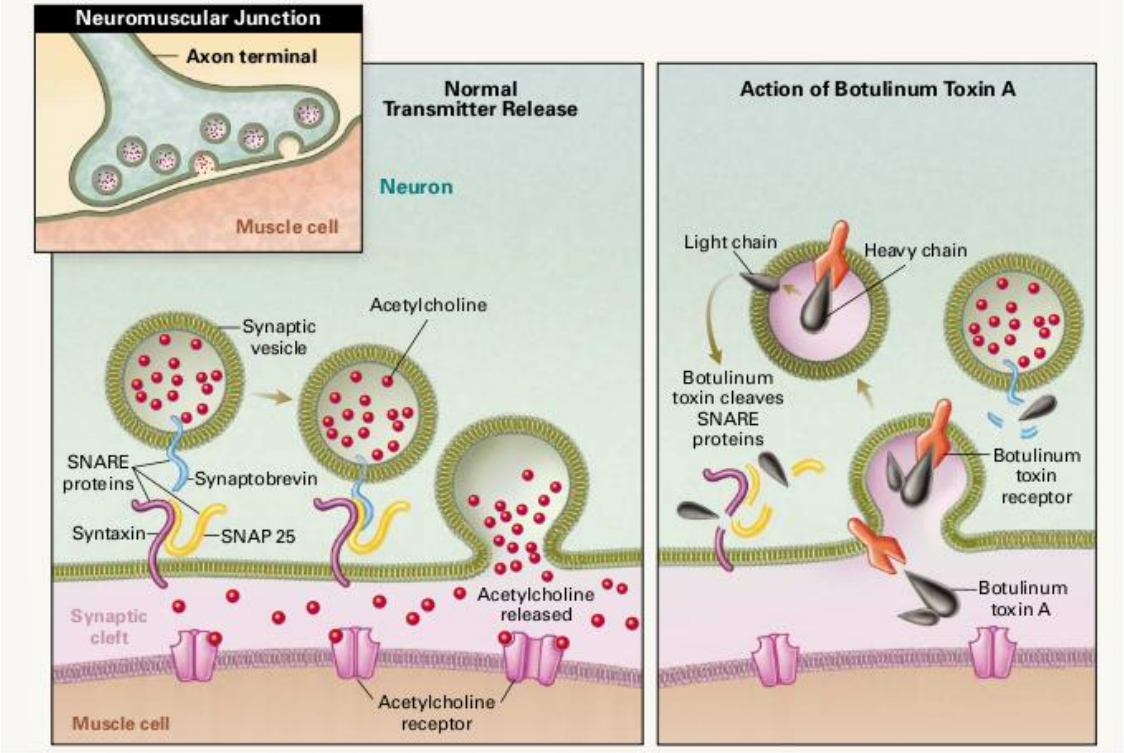

Figure 1: Left panel shows the normal acetylcholine release from the synaptic vesicles. In order to be released, the vesicles get attached to the pre-synaptic membrane through the SNARE proteins, fuse to the membrane, release acetylcholine and the muscle contracts normally. When BoNTA is present (right panel), the botulinum toxin is endocytosed to the motor neuron; the light chain of the toxin is translocated to the cytosol and cleaves the SNAP-25, one of the membrane proteins that form the SNARE complex. As a result, the vesicle containing acetylcholine will not fuse to the membrane, will not release acetylcholine, and the muscle will not contract. This figure shows vesicles containing acetylcholine, but it is believed that the same occurs for other neurotransmitters (e.g. glutamate and CGRP) that are also stored in vesicles and are released through the same mechanism. Reproduced with permission from (73), Copyright Massachusetts Medical Society.

For a long time, it was widely assumed that when injected peripherally, BoNTA wouldn't leave the synaptic terminal and its effects would be confined to the injection site. However, evidences are accumulating for potential central effects of BoNTA when it is peripherally administered. Some animal studies provide direct and indirect evidence that when injected peripherally, BoNTA is retrogradely transported through axonal migration and neuronal transcytosis from the injection site to the centrally located centers of the nervous system (74-77). Further investigation is needed in order to better elucidate the exact mechanism for this transport and whether it is the toxin molecule or the already cleaved SNAP-25 that migrates and exerts its

action. Awareness is being brought to this issue since it can change the clinical applications of BoNTA. Anyhow, it is important to consider that either way, peripheral treatment with BoNTA will have a combination of peripheral and central effects, by either reaching central part by retrograde transport or by indirectly influencing central sensitization. It is proposed that BoNTA blocks the release of nociceptive substances and inhibits peripheral sensitization, which would then result in an indirect decrease of the central sensitization (8,78). This might be, at least in part, one of the underlying mechanisms for analgesic effect of BoNTA in chronic migraine. Most experimental knowledge regarding the antinociceptive mechanisms of BoNTA is based on *in vivo* animal models (79-81) and *in vitro* culture systems (78,82-86). A detailed schematic of BoNTA action in the peripheral and nervous system is exemplified in figure 2.

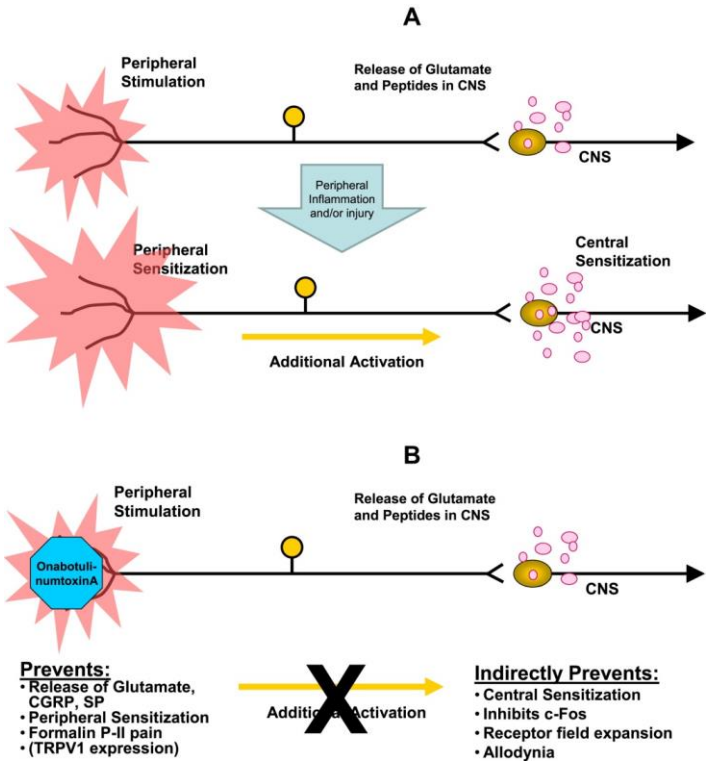

Figure 2: Peripheral and central effects of BoNTA. The upper part of the figure (A) shows the sensitization of the peripheral and central system under normal conditions. When the tissue undergoes an injury or other stimuli, as a response, neuropeptides and inflammatory mediators are released resulting in the peripheral sensitization, which will result in an increased amount of signal through the spinal cord or trigeminal nucleus, inducing the sensitization at the central nervous system (CNS). The lower panel (B) shows how BoNTA influences the peripheral and central sensitized states. When present, BoNTA directly inhibits the peripheral sensitization by blocking the release of neurotransmitters, which, in turn, will indirectly impact and inhibit the central sensitization. Reproduced from (8) with permission from Elsevier.

4.4.2. Analgesic effect

It is believed that BoNTA suppresses the release of substances involved in pain and vasodilation, e.g. glutamate and CGRP (8). The analgesic effect of BoNTA is supported through many experimental pain models in animals and humans that present positive results for BoNTA's antinociceptive or analgesic efficacy (8,9,78,80,87,88). However, efforts are still being made to further investigate BoNTA's effects and elucidate unknown information, e.g. the optimal dose and interval, best route of administration, how far the toxin molecule spreads and its consequence, among many unanswered questions. If a modulatory role is confirmed in pain pathways both in the periphery and possibly at the central levels, BoNTA can actually be an option for many other painful conditions besides chronic migraine, blocking pain transmission and its maintenance.

The beneficial effect of BoNTA in migraine was confirmed through multiple clinical trials and OnabotulinumtoxinA has been recently approved for chronic migraine. The recommended dosing from the manufacturer (Allergan®) is 155 Units divided into 31 injection sites along 7 different muscles, including the craniocervical muscles such as the temporalis, frontalis and trapezius muscle. Even though, the exact mechanism of BoNTA in migraine still remains unknown. Recently, a study indicated that injection of BoNTA into the temporalis muscle, a site for administration of BoNTA for migraine prevention, decreases the mechanical sensitivity of temporalis muscle nerve fibers in female rats (39). It is also believed that the sensitization in the trigeminovascular system may play an important role in migraine pathophysiology (89,90). BoNTA may eliminate local, painful pericranial muscles (migraine triggers) to avoid further attacks. However, pain relief occurs prior to muscular relaxation or in areas with no muscle tension (91). Therefore, muscle tension and relaxation by BoNTA cannot be the sole responsible cause, and prolonged muscle sensitization might be responsible for indirect cause or maintenance of central sensitization.

Even though the efficacy of BoNTA for pain treatment is prevailing, there is still divergence between the analgesic efficacy of BoNTA (92-94) and the time course of effect when comparing clinical and experimental studies. While in the experimental settings the differences are seen from as early as 3 hours after the BoNTA treatments (39) and peaks 1 week after (42), at the clinic, physicians start to notice the analgesic effects later on, and the greatest effect is believed to peak around 1 month after the treatment (62,63).

One of the main advantages of BoNTA can be its long-lasting effect when compared with other analgesics that are limited to frequent intake. This is especially valuable when treating elderly patients or those on polypharmacy where other comorbidities are present (e.g. difficulty in swallowing several pills). Even though injection is the only available route of administration of BoNTA at the present time, which may limit its use due to patients' compliance for injections in

general, that can bring the advantage of reducing the oral intake medication and decreasing the risk of over or under dosage. Besides, the treatment is also considered to have a reasonable safety profile (9,95).

For a long time, it was believed that only neurons were involved in pain pathways, however, in the past years, non-neuronal glial cells became a novel and promising target for pain research and treatment (96,97). Previously, glial cells were acquainted for mainly support of the neurons in the central and peripheral nervous system. Now, however, they are being linked as an important component to pain initiation and its maintenance. By targeting these non-neuronal cells, alterations can be seen at structural levels and by loss of release of pain mediators and pro-inflammatory substances (96,98). Nevertheless, researchers have mainly focused on astrocytes and microglia, the main residents' glial cells of the central nervous system, in connection to pain. However, peripheral glia, e.g. satellite glial cells (SGCs), is also known to contribute to pain transmission. To explore potential role of SGCs and their activity modulation by BoNTA, the study III of this Ph.D. project was designed. It was hypothesized that SGCs can release glutamate through vesicular mechanisms and that this phenomenon can be blocked by BoNTA via interacting on SNARE proteins (SNAP-25 and 23) presented in SGCs.

Taken together, although some aspects of antinociceptive and analgesic actions of BoNTA are known, there are still several details on underlying mechanistic points to be explained. Those aspects include (but are not limited to) its direct and indirect effects both at neuronal and non-neuronal levels and further elucidation on reasons for mismatch between clinical and experimental time-course of effects. Therefore in this Ph.D. project, an attempt was made to contribute to addressing some of these points with a focus on inhibitory role of BoNTA in association with glutamate in pain mechanisms.

5. The Ph.D. Project

5.1. Aims

The main focus of this Ph.D. was to further investigate the analgesic/antinociceptive effect of BoNTA through direct and indirect methodological techniques. To achieve this goal, three different and novel studies were proposed to elucidate potential analgesic mechanisms of BoNTA. The first study was a psycho-physic methodological study that investigated the time-course analgesic effect of BoNTA on a glutamate-induced experimental pain model in humans. A dermal microdialysis technique, in study II, was used as a tool to directly investigate whether BoNTA could alter glutamate release when it was evoked by an experimental pain model of topical capsaicin combined with mild heat. In these two studies, pain, vasomotor responses and, sex-related differences were also investigated. Study III explored the potential inhibitory effect of BoNTA on vesicular release of glutamate from non-neuronal SGCs isolated from the rat trigeminal ganglia (TG). Imaging techniques (IHC and ICC) were also used in this study to investigate the presence of SNAP-25 and 23, the docking proteins that are cleaved by BoNTA.

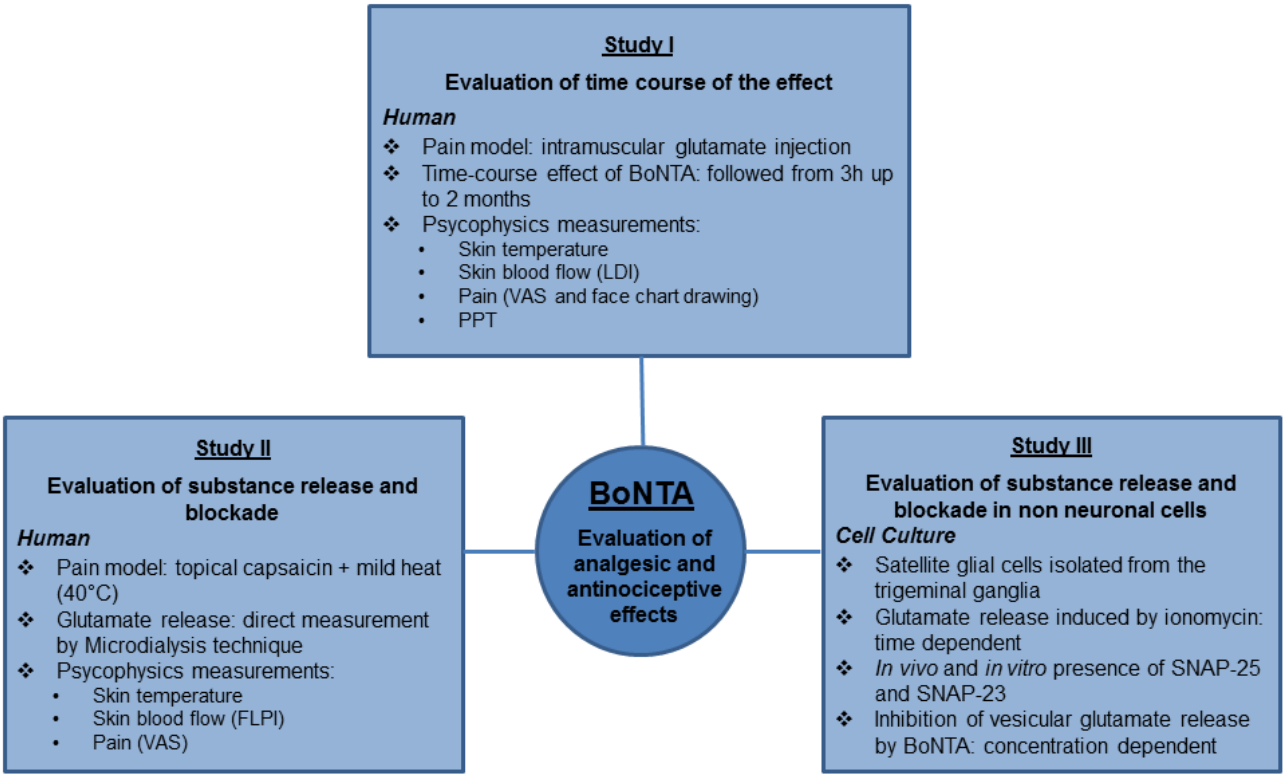

Figure 3: Outline of the Ph.D. project. The analgesic effect of BoNTA was the common ground for the 3 studies.

5.2. Study hypothesis

It was hypothesized, that BoNTA would decrease pain and vasomotor reactions induced by glutamate and capsaicin combined with mild heat, in study I and II, respectively. In these two human experimental studies, an indirect approach was taken to assess the time course and sex-based differences of potential analgesic effects of BoNTA. Besides, in study II, a direct measurement approach was also applied (microdialysis), and it was proposed that BoNTA would reduce or block the glutamate release in correlation with pain or vasomotor reductions. In study III, a cell-based platform was used in which it was suggested that SGCs would have expressed SNAPs (SNAP-25 and SNAP-23). This is the membrane protein to be cleaved by BoNTA and that would reduce the release of glutamate from the cultured trigeminal SGCs, when they are activated by ionomycin, a pharmacological tool to evoke calcium-dependent glutamate release.

6. Description of the studies

6.1. Study I

Time course analysis of the effects of Botulinum neurotoxin type A on pain and vasomotor responses evoked by glutamate injection into human temporalis muscles.

6.1.1. Aims and hypothesis

The aim of this study was to examine the time-course of events in the human experimental pain model induced by injection of glutamate into temporalis muscle of healthy men and women in presence and absence of BoNTA. Glutamate-evoked pain model in temporalis muscle of humans was used to mimick some aspects of trigeminal pain and sensitization seen in craniofacial pain conditions, e.g. headaches. This was a translational study from a similar animal experiment in order to investigate similarities and differences in time course of events seen in this model between rat and human.

We hypothesized that the glutamate injection would elicit pain, induce a mechanical sensitization, and increase the temperature and blood flow when compared to the saline treated side. We also proposed that the pre-treatment with BoNTA would decrease the pain, increase the mechanical thresholds, and would reduce the glutamate-induced vasomotor reactions in the human temporalis muscle. These expected results would be a consequence of suppression release of neurotransmitters from peripheral nociceptive nerve endings in the treated muscle by BoNTA and that this response might be different in men and women.

6.1.2. Main findings

The intramuscular injection of glutamate into the temporalis muscle proved to be effective in eliciting a short-lasting trigeminal human pain model, besides increasing the skin temperature and blood flow in the treated area. However, the glutamate injection did not cause a detectable muscle mechanical sensitization, limiting the possibility to test the reversibility by BoNTA. But it was found that the BoNTA pre-treatment of the temporalis muscle decreased the glutamate-induced pain and vasomotor responses with no side effects from as early as 3 hours after its treatment and its peak was seen at day 7. Glutamate itself induced more pain in women, and when pre-treatment with BoNTA was made, women had a higher skin temperature while, men presented a higher skin blood flow (for details of this study please refer to Paper I).

6.1.3. Conclusion

This study revealed that the analgesia due to intramuscular injection of BoNTA may occur within a few hours (3 hours), even though clinical data show that this onset is present only later on. The time course of the events in this study was very well matched with our previous animal study

and suggests that most likely under acute experimental pain conditions both in humans and animals, BoNTA acts rapidly within few hours. It is therefore suggested that the rapid onset, at least partially, might be due to direct action of BoNTA in blocking vesicular release of neuroactive substances on peripheral nociceptors, and altered sensory processing in pain patients might need longer duration to be modulated by the toxin or the central effect of the toxin in chronic pain conditions develop over time, directly or indirectly. With current technology and methods applied in human studies, it is not easy to speculate what the reasons are for a delayed effect of the toxin analgesic effects in pain patients in comparison with animal and human experimental findings. We also found sex-related differences, which were limited to vasomotor responses. There is no report available on sex-based responsiveness in patient population. In chronic migraine, due to the disorder nature occurring three times more in females than males, it might not be easy to interpret if there is any sex-related response to the toxin, so it needs further investigation.

6.2. Study II

Blockade of glutamate release by Botulinum neurotoxin type A in humans: a dermal microdialysis study.

6.2.1. Aims and hypothesis

The aim of this study was to investigate the mechanism of BoNTA on the pattern and level of glutamate release induced by a topical capsaicin patch (8%) with mild heat in the human skin. The study further investigated the effect of BoNTA on pain response in capsaicin-sensitized skin, including the vasomotor response. This study was the first in humans to use the direct approach for following the blockade of glutamate release by BoNTA in human skin. Previously, a similar result was shown with the formalin model in the hind paw of rats. We found the dermal microdialysis as the best approach to investigate release responses and modulations in a minimally invasive way, and still translatable to what has been already shown in animals.

We hypothesized that a subcutaneous injection of BoNTA would alter the pattern of release of glutamate, which is potentially involved in the transmission of pain following a challenge test using capsaicin and mild heat. These changes were proposed to be detectable by dermal microdialysis in the human skin. It was also proposed that BoNTA would decrease pain sensation and vasomotor responses in correlation with glutamate release blockade.

6.2.2. Main findings

The BoNTA pre-treated side showed positive results in decreasing the pain intensity and suppressing the skin blood flow after the capsaicin plus mild heat pain model. BoNTA also significantly lowered the release of glutamate in the treated area when compared to the saline side.

These responses were sex-independent. This was the first evidence in humans that BoNTA attenuated glutamate release in the human skin and supports the previous findings in animals. We found a correlation between the increased glutamate levels and skin blood flow, but no correlation was found between glutamate levels and pain or skin temperature. Hence, this study provided the first evidence that blockade of glutamate release may contribute, partly, to the peripheral analgesic effects of BoNTA (for details of this study please refer to Paper II).

6.2.3. Conclusion

This study demonstrated that BoNTA attenuated the release of glutamate in human skin when it was provoked by capsaicin plus mild heat. Although BoNTA inhibited the provoked pain, only an association between BoNTA reduction in glutamate release and provoked blood flow response was found, not pain per se. There might be some pathways involved in pain response distinct from vasomotor responses to BoNTA in association with glutamate, which calls for further investigation.

6.3. Study III

Botulinum neurotoxin type A modulates vesicular release of glutamate from trigeminal satellite glial cells.

6.3.1. Aims and hypothesis

The present study aimed at finding whether SNAP-25 and SNAP-23 are expressed in cultured trigeminal SGCs, if those cells would release glutamate in a time- and calcium-dependent manner following stimulation by ionomycin, and if BoNTA would block or decrease the vesicular release of glutamate from these cells. The purpose of study III was not only to study possible role(s) of peripheral non-neuronal cells (SGCs) in trigeminal sensory transmission, but also to advance our understanding about mechanism(s) of analgesic action of BoNTA at the level of sensory ganglia.

It was hypothesized that SNAP-23 and SNAP-25 would be present in the isolated cells and in the trigeminal tissue section. This was based on the fact that SNAP 25 is well known to be present on neurons, but the presence of SNAPs on non-neuronal glial cells is not well studied and there was limited information on central glia for the expression of these membrane proteins. The intention was to see if SGCs in the peripheral sensory ganglia can modulate pain via vesicular release of glutamate. Therefore, it was proposed that ionomycin would be able to induce calcium-dependent glutamate release from these cells and that BoNTA would be able to reduce or block the vesicular release of glutamate from the cells based on the action on SNARE proteins.

6.3.2. Main findings

It was demonstrated that the cell membrane docking proteins SNAP-25 and SNAP-23 were present in the primary cultures of trigeminal SGCs and in the TG tissue. These are the proteins that BoNTA cleaves to exert its effect. Therefore, it is possible to study the antinociceptive effect of BoNTA at the level of sensory ganglia. Moreover, it was verified that cultured SGCs, when stimulated with ionomycin, can release glutamate, which is most likely based on a vesicular release. Besides, BoNTA was able to inhibit this vesicular release of glutamate, possibly by blocking the docking proteins presented in those cells. Up to date, this is the first evidence showing that trigeminal SGCs contain SNAPs and, most likely through cleavage by BoNTA, glutamate release can be inhibited and influence the pain transmission at this level (for details of this study please refer to Paper III).

6.3.3. Conclusion

The results from study III shed light on possible role of SGCs in trigeminal pain transmission and the antinociceptive action of BoNTA at the level of trigeminal ganglion, with an emphasis on the role of non-neuronal cells. Further investigation is required on how BoNTA can reach the sensory ganglia after peripheral injection and whether it is the toxin itself or the cleaved format and under which conditions it can exert its potential effects. The safety aspect of such modulation needs to be further investigated.

7. General methodological approaches

The local ethics committee (Region of North Jutland, Denmark) approved the 2 human studies (studies I and II). The same investigator (author) performed the experimental procedures following the Good Clinical Practice (GCP) guidelines and the Declaration of Helsinki. Before both experiments, all subjects, who were recruited through on-campus advertisements at Aalborg University, Denmark, were screened by the responsible physician to assess the fulfillment of studies criteria. For the cell study (study III), all procedures were conducted according to ethical guidelines defined by the Danish Animal Experiments Inspectorate in accordance with the guidelines set by International Association for the Study of Pain (IASP) for use of laboratory animals in medical research. The following section provides details on the methodological approaches used in the course of this Ph.D. project with a main focus on the details that are not provided in the papers (I, II, III). In addition, methodological considerations and limitations are presented further on item 9.

7.1. Study I

This study included healthy young volunteers from both sexes to provide a most homogenous group with a balance in number, age and sex of the participants (thirty subjects, 15 men (mean age ±SEM: 23.6±0.51 years) and 15 (23.3±0.62 years) women). Besides the session that the pain model was validated (glutamate-induced experimental pain model), the volunteers participated in 5 experimental sessions after the screening, including a treatment session (BoNTA and saline) and four follow-up sessions (3 hours and 7, 30 and 60 days following BoNTA). This design allowed the investigation of time course of events (glutamate-evoked pain and vasomotor responses) in presence and absence of BoNTA.

7.1.1. Glutamate-evoked pain model

Glutamate is one of the most important and abundant excitatory transmitter in the nervous system and its injection in the different tissues, e.g. skin and muscle, has been used as a pain model in animal and human studies (30,38). As explained in item 4.1, there are several available options of experimental pain models. The first study in this Ph.D. was a translational experiment from a previous animal study from our group where glutamate injection into the rat temporalis muscle was also used as an experimental pain model (39). Therefore, an injection of glutamate into the temporalis muscle was used as an evoked pain model in humans, which carries a safe profile; it is short lasting and is also a well-tolerated substance to induce an artificial pain state. The first part of the study was designed as a placebo controlled study (glutamate *versus* saline). This way glutamate and isotonic saline were injected randomly (left *versus* right) into the temporalis muscle. This study was also designed as a blind study, in which subjects were unaware of the content of

the syringes. The second part consisted of the investigation of the time-course of BoNTA as an analgesic compound followed by glutamate injection on both sides of the temporalis muscle.

7.1.2. BoNTA

BoNTA was the main investigational compound and was used in all of the 3 studies. For the first study, each vial of BoNTA (BOTOX®, Allergan, Inc., Irvin, CA; 200 U/vial) was reconstituted with preservative-free 0.9% Sodium Chloride Injection. Each subject received one single injection of BoNTA into the anterior temporalis muscle (5 U/0.1 mL). The same volume of sterile physiological saline (0.1 mL, 0.9%) was injected into the contralateral anterior temporalis muscle to serve as a vehicle control injection. BoNTA injections were given at the Neurology Department, Aalborg Hospital, where all safety precautions were implemented. The injection sides were randomized and both the experimenter and subjects were blinded to the content of the injections. Electromyographic (EMG)-guided injections with disposable needle electrodes were used in order to ensure intramuscular injection. In order to ensure that the BoNTA pre-treated site was identified for the subsequent sessions, a transparent sheet was used and mapped with ink.

7.1.3. Assessment of pain

Pain is described as an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage or described in such terms (99). It can also be influenced by personal and emotional feelings that can override the actual experience. Since pain is a subjective measurement, a self-rating from patients' remains as the most used and direct approaches to try and quantify pain in experimental settings and also in daily clinical practice. VAS (visual analogue scale) is a common tool used in experimental and clinical settings to help researchers and clinicians to follow induced or spontaneous ongoing pain (100). These scales can be presented as simple as a numerical scale or as an electronic version, but both are rated from 0 to 10 or 0 to 100, where the 0 usually means "no pain" and the other extremity means "the most pain imaginable". Pain can be rated continuously or at specific time points. From the subjects' ratings it is possible to extract different outcomes that can be used and analyzed separately: peak pain (the highest VAS score), duration of pain and AUC [AUC VAS (cm × sec)]. For the present study, glutamate-evoked pain was rated continuously by the subjects on an electronic scale (from 0 to 10) for 10 minutes after the injections and peak pain intensity and the area under VAS curve were extracted and used for analysis. Besides VAS, another simple method to graphically represent pain is drawing the perceived pain. This experiment used a face chart drawing that was given to each subject after resolution of the glutamate-evoked pain. After being shown where they received the glutamate injection, they were asked to draw the area of perceived pain on the chart. VistaMetrix (SkillCrest,

LLC, version 1.3) was used for quantitative data extraction from graphics and used to calculate the area in arbitrary units.

7.1.4. Pressure pain threshold

PPT is one of the biomarkers that can be used for pain assessment, diagnostic purposes, and also evaluation of response to analgesic treatments. Pressure algometry is a technique that applies an increasingly constant pressure (in kPa/s), and it is used to assess the mechanical sensitivity of muscle and deep tissues. Both threshold and tolerance can be measured for different purposes. In study I, PPT was used to measure the mechanical sensitivity of the temporalis muscle after the glutamate injection and compared to the baseline. Subjects were instructed that the PPT is 'the point at which the pressure sensation just becomes painful'. The pressure was delivered at a constant rate while the algometer tip was applied perpendicularly at the target tissue.

7.1.5. Thermography

Thermo imaging is a refined and well-known technique for non-invasive monitoring of tissue temperature surface (101). This is a direct measurement that enables the investigator to follow temperature changes in the skin, from the superficial muscle or tissues below that will have the temperature changes reflected in the skin. These changes will most likely arise from the dilatation or constriction of the arterioles that lay bellow the skin and control the skin temperature (102). These measurements were performed by means of a non-contact infrared thermographic camera (FLIR Systems, Inc., -Stockholm, Sweden) that captures infrared wavelength (700 nm – 1 mm).

7.1.6. Blood flow (LDI)

Laser Doppler imaging (LDI) is a sophisticated and well stablished scanning technique for non-invasive monitoring of microvascular perfusion often referred to as microvascular blood flow or red blood cell flux (101,103). This technique enables a real time measurement of the moving red blood cells inside the vessels, like arterioles and veins, in the skin surface. LDI works through a laser beam that scans the skin without contact through a moving mirror. While the low power laser source is directed to the targeted area through this moving mirror and scans the surface of the skin, its frequency is then shifted when it encounters moving elements, like the red blood cells. This frequency, changed or not, is detected by a photodetector and collected by the sourced computer that generates a color coded image that represents the blood flow from the scanned area (103).

7.2. Study II

Healthy volunteers from both sexes were recruited for this study in order to provide a most homogenous group with a balance in number, age and sex, and they consisted of 6 men (mean

age ± SEM: 25.0±1.5 years) and 6 women (25.7±0.72 years). Potential subjects were asked to attend a screening session where they were challenged with the intended pain model. This was performed, because previous pilot studies showed that some participants did not present any response after capsaicin stimulation. For the present study, only the participants that developed a visible flare and rated the capsaicin-induced pain intensity above 5 (VAS 0 to 10) were included. In order to have a more homogeneous group regarding body composition, weights and heights were checked and only participants with body mass index (BMI) between 19 kg/m^2 and 35 kg/m^2 were included. After that, the experimental sessions followed with the first as BoNTA and saline (control) treatment injection in each forearm in a random blinded fashion. One week later, subjects were challenged by topical capsaicin patch (8%) plus mild heat, while having a microdialysis membrane in the pre-treated area.

7.2.1. BoNTA

Similarly to the study I, each vial of BoNTA (BOTOX®, Allergan, Inc., Irvin, CA; 100 U/vial) was reconstituted with preservative-free 0.9% Sodium Chloride Injection. Each participant received a single injection of BOTOX® (10 U/0.2 mL) with disposable needle intradermally in the volar part of the forearm, 5 cm distant from the cubital fossa. The equal volume of sterile physiological saline (0.2 mL, 0.9%) was injected as control in the contralateral forearm. The sites of injections were mapped on a transparent sheet for further location tracking for the microdialysis membrane insertion.

7.2.2. Capsaicin + mild heat-induced pain model

Capsaicin is a well-known model causing a short burning artificial pain. The capsaicin-heat model is also well-established and presents some useful aspects, like mimicking both neuropathic and inflammatory aspects. This model was selected because of its stability, besides the combination of topical application and lack tissue injury. Before the application of the capsaicin patch, the area was pre-sensitized with a heat stimulation of 45°C for 5 minutes. The patch was cut as a rectangle that covered the pre-treated BoNTA site and was applied for 1 hour to the just sensitized skin, right in the middle of the 2 probes to ensure the stimulation was in the best place to capture the changes. In order to increase the response, a mild heat (40°C) was used, avoiding the microdialysis membrane to ensure it would not damage it.

7.2.3. Assessment of pain

Pain measurement technique by VAS was explained in item 6.1.3. In study II, pain scores were recorded right after the membrane insertion, following the heat sensitization period and every 15

minutes from the capsaicin patch application. The peak pain intensity scores were manually recorded and used for statistical analysis.

7.2.4. Thermography

The thermography technique is explained in item 6.1.5. For study II, measurements were performed before the membrane insertion (baseline) and every hour, for 3 hours after the insertion. While the baseline measurement was performed with the intact and uncovered skin, the subsequent measurements were taken after probe insertion or placement of patch on the canulated area.

7.2.5. Blood flow (FLPI)

Similarly to study I, skin blood flow was also measured; however, a laser speckle contrast imager (Moor Instruments, Devon, UK), which consists of another device to monitor vasomotor changes in the treated area, was used. This full-field laser perfusion imager device (FLPI) uses a full field laser technique and provides real-time and high-resolution images of blood flow. The technique is non-invasive and is based on a random speckle pattern that is generated when tissue is illuminated by the laser light to capture the blood cells' movements.

7.2.6. Microdialysis

Microdialysis is an *in vivo* technique that can be used to continuously monitor the release of substances and responses to drugs and procedures in virtually any tissue of the body. In study II, this approach was used in the dermis of the forearm to measure the pattern of glutamate release. Before the membrane insertion, an ice pack was used in the intention area for 5 minutes to cause a numbness of the skin and decrease the amount of pain. Two linear probes (10 mm length, 100 kD cut-off) were inserted in each of the subjects' arms with the BoNTA pre-treatment injection in the middle. Figure 4 shows in details the schematic and disposition of probe placement and insertion along with the pain model application site. After the probes were in the correct place, they were attached to an adjustable pump that was set to 3 µL/min, as a perfusion rate. Samples were collected overtime from both arms. The glutamate recovery of the experimental system was evaluated in our own preliminary trials in human skin with the same probe and pump and at the similar flow rate. When the experiment was finished, the probes were removed from the subjects' arms.

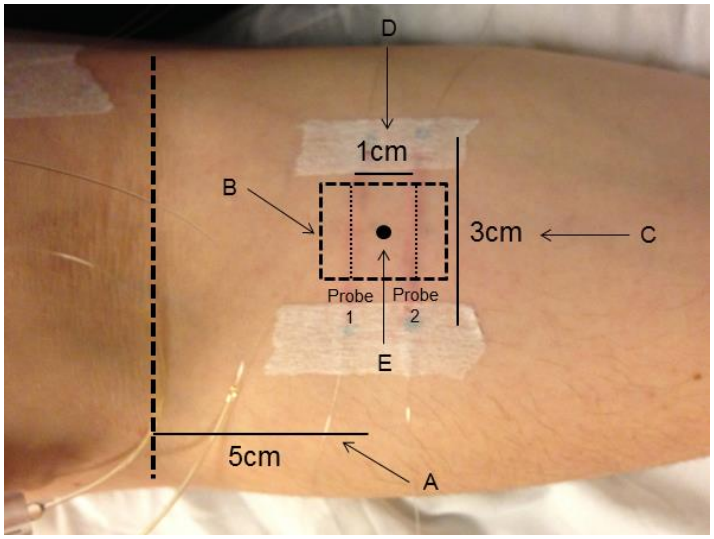

Figure 4: Schematic of the experimental set-up. Arrows indicate: A – Distance between the cubital fossa and the BoNTA injection point (5cm); B – Capsaicin patch application covering probe 1 and 2; C – Distance between probes inlet and outlet (3cm); D – Distance between probe 1 and 2 (1cm); E – BoNTA and saline injection point. Figure shows the schematic of the ROI of all interventions. BoNTA (10U) and control pre-treatment injections (E) were made approximately 5cm away from the cubital fossa (A). The guided needle was inserted across the skin for 3cm (C) for helping the insertion of the probes, which were inserted 1cm apart from each other (D), having the pre-treated site in the middle. The capsaicin patch was placed in a way to cover both probes (B). This figure was originally published in *Pain Res Manage* 2014;19(3):126-132 (104) and reproduced with permission from Pulsus Group Inc.

7.2.7. Glutamate analysis

Samples of approximately 10µL each were analyzed for their glutamate content to further investigate the glutamate changes over the time-course of the trial. ISCUSflex Microdialysis Analyzer (M Dialysis AB, Sweden) was used and through enzymatic reactions and colorimetric measurements can determine the content of different substances, including glutamate. The final results compare the glutamate concentration (in µM) between BoNTA and saline pre-treated sides.

7.3. Study III

7.3.1. Animals

The study was performed using 20 adult male Wistar rats (2 to 4 months old) provided through the Animal Research Facility, Department of Pathology, Aalborg University Hospital, Denmark. Animals were housed in groups of three rats per cage, in temperature-controlled rooms, on a 12h

light/dark cycle with access to food and water *ad libitum*. All animals were deeply anesthetized and then euthanized prior to all experimental procedures.

7.3.2. Primary cell culturing technique

Primary cell cultures are broadly used to investigate *in vitro* neurobiological studies, which provide and enable further experimental investigation of cellular pathways, cell-cell interactions, besides physiology and morphology of the isolated cells. For the present study, animals were deeply anesthetized and euthanized by cervical dislocation before the initiation of the isolation. The trigeminal ganglia were exposed and carefully removed from the connective tissue and the rat skull. The isolated ganglia were then cut into smaller sections and after a number of steps, the trigeminal glial cell solution was added to uncoated culture flasks and then placed in the incubator (37°C - 95% air/ 5% CO_2). The supplemented growth medium was changed after 3 and 24 hours in the first stage, and then continuously every 2 to 3 days prior to the experimental procedure. This isolation procedure has been standardized in previous studies (105). Whereas, the isolation of the glial cell from the neuron enables the investigation of specific issues, there is still incongruity that this procedure maintains its original function (97,106). It is hard to speculate how and to what level the responses form the isolated SGCs differ from the response of the whole TG, when the *in vivo* SGCs and neurons are intact.

7.3.3. Immunohistochemistry (IHC)

Immunohistochemistry is an important and largely used research and clinical tool to identify the distribution and localization of an antigen or a specific cellular element in the targeted tissue. The first step is to prepare the slides with sections of the tissue, followed by the staining and the interpretation of the results. The preparation of slides can be after the tissue has been fixed in formalin and embedded in paraffin or can be frozen and stored in -80°C. This is followed by the slicing of the tissue and placement of these slices to the slide. Then comes the staining itself that is based on the antigen-antibody binding reaction and can be a direct or indirect staining. In the direct one, the antigen binds to the antibody that is already conjugated with an enzyme for further detection. However, the indirect staining is the most commonly used and can be described in a few steps. Firstly, the specific antigen binds to the primary antibody, then after incubation with a secondary antibody that is conjugated with and enzyme, it binds to the primary one. In order to reveal the binding sites, a substrate is added to catalyze and generate the color to make the antigen visible. The slides are usually mounted after that to preserve the staining for further analysis. The last step is the interpretation of the staining under the appropriate microscope (107,108). For the present study, the TG was appropriately removed from the rat skull and treated to later be frozen in a cryo-mold. Each ganglion was sectioned in slices, of 10µm thickness, on a

cryostat and mounted on polysine coated glass slides. Then, the immunostaining was done by the indirect way, as described above. After incubation of primary and secondary antibodies, the slides were mounted with glass cover slips and images were obtained using a Nikon microscope (Az100, Nikon, Tokyo, Japan) equipped with a fluorescent illuminator (L200/D, Prior Scientific, Rockland, MA, USA) and a digital camera (DS-Vi1 Nikon, Tokyo, JP) connected to a personal computer. Image J was used for further analysis and noise-to-signal ratio adjustments.

7.3.4. Immunocytochemistry (ICC)

Immunocytochemistry is a common technique used as a laboratory tool to identify specific proteins or antigens in cell culture or suspension. Each kind of cell requires a slightly different procedure preparation in order to fix it to the slide and make it permeable for the antibodies. Basically, the cells need to be attached to the microscope slide that can already have adherent cells on them. After the fixation, the procedure is similar to the IHC explained above. Firstly, the slide will be incubated with the primary antibody, followed by the secondary one that has the conjugated enzyme and was treated with a substrate for the colorimetric enzyme reaction. Then, it will be mounted and interpreted under the appropriate microscope (109,110). For the present study, cells were fixed to the microscope slide and the procedure performed was exactly as just described above. After the staining was finished, the slides were mounted with glass cover slips. Images were obtained using a Nikon microscope (Az100, Nikon, Tokyo, Japan) equipped with a fluorescent illuminator (L200/D, Prior Scientific, Rockland, MA, USA) and a digital camera (DS-Vi1 Nikon, Tokyo, JP) connected to a personal computer where the analysis and noise-to-signal ratio adjustments were completed.

7.3.5. ELISA

The enzyme-linked immunosorbent assay (ELISA) is an *in vitro* technique, which uses antibodies and change of color to identify and quantify an analyte, the substance being investigated. This technique works with a stationary solid phase that is usually on the bottom of the ELISA plate and this ligand will bind to the substance being analyzed, forming the antigen-antibody interaction. Depending on the type of ELISA technique being used, a detection enzyme will be linked to a primary or secondary antibody that will be bind to the antigen. After being in contact with the substrate, there will be a change of color that will be read by spectrophotometer, fluorometer, or luminometer (111,112). This technique can be either used to identify a substance, so a change of color will answer either yes or no, or to quantify the antigen, having the reading in the end. This is a well-established and straightforward technique; even though, few challenges can surface, like timing, number, and volume of samples. The volume is an important issue when considering

ELISA as an analytical tool, since kits usually ask for 50 or 100µL samples. Both experiments detailed below used an ELISA kit for the detection and quantification of glutamate.

7.3.6. Time-dependent glutamate release by ionomycin

One of the interests of this study was to investigate if the isolated SGCs would release glutamate. In order to follow that, ionomycin was used as a pharmacological tool to increase intracellular calcium levels, leading to glutamate release. In order to test its functionality, a time-dependent experiment was made with time-points at 4, 8, 12, and 30 minutes with one concentration of ionomycin (5µM). Once cell confluence was ≈ 90%, the supplemented growth medium was washed off and replaced with the glutamate-free Dulbecco's Modified Essential Medium (DMEM) and left overnight in the incubator. The glutamate-free medium was an important step since glutamate was also the investigated substance. On the day of the experiment, after the medium was replaced by a fresh one, the cells were treated with ionomycin for all the specific time-points, and samples were collected. The glutamate concentrations in the collected samples were determined using a competitive immunoassay for quantitative determination of glutamate in biological samples (BA E-2300, Labor Diagnostika Nord, Nordhorn, D).

7.3.7. Modulatory effect of BoNTA on glutamate release from cultured SGCs

After testing the efficacy of ionomycin in releasing glutamate and finding the best time-point, BoNTA was used to investigate its modulatory effect in blocking the calcium dependent glutamate release stimulated by ionomycin. As soon as the cultured cells' confluence reached ≈ 90%, the supplemented growth medium was washed off and replaced with the glutamate-free medium and left in the incubator overnight. The overnight medium was replaced with pre-treatment medium containing 0.1pM, 1pM, 10pM and 100pM of BoNTA (Botox, Allegan, CA, USA) or glutamate-free medium and placed in the incubator (37°C) for 24h. After that, the cultures were treated with 5µM ionomycin for ionomycin alone, glutamate-free medium for control and a mixture of 50µM BAPTA plus 5µM ionomycin for BAPTA as a positive control. BAPTA is a well-established positive control for BoNTA (113). Samples of the cultured medium were collected after 30 minutes of incubation. Glutamate concentrations in the collected samples were determined using a competitive immunoassay for quantitative determination of glutamate in biological samples (BA E-2300, Labor Diagnostika Nord, Nordhorn, D). After analyzing the results of the present study, a new interval could be investigated since 10pM showed no result and 100pM did. The optimal dose is always the lowest one that presents a positive effect without adverse effect. In order to check BoNTA's blocking effect, it is also possible to quantitatively measure cleaved SNAPs (SNAP-25 and/or SNAP-23) using western blot technique. This was not performed due to time and technical issues, even though, it is valued information that requires further investigation.

8. Discussion

The effect of BoNTA on glutamate release was undoubtedly the common aspect for all studies in this Ph.D. project. Glutamate is an excitatory amino-acid that is known to be involved in pain transmission, besides maintenance of pain and sensitization states both at the peripheral and central levels. Exogenous glutamate injected into the temporalis muscle efficiently evoked pain (study I) that was diminished by BoNTA pre-treatment. Endogenous interstitial levels of glutamate (induced by capsaicin in combination with heat) were also decreased by pre-treatment of BoNTA, as documented by real-time dermal microdialysis technique (study II). Besides, ionomycin-evoked calcium-dependent glutamate release was also blocked by pre-incubation of BoNTA and isolated SGCs from rats' TG (study III). The next session discusses the main outcomes of the studies presented in this thesis.

8.1. Glutamate release in association with pain

For a long time, glutamate was mainly considered to be involved in the central processing of pain (19); however, studies have shown its contribution and importance to the peripheral pain transmission (21). Glutamate is stored in synaptic or neurotransmitter vesicles, and this property makes it a suitable target for BoNTA to possibly modulate its vesicular release. Vesicles containing neurotransmitters need a membrane protein to assist their attachment and further substance release. BoNTA is known to cleave selectively a protein (SNAP-25) that is a part of a functional protein complex (SNARE), responsible for attaching these neurotransmitters' vesicles to the cell membrane (8,69,85). The three studies in this Ph.D. approached potential analgesic and antinociceptive properties of BoNTA from different angles.

Glutamate injection in the temporalis muscle induced pain and increased vasomotor responses (study I). This result is in line with previous studies of several groups that have been confidently using this model for years (37,114). Glutamate, when injected intramuscularly, elicits pain that is partly mediated through peripheral NMDA, receptors located on small fibers in the peripheral tissues, e.g. skin and muscle (37,115). Besides, glutamate injection has been shown to sensitize the peripheral tissues under investigation (32,37); however, when it was injected to the temporalis muscle, no significant sensitization was found in the present project in comparison with saline. This might have been due to several reasons, e.g. high variations among the responders or the assessment technique, which will be discussed further in the next section.

Capsaicin in combination with heat was able to provoke pain, besides increasing the vasomotor reactions in the skin. Capsaicin has been used as an efficient experimental pain model and several formulations are available containing different doses (e.g. up to 8%) and for different routes of administration (e.g. for injection or topical administration). When applied, capsaicin interacts with sensory C fibers through activation of TRPV1 receptors, causing a burning pain

response due to neuropeptides release, e.g. glutamate, CGRP, and SP (44,45). It is common to use injection and some topical formulations (116), but in this PhD project, for the first time, the clinically approved capsaicin patch (Qutenza, 8%) was used to elicit pain. Since there were no previous studies available to base our methodology, some pilot studies were conducted in order to reach a potent and yet stable pain model (item 9.1). The final model (capsaicin plus mild heat) yielded satisfactory and similar results as previous studies, in regard to pain and vasodilation (52,117,118), besides being effective in releasing glutamate.

Recent evidences of non-neuronal cells being involved in pain transmission and maintenance (96,98) broaden the idea to investigate the release of glutamate from SGCs isolated from the TG of rats; this particular type of cells had not been studied before. The results were adequate, presenting a concentration-dependent glutamate release evoked by ionomycin, revealing a calcium-dependent mechanism. Previous studies in different types of glial cells had already successfully showed similar results (29,119,120). This study linked neuronal (study I) and non-neuronal (study III) aspects, both targeting peripheral cells innervated by the TG and their contribution to glutamate release and effect.

Taken together, three models were successfully established with connections on pain/nociception and glutamate. Consequently, it was, then, possible to investigate BoNTA modulatory effect on these tissues and cells, together with glutamate release. The next topic of this thesis presents BoNTA's effect on the above mentioned studies, with a focus on evidence and assumption of glutamate release and its blockade by BoNTA.

8.2. Blockade of glutamate release by BoNTA

BoNTA's effect on pain and its modulation on glutamate release was the focus in this Ph.D. project. As already proposed, BoNTA selectively cleaves SNAP-25, a synaptosomal membrane protein that is part of a complex that enables vesicles containing neurotransmitters to attach and fuse to the pre-synaptic neuron membrane to further release of the substances. This mechanism has already been thoroughly studied in regard to acetylcholine and muscle contraction; however, it is not yet fully elucidated for other substances and tissues. There are also evidences showing that non-neuronal cells are involved in pain and neurotransmitter release and re-uptake. Hence, these cells might also express SNAP-25 and SNAP-23 (an analogue of SNAP-25) and a similar cleaving mechanism might be involved when BoNTA is exposed to those cells.

The analgesic effect of BoNTA was investigated in both human studies (I and II). A number of direct and indirect approaches were used in order to follow the responses in glutamate release or modulation by treatment of BoNTA. The most prominent and direct finding came from the microdialysis study (study II), where a decreased level of glutamate was found in the BoNTA pre-treated site opposed to the control one. It is believed that BoNTA partly blocked the release

through the mechanism cited above. Even not having a direct evidence of glutamate or other substance blockade in study I, the decreased pain and reduced vasomotor responses are believed to result from BoNTA effect in blocking substances involved in pain and vasodilation, including glutamate, CGRP, and SP. There are still divergences about analgesic effect of BoNTA in particular in human experimental studies, with mostly positive results (71,80,121,122); however, negative outcomes have also been reported (92-94).

Besides the studies showing transmitter release from neurons and the inhibitory effect of BoNTA, in study III, we aimed at cell-based platform of non-neuronal cells to investigate whether BoNTA can yield similar effects on vesicular release of substances from these cells. This is a new concept as there are only few evidences proposing that BoNTA moves from the peripheral site of application, e.g. muscles, and can reach the central nervous system via retrograde transportation. If this hypothesis is correct, then BoNTA can reach the sensory ganglia and can yield effects on non-neuronal cells to inhibit substance release, which are involved in sensory transmission and pain. In study III, besides the investigation of glutamate release blockade by BoNTA, the presence of SNAP-25 and SNAP-23 was also investigated to shed light of possible mechanisms involved. It was observed through IHC and ICC that SGCs present the SNAPs similar to neurons and that BoNTA was able to reduce the calcium-dependent vesicular glutamate release when stimulated by ionomycin. Since SNAP-23 was already shown to be present on astrocytes, we expected to find similarities with SGCs. However, besides SNAP-23, SNAP-25 was also identified both in the tissue of the TG and in the isolated cells, suggesting that BoNTA is able to modulate vesicular release of neurotransmitters, e.g. glutamate, both in neuronal or non-neuronal cells.

Further investigation is needed to better elucidate other aspects, like the percentage and presence of the cleaved SNAPs. Retrograde transport of BoNTA needs further investigation at both molecular and basic pain models in animals. For a long time, it was believed that, when injected peripherally, BoNTA wouldn’t leave the injection site. However, evidences are showing central effects from peripherally treated BoNTA in animal and human studies, but there are still a huge amount of controversies and missing points, indicating the need of further research. Taken together, the studies in this Ph.D. project were designed to address one common ground in targeting glutamate at peripheral neuronal and non-neuronal pathways by BoNTA.

9. Methodological considerations and limitations

This section presents some discussion aspects relating to the methodology that has not been discussed in the papers and should also be considered.

9.1. Experimental models of pain

Glutamate injection in the temporalis muscle of healthy humans was used in study I as the pain model, and it was effective to evoke reliable pain and vasomotor responses with no safety issues to be reported or recorded. It was also expected that the applied model would also sensitize the muscle tissue reflected on the mechanical sensitivity to pressure being higher after glutamate, according to previous studies (38,123). However, this was not found in study I, becoming irrelevant to investigate any potential effects of BoNTA on muscle sensitization. It can be suggested that the assessment technique was not the ideal one, considering the targeted tissue. This is discussed further in item 9.4. The experimental pain model used in study II was a capsaicin patch in combination with heat (40°C) after an acute sensitization period (45°C/5 min). Even though the expected response was successfully achieved with the above mentioned model, a number of experimental pilots were done. To elicit pain, a capsaicin solution of 1% was used in a small chamber that only caused a small flare. At that time, the patch had just been approved to treat peripheral neuropathy and no record was found for its use as a potential inducer for an experimental pain model in humans. It is well known that single application of capsaicin evokes burning sensation and pain due to release of neurotransmitters contributing in pain pathways. However, repeated applications cause desensitization of sensory and mechanical nerve cells and result in pain relief (49). This dual effect makes capsaicin an ideal substance for studying pain as a model. A pilot study was performed with the application of the patch alone, but still the results were not satisfactory in terms of pain induction or vasomotor responses. It was noticed that some subjects did not react at all to the capsaicin patch and the search for a more stable, stronger, but yet not unbearable stimulation was continued until we tested the pre-sensitization stimulation with heat application on top of the capsaicin patch, resulting in the used pain model for study II.

9.2. BoNTA

BoNTA injections were always performed at the hospital by the neurologist responsible for the studies. For study I, the exact point of injection was determined through palpation of the contracted temporalis muscle after jaw clenching. This had to match a part of the temporalis muscle without hair for later observation of vasomotor changes. This means that it needed to be the anterior part, where the muscle is thinner. A careful analysis of the intended injection area was made in order to avoid the frontal branch of the superficial temporal vein. In study II, the intradermal injection of BoNTA was chosen, because studies show that pain relief occurs prior to muscle paralysis (91),

when injected in the muscle. In a tentative way to exclude this co-founding factor that occurs in the muscle, the skin was selected. The forearm skin was selected because it was easily accessible compared with the facial dermis, and the forearm in humans has been subjected to many pain studies and somewhat it is a standard site. Besides, a careful training was taken in order to ensure that the injections, and later on the probes, were applied in the dermis layer of the skin, which is where the capillaries and nerve endings are present and, consequently, where the neurotransmitters are released.

9.3. Pain assessments

Besides VAS and the mapping of pain area distribution, there are a number of other techniques that can be used, and one of them is the McGill Pain Questionnaire (MPQ), a broadly used and reliable technique where patients and subjects choose words in order to characterize their pain by aids of word descriptors (124). MPQ was not used in these studies due to methodological issues, but it could be included when designing a study. Besides, considering that pain is a subjective measurement, it is a good idea to match subjective measurements (VAS, MPQ) with objective ones (thermo-imaging, LDI, FLPI).

9.4. Pressure pain threshold

Pressure algometry is a technique that is non-invasive and easy to apply; however, one of the main disadvantages of this method is high variability in responses, even though each person can be normalized to his/her own baseline assessments. When assessing the sensitivity changes in the muscle, we cannot exclude the skin influence in the result and net pressure in the muscle (125). The location can also influence the assessment and make the inter individual and intra individual variation higher, confounding the results. Another device called palpometer can be used to detect the mechanical sensitivity. The palpometer consists of a small plastic cylinder with a probe in the tip that is calibrated to a specific pressure load to be delivered to the tissue and detect its sensitivity (126,127). The hand-held algometer has been shown to work in research settings, even though there are still issues about high variations, calibration issues, and the need for a power supply. In comparison, the palpometer was developed to be an easy-to-use, low-cost, and purely mechanical device.

9.5. Vasomotor response assessments

This section includes methodological considerations not covered in papers for the techniques thermography and blood flow assessments in both studies I and II. All measurements were done in a standardized manner, to ensure an optimal room temperature and lightning in order to decrease any influence in the results. Subjects were asked to refrain from exhausting activities, caffeine

and/or drinks, and alcohol for at least 24h prior to the experiment; these are all well-known activities and substances that increase the skin blood perfusion and temperature, causing flushes especially in the neck and face area (128). Besides, on the day of the experiment, the study participants were allowed - at least 15 minutes before the start - to adapt to the lab environment. Moreover, a supine position was ensured in order to decrease the body heat loss (129). Other important factors were the distance between devices and tissue and the definition a region of interest (ROI). The distance between the camera lenses and the tissue to be assessed determines the area that will be captured. The longer the distance, the larger will be the portion of the tissue assessed, and after finding the optimal distance, it was kept constant for all participants. The ROI was also defined for each measurement and used to provide a more precise and localized information on the changes. These adjustments can provide the opportunity to see the temporal profile of the changes as well as the confined changes right in the treatment site. Measurements were performed over time after each injection. In the presented studies, the same thermocamera was used; however, two different devices to assess the blood flow were used. LDI is a sophisticated and well-stablished scanning technique for non-invasive monitoring of microvascular perfusion often referred to as microvascular blood flow or red blood cell flux (101,103). This technique enables a real time measurement of the moving red blood cells inside the vessels, like arterioles and veins, in the dermis. Alternatively, Laser Doppler Flowmetry (LDF) and full field laser technique can also be used to follow the real time assessment of changes in blood flow. However, LDF is based on a single point measurement and the probe needs to be in contact with the skin and sometimes it’s hard to interpret, due to a great variability and changes in one single point (130). This technique is also used in clinical settings to assess skin inflammation, vascular disorders, and to follow burns and wound healing processes (131,132). FLPI is another possibility that captures the whole area in one single shot, allowing very rapid changes to be followed. When comparing both devices used, FLPI and LDI, they are easy to use, fast and reliable. However, FLPI can be advantageous if the purpose is to follow rapid changes in the vasomotor response since it can take one frame per second that covers a large area. On the other hand, LDI can take from few seconds to minutes to scan the intended area.

9.6. Microdialysis

Microdialysis technique was used in study II and many issues that should be considered are presented here. The microdialysis membrane that is inserted in the targeted tissue acts as an "artificial blood vessel", which means that it is permeable to fluids and substances. The concentration gradient is what governs the direction of migration of the targeted substances to and from the membrane lumen and the interstitial space where the microdialysis probe is inserted. At the same time that these substances move through this membrane and tissue, a sample is taken

at the end of the cannula. It is through these fluid samples that one can measure the level of many substances if a reliable measurement technique is available. Figure 5 shows in details how the mechanism of the diffusion of molecules works.

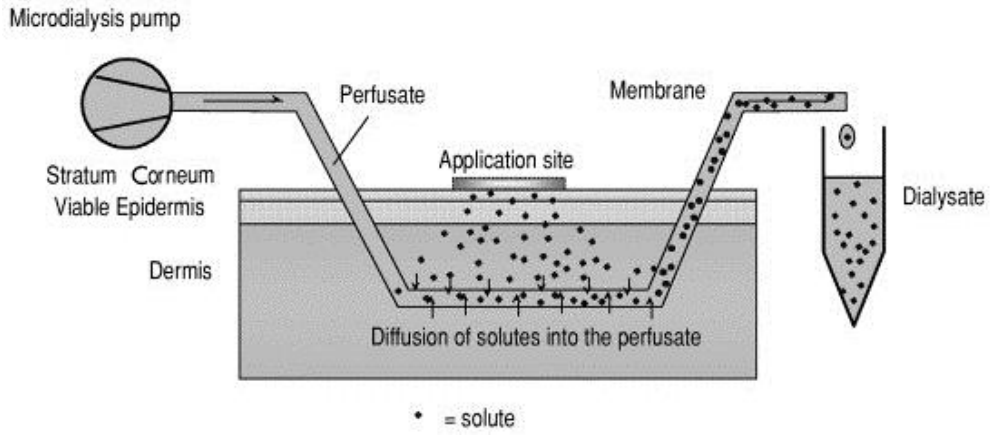

Figure 5: A linear microdialysis membrane is inserted into the dermis layer of the skin with the help of a needle that is removed afterwards. The membrane is attached to a microdialysis pump, which drives a perfusate solution through the cannula in a chosen perfusion rate. When the perfusate reaches the permeable membrane, substances and fluid, driven by the concentration gradient, run freely from the lumen of the membrane and the interstitial space. At the same time that this exchange is happening, the perfusate solution is running from one end (pump) to the other end, where a sample is collected in a vial. After collection of the sample, it can be stored and analyzed for the targeted substance. Reproduced from (133) with permission from Elsevier.

This technique has been extensively used in the clinic as well as in the experimental field, from peripheral tissues, like the skin, to deeper muscles, organs, and brain (134-137). It is a valuable and flexible tool that can be used for a short period of time, similar to what was used in study III (5 hours), but it can also be used for a longer period. It is also common to be used to track patients before and after surgery, in order to monitor the levels of different biomarkers to better follow patients' recovery (138,139). Microdialysis is also thoroughly used in drug development and dose response studies to follow the pharmacokinetics and pharmacodynamics of a specific drug (137,140). When setting up a microdialysis experiment, besides finding the appropriate measurement technique, many other aspects need to be taken in consideration, such as:

- Type of membrane – a linear flexible probe might be suitable for peripheral tissues, like skin or muscle, while a stiff probe is needed for a brain microdialysis;
- Length of membrane – a longer membrane enables a better recovery of the substances, however, this will be evaluated in accordance with the size of the targeted tissue;

- Membrane cut-off – this will determine which substances will be recovered. For instance, the molecular weight of the targeted substance to be collected needs to be smaller than the membrane cut-off;
- Targeted tissue – the location of the tissue will influence the accessibility and the technique for the probe insertion, in order to maintain its optimal function – some probes are really fragile and any inadequate procedure might damage the probe;
- Perfusion flow – a high flow will remove or introduce as many molecules as possible, whereas a low flow will have a more concentrated dialysate and will not disturb the physiology of the tissue as much as a high flow. The chosen flow will also influence the volume of the dialysate; the lower the perfusion rate, the lesser the sample volume is.

In study II, microdialysis was used to monitor continuous changes over time for the interstitial glutamate level in a dynamic way to follow the analgesic effect of BoNTA on the pattern of glutamate concentration, after challenging the tissue with the capsaicin + mild heat pain model. This method was chosen because it has the capacity to show small and localized changes, since the probes are placed right below the stimulated area. It is likely that such small and localized changes in the glutamate release, for example, wouldn't reach the systemic blood circulation, disabling the possibility of following the changes through blood samples. Since the probe insertion is not a painless procedure and does cause damage to the tissue, some experiments tend to use topical anesthetic before its insertion (141). Since the main objective of study II experiment was to follow the glutamate level changes, a substance involved in pain sensation and transmission, we chose to use only an ice pack for 5 minutes to cause transient numbness in the skin to decrease the potential pain due to probe insertion. The length of the used membrane was 10mm and the size also interferes on the recovery of the substances; a longer probe will recover more molecules (136,142). Because of the insertion procedure and the available area in the forearm, a longer probe could not have been used. When inserting the membrane, 1cm was left on each side to better accommodate the membrane and also to allow more space to apply the pain model in order to avoid the in- and out-lets of the probe insertion. Sample volume was one of the limitations of this study since we had small volume samples and more sophisticated and sensitive tool, as High Performance Liquid Chromatography (HPLC) was not available. However, the analytical tool used needs to match the acquired volume. Considering this, the volume of the sample and the analytical tool also influence how many substances can be analyzed for the same experiment. In order to increase the volume, several membranes and pumps can be used simultaneously or the perfusion rate can be enhanced. However, if the rate is too high, it may not allow the molecules to reach the equilibrium with the tissue and the sample will be unsuitable.

9.7. Primary cell culture

For study III, some methodological issues can be outlined. Ionomycin was the pharmacological tool used to promote calcium-dependent glutamate release from the SGCs culture. Besides ionomycin, other substances like bradykinin (28), PGE_2 (143), and ATP (144) could be used for the same purpose. Ionomycin was chosen due to the positive outcomes in previous studies in astrocytes, where they also showed a time-dependent response (29). Another concern was the maintenance of the characteristics of the studied SGCs, since they were removed from the sensory ganglia where they surround neurons and interfere in their response. The knowledge about this close and complex interaction between neurons and glial cells is very limited and it is also hard to predict how similarly the response from the *in vitro* studies to the *in vivo* tissue is. However, we were able to show that expression of the SNAPs remained unchanged in the tissue and in isolated cells. A study made by Poulsen and colleagues analyzed these isolated SGCs up to 21 days of *in vitro* culture and concluded that this method can be a valid and useful cell-based platform for studying glial activity and modulation (105). The same procedures were followed in study III. Besides the mentioned results and methodological issues discussed above, there are other considerations that could further enrich this study. Providing results on the ability of BoNTA to cleave SNAPs would be highly relevant. BoNTA ability to cleave SNAP-25 has already been demonstrated in astrocytes by immunocytochemistry after retrograde transport of BoNTA in the spinal cord. Therefore, additional experiments could be performed in order to investigate whether cl-SNAP-25 and 23 can be found in the trigeminal satellite glial cells. Besides, the percentage of the expression and cleavage is also highly important. The condition under which the inhibitory effects of BoNTA occur should also be addressed. There are a couple of important factors: type of the toxin, treated cells (type, age, etc), concentration, and time of the toxin incubation. The presented result from the toxin modulation seemed to have a possible effect of *"all or nothing"*. It seems that low doses of toxin provide full analgesic effects (from the lowest effective dose), without any motor effects. For example, at 3 U/kg, BoNTA seems not to affect the carrageenan- and capsaicin-evoked pain, but at slightly higher dose (3.5 U/kg), and further increased doses (5 and 7 U/kg) BoNTA exerts similar and maximal analgesic activity (80). Similar antinociceptive effect of 3.5, 7, and 15 U/kg BoNTA doses was reported in formalin test (88). In few studies, increased analgesic effects have been shown to occur at higher doses of the applied toxin (20–40 U/kg) (60,88). However, systemic spread of BoNTA impaired the animal motor performance, which most likely interfered with the ability to produce a nocifensive reaction (88). Up until now, clinical trials have not addressed a clear dose-response of BoNTA, and the employed doses are based on the clinical effect, only empirically (145). These unanswered questions can help elucidate and better describe the antinociceptive/analgesic effects of the toxin at the level of sensory ganglia.

10. Conclusions and future perspective

This Ph.D. project provided both direct and indirect evidence of potential mechanisms underlying analgesic/antinociceptive effects of BoNTA by modulatory effect on glutamate release. For the first time in humans, an analgesic effect within hours after BoNTA treatment was shown. Another novelty was the use of the microdialysis technique in humans to dynamically and directly show a decrease in evoked glutamate levels following administration of BoNTA. Hence, it was also presented for the first time that BoNTA can modulate the vesicular release of glutamate from non-neuronal cells of the trigeminal ganglia in the peripheral nervous system, and that this effect was concentration-dependent and most likely through cleavage of SNAPs. It was concluded that BoNTA exerts its analgesic effect, somehow, through modulation of glutamate.

Outcome from these three studies shed light on potential mechanism of action of BoNTA for pain relief and also advanced the understanding on potential contribution of SGCs in nociception. The effect of BoNTA on glutamate release was the common point over the studies in this Ph.D. project and it is proposed that BoNTA, at least in part, blocks or reduces the release of glutamate, which is one of the potential players in pain pathways. Even though glutamate was the main neurotransmitter investigated, other pain mediators should also be considered and investigated deeper. And yet, much is still unknown about the antinociceptive and analgesic effects of BoNTA, indicating a need for further research to better expand its benefits in a safe and reliable approach. Increasing the evidences on BoNTA antinociceptive mechanism can facilitate its approval for other painful conditions in addition to migraine. Brain imaging techniques, advanced methodology in *in vivo* animal studies, investigation of other substances involved in pain by means of microdialysis technique and translational studies in patients are some of the prospective studies that could assist in expanding our knowledge.

11. Papers

Paper I

TIME COURSE ANALYSIS OF THE EFFECTS OF BOTULINUM NEUROTOXIN TYPE A ON PAIN AND VASOMOTOR RESPONSES EVOKED BY GLUTAMATE INJECTION INTO HUMAN TEMPORALIS MUSCLES

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Paper II

BLOCKADE OF GLUTAMATE RELEASE BY BOTULINUM NEUROTOXIN TYPE A IN HUMANS: A DERMAL MICRODIALYSIS STUDY

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Keywords: Botulinum neurotoxin type A; Capsaicin; Glutamate; Human experimental pain model; Microdialysis; Vasodilation.

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Paper III

BOTULINUM NEUROTOXIN TYPE A MODULATES VESICULAR RELEASE OF GLUTAMATE FROM TRIGEMINAL SATELLITE GLIAL CELLS

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