Nicotine-induced escalation of ethanol intake – an effect mediated by the vagus nerve?

Degree project thesis in Medicine

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Abstract

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There is a clear connection between alcohol abuse and smoking. Previous studies show that 83% of those with alcohol abuse also smoke and that alcoholism is 10 times more common in smokers than in non-smokers. This clinical observation also finds support in pre-clinical animal studies where nicotine treatment has shown to increase ethanol intake in rats. Current research shows that central nicotinic acetylcholine receptors (nAChR) play a vital role in the dopamine releasing effects of ethanol. However, other studies have demonstrated that blockade of peripheral ganglionic nAChR invokes some kind of adaptations, which in turn escalates ethanol intake. We have reason to believe that both peripheral and central nAChR are involved in ethanol consumption. Since the vagus nerve receives input via nAChRs and is a big contributor to the autonomic nervous system, our hypothesis is that this nerve mediates the nicotine-induced escalation of ethanol intake. Thus this present study focused on designing a method that allowed us to surgically ligate certain branches of the vagus nerve in the rat thus removing the peripheral nAChR influence of ethanol intake. If we manage to establish a successful method we will later be able to let vagotomized rats undergo an ethanol-consumption study where we can evaluate if the abolished influence of the vagus nerve changes nicotine-induced escalation of ethanol intake.

A total of 16 animals across 4 trials were operated on during the course of this study. 6 animals were judged to have a successful vagotomy, resulting in a 40% success rate for the surgical procedure. However, we faced great challenges with stabilizing the animals in the post-operative phase due to persisting weight loss, but were finally successful in stabilizing the weight in trial 4 with a liquid diet. All in all, we managed

to achieve a successful gastric vagotomy with a stable post-operative weight gain in only 1 animal of the 16 operated. Conclusively, more surgical practice is needed before achieving an optimal method. However, we believe that with practice, a higher success rate of vagotomy can be achieved and along with a post-operative diet based on liquid diet we will have vagotomized animals stable enough to undergo a voluntary ethanol intake paradigm.

Key words: Ethanol intake, nicotine, vagus nerve, vagotomy.

Introduction

According to the 2014 WHO Global status report on alcohol and health approximately 5.9%, or 3.3 million, of all global deaths were caused by harmful use of alcohol. In 2010 alcohol was the third largest risk factor of global disease and burden, resulting in 139 million disability adjusted life years (DALY) worldwide [1]. Furthermore, the same study also stated that tobacco smoking was the second largest risk factor of global disease. Together tobacco and alcohol use stood for 11.8% of the global disease burden, meaning that more than one in ten people had a disease caused by either tobacco or alcohol. This exemplifies the huge importance of finding strategies to prevent and treat addiction to these substances, thus research in addiction biology is a vital part of decreasing the global disease burden.

One of the biggest scientific achievements in addiction biology has been the discovery of the role of dopamine in the brain reward system, which is a key structure for development and maintenance of dependence to drugs [2]. The brain reward system is a circuitry between several areas in the brain, including the ventral tegmental area (VTA), nucleus accumbens (nAc), prefrontal cortex (PFC), basal ganglia, dorsal striatum (DS), amygdala (AMG) and hippocampus (HPC) [3]. It has been shown that all these areas play a different role in drug dependence but that the pathway between VTA and nAc, also known as the mesocorticolimbic dopamine system, is the most central part of the reward system [4]. The VTA contains a cluster of dopaminergic neurons with efferent projections to the nAc. Activation of these dopaminergic cells can cause and outflow of dopamine in the nAc. This extracellular

increase of dopamine in the nAc mediates the rewarding effect of different stimuli. These include natural stimuli such as food and sex as well as drugs. The increased dopamine levels creates a sense of reward motivating us to repeatedly exposing ourselves to such stimuli, when this gets out of control a dependence to the stimuli is evolved [3]. Previous studies show that drugs of abuse, such as ethanol, nicotine, cocaine, amphetamine and opiates, all share the common ability to activate the mesocorticolimbic dopamine system and that an increase in dopamine levels in this area maintains the dependence on drugs [5]. The exact mechanism for how some of these different drugs activate the dopamine neurons is still subject for investigation. There seems to be both a direct and indirect modulation of these dopaminergic neurons. For example psychostimulants such as cocaine and amphetamine exert their dopamine increasing effect in the nAc by manipulating monoamine reuptake transporters in presynaptic terminals in the nAc. These transporters have the function to pump back extracellular neurotransmitters such as dopamine into the presynaptic terminals. Cocaine binds to these transporters inhibiting their function, resulting in greater levels of extracellular dopamine. Amphetamine both inhibits the reuptake transporters and also reverses their transport function creating an outflow of dopamine from the presynaptic terminal, resulting in greater extracellular levels of dopamine [3]. Opioids seem to exert their reinforcing effect through μ-receptors. Stimulation of μ-receptors increases firing of dopaminergic neurons in the VTA resulting in dopamine release in the nAc [6]. Also, it has been shown that opioids act on μ-receptors on GABA interneurons resulting in a disinhibition of dopaminergic neurons. Nicotine also stimulates dopamine release by increasing firing of dopaminergic neurons. Nicotine acts by stimulating nicotinic acetylcholine receptors (nAChR), which can be found on dopaminergic neurons in the VTA [6]. Nicotinic receptors can

also be found on GABA interneurons as well as glutamatergic neurons. Nicotine acts on these and subsequently disinhibits respectively increases activity of dopaminergic neurons [6]. When it comes to ethanol the mechanism for dopamine release is still not clearly understood and several different hypotheses have been forwarded. One of these hypotheses comes from Gothenburg, where the Addiction Biology Unit has suggested that ethanol increases dopamine in the nAc by activating a network of neurons. In a long series of studies they have found that ethanol primarily, directly or indirectly, act on glycine receptors in the nAc, which in turn disinhibits GABAergic neurons projecting to the VTA. This produces a decrease of inhibitory GABAergic tone in the VTA allowing increased release of acetylcholine in the dopaminergic cell body region. This increase of extracellular acetylcholine activates nAChR located on dopaminergic neurons projecting to the nAc producing an increased dopamine release in the nAc [7].

Knowing that dopamine release in the mesocorticolimbic dopamine system seems to be the common denominator for all drugs of abuse to produce brain reward gives a clearer understanding about why people with addiction often are prone to abuse multiple substances. An excellent example of this phenomenon is seen in the co-abuse of tobacco and alcohol. The fact that there are an elevated number of smokers in a population consisting of people with alcohol abuse has been known for a long time [8]. A case-control study comparing smoking in alcoholic and non-alcoholic individuals showed that 83% of those with alcohol abuse also smoked, compared to 34% of those in the control group. The same study also stated that alcoholism was 10 times more common in smokers than in non-smokers and that only 7% of the

alcoholic population was able to quit smoking compared to 49% of the non-alcoholic population [9].

Thus it is obvious that there is a clear connection between smoking and alcohol consumption. Now the question becomes why? Many would argue that environmental factors such as socioeconomic status play a big role. Also a genetic predisposition for developing dependence to drugs of abuse may be a part of the explanation. While these factors may well explain a positive correlation between tobacco and alcohol, it would also suggest that this correlation exists between all addictive drugs, which is not the case. Rather, it has been shown that there are physiological/pharmacological mechanisms that contribute to the co-abuse of alcohol and tobacco. As mentioned above, it has been shown that nAChR play an important role in the dopamine releasing mechanism of ethanol. As previously explained activation of nAChR on dopaminergic neurons in the VTA produce dopamine release in the nAc following both ethanol and nicotine administration [10]. Since it was shown that nAChRs play an important role in the dopamine elevating properties of ethanol it was hypothesized that these receptors also participate in ethanol consumption. Knowing that ethanol - induced dopamine release decreases by blocking central nicotinic receptors it is only logical to think that a stimulation of these receptors should result in an opposite effect [11]. If so, this could be a possible physiological explanation for the correlation between smoking and alcohol consumption as mentioned earlier.

To gain more knowledge about this mechanism studies have been carried out on rats in controlled conditions where the influences of other factors are minimal. In these studies the animals were given a free choice between ethanol and water during a

period of time prior to being exposed to subchronic nicotine treatment. When reintroduced to the free choice between ethanol and water it was shown that animals that had been pretreated with nicotine significantly increased their ethanol intake. Nicotine treated rats were also more likely to consume ethanol than water, indicating an increased preference towards ethanol. This nicotine-induced increase of ethanol consumption could be reversed by pre-treatment with mecamylamine, a nAChR antagonist [12]. Using microdialysis in freely moving animals, it was also shown that the release of dopamine in the limbic forebrain of rats exposed to acute nicotine or ethanol increased if they had been subchronically pre-treated with nicotine [12]. Thus nicotine treatment seems to facilitate the effects of ethanol, resulting in a greater ethanol intake in an animal exposed to nicotine. An interesting observation in this study was also that these effects lasted several weeks after nicotine treatment was seized. This indicates that subchronic nicotine treatment induces some kind of neuroplasticity in the brain [12]. In previous studies [13,14] it has been suggested that chronic exposure to nicotine may upregulate central nAChR, resulting in an increased dopaminergic release. Another explanation might be that nicotine sensitizes the mesocorticolimbic dopamine system, which would result in a greater release of dopamine when exposed to nicotine/ethanol. Thus, it was concluded that (1) blocking central nAChR prevents ethanol from increasing nAc dopamine and (2) acute blockade of nAChRs results in a decreased intake of ethanol.

As mentioned above previous microdialysis studies show that it is the central nAChR that mediates the dopamine releasing effects of ethanol and not the peripheral receptors. When it comes to ethanol intake though, an interesting observation was made in a study from Gothenburg [15]. In this study rats in a voluntary ethanol

paradigm were subchronically treated with nicotine, mecamylamine (a nAChR antagonist that can pass the blood-brain-barrier), hexamethonium (a nAChR antagonist that can´t pass the blood-brain-barrier) or vehicle during 15 days. After treatment the rats were reintroduced to voluntary ethanol consumption. An increase in ethanol intake and preference was unexpectedly seen in all animals except the vehicle group. Treatment with nicotine + mecamylamine or nicotine + hexamethonium did not counteract the nicotine-induced escalation of ethanol intake, on the contrary treatment with nicotine + hexamethonium seemed to increase the ethanol intake more than nicotine alone.

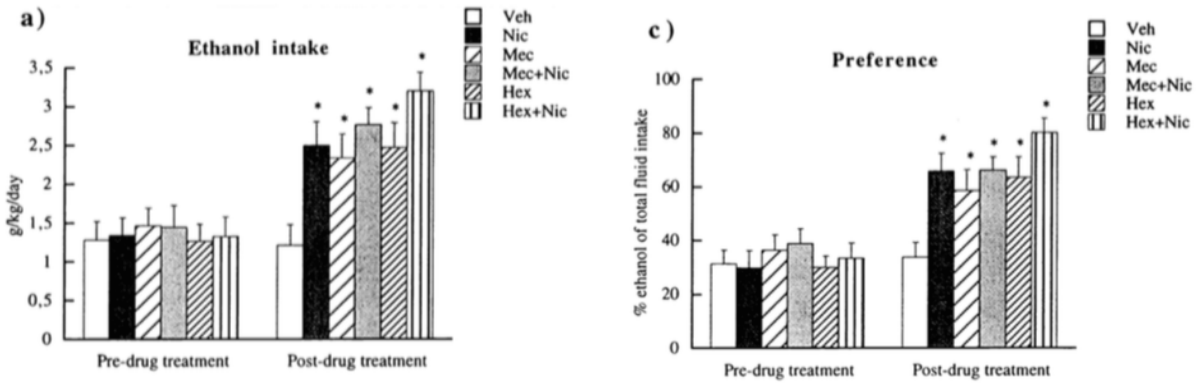

Fig.1 Shows the effect on ethanol intake and preference after administration of nicotine, mecamylamine and hexamethonium.
Source: Ericson, Jörgen A. Engel, Bo Söderpalm. 1999. Peripheral involvement in nicotine-induced enhancement of ethanol intake. Alcohol, volume 21.

The common denominator in all of these substances (nicotine, mecylamine and hexamethonium) is the fact that they all induce a blockade of peripheral nAChR. Nicotine, although known as an agonist at the nAChR, does so by binding to the nAChR and after stimulation the bond between the nicotine molecule and the receptor remains for a period of time, causing a blockade, desensitization, of the nAChR. Thus it seems like it is the blockade of peripheral nAChR that causes a state where the animals increase their ethanol intake. This effect also remains after treatment has been

ceased, yet again indicating some kind of neuroplasticity in the brain, which given the results from this study seems to be caused by the peripheral blockade of nAChR. The same study also showed that treatment with peripheral anti-muscarinic drug significantly reduced the ethanol intake during the first 3 days in nicotine-pretreated animals. After 3 days this effect was gone, which can be explained by the rapid tolerance development known for anti-muscarinic drugs. Thus it may well be that peripheral blockade of nAChR causes an autonomic compensatory mechanism where increased peripheral autonomic activity stimulates the ethanol reinforcing effects of central nAChR rather than direct stimulation of central nAChR. This gives support to the fact that peripheral neurotransmission seems to be involved in the enhancement of ethanol intake [15].

To summarize, it is hypothesized that central nAChR are involved as one of the steps of how ethanol induces dopamine release in the nAc. Studies show that by blocking central nAChR the dopamine releasing effect of ethanol can be prevented. In addition, acute antagonism of nAChRs decrease ethanol intake in rats. Furthermore, it has been shown that subchronic treatment with nicotine increases ethanol intake in the rat, but interestingly this effect can't be counteracted with simultaneous chronic administration of central or peripheral nAChR antagonists. On the contrary previous results suggest that subchronical blockade of peripheral nAChR rather increases ethanol intake and that this effect remains after cessation of the antagonist. Why this occurs is unclear. One theory is that perhaps a signal pathway exists between the peripheral nAChR and the brain reward system, where blockade of the peripheral receptors causes neuroplastic adaptations, which in turn increases the ethanol reinforcing effects. This communication between peripheral and possibly the

mesolimbic dopamine system may be mediated by increased autonomic activity since a blockade of muscarinic receptors has shown to decrease ethanol intake in the nicotine-pretreated rat.

The aim is now to find out where this signal pathway lies in the body. In mammals nAChR are divided into two principal groups: muscle and neuronal type. In turn the neuronal type nAChR can be found in the central nervous system (CNS) and in autonomic ganglia (autonomic nervous system). The peripheral nAChR in the autonomic ganglia are the ones that are thought to play a role in the nicotine-induced increase of ethanol intake mentioned above, since they play a central role in autonomic neurotransmission [16]. The autonomic nervous system (ANS) is known to have both efferent and afferent function. In our case afferent function is of interest since we are looking for a pathway that mediates signals from the peripheral ganglionic nAChR to the brain reward system. As mentioned above this communication seems to be mediated by an increased autonomic activity. Given this information, hypothetically the vagus nerve may be a likely candidate for mediating these signals since it is known to play a substantial role in the ANS. The vagus nerve is the 10^{th} cranial nerve (n.X) of the body and the main contributor to the parasympathetic system. The nerve extends from the medulla oblongata and proceeds through the thoracic cavity and the abdomen, giving out branches to several visceral organs such as the heart, lungs, liver, pancreas, spleen and the gastrointestinal canal. The vagus nerve exhibits both an efferent and afferent function, using acetylcholine as its main neurotransmitter. In the case of afferent signals, up to 80% of the vagal fibers in the abdomen are thought to be afferent [17], which makes this location interesting for our hypothesis. Involvement of the vagus nerve in ethanol consumption in the rat

is also supported by an older study that investigated the role of gastric branches of the vagus nerve on voluntary alcohol intake. This study found that ligation of the gastric vagus nerve decreased alcohol intake in the rat and hypothesized that this part of the nerve may relay information regarding alcohol consumption to the brain [18]. We believe that if we can ligate the afferent signal transmission in the gastric branches of the vagus nerve, we may abolish the increase of autonomic activity that is believed to mediate the communication between peripheral nAChR and the brain. This should then prevent the escalation of ethanol intake in rats exposed to subchronic nicotine. To test this hypothesis we first need to establish a method where we successfully can manipulate the vagus nerve. In humans, manipulation of the vagus nerve was a standard procedure during the 1980s when vagotomy was used for treatment of peptic ulcer. Although this procedure is now outdated following the introduction of Histamine H2 receptor-blockers, there are still a lot of material left from animal studies of that era describing the vagotomy procedure. The purpose of this study was to use that material on operational guidelines and try to recreate a method to perform gastric vagotomy in the rat.

Hypothesis and Aim

Our hypothesis is that peripheral ganglionic nAChR communicates with the brain reward system through vagal transmission. We believe that once the vagal transmission is abolished, nicotine treatment will no longer produce an increase of ethanol intake in the rat.

The aim of this present study was to develop a method that allows us to ligate the gastric branches of the vagus nerve in the rat. The goal was to have vagotomized rats stable enough to undergo a future ethanol-consumption study where we can evaluate if nicotine-treatment still produces an escalation of ethanol intake.

Ethics

To carry out experimental studies on animals it is required to have an approved ethical application that applies with the laws of animal protection. The present study was approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden according to the ethical laws for animal studies.

Method

Animals

A total of 16 animals were used across 4 different trials. The animals consisted of male Wistar rats weighing approximately 200 g and were supplied by Taconic (Ejeby, Denmark). Upon arrival in the animal department, all four animals were housed in one cage (55 x 35 x 20 cm) at constant room temperature (22°C) and humidity (65%) for 2 weeks to adapt to the novel environment. After surgery the animals were put in individual single-cages. The animals were kept under artificial light ± dark conditions (light on at 9:00 P.M. and off at 9:00 A.M.) and had free access to rat and mouse standard feed (Beekay Feeds) and tap water prior to surgery. In trial 2-4 the animals were put on a special diet 3-5 days before surgery for adaptation purposes.

Practice period

In order to gain better understanding of the anatomical outlay in the abdomen of the rat, practice was undertaken in sacrificed rats from other ongoing trials in the lab before proceeding with live animals. A total of 8 rats served as subjects during this practice period. A simulation of the whole surgical procedure was performed, including attempts to identify the abdominal parts of the vagus nerve and its gastric branches. This made it possible for the operator to gain a clear visualization of the different steps in the procedure and a realistic appreciation of the location of the different abdominal organs.

Anesthesia

The fundamentals of anesthesia were based on the method used for probe-operation for microdyalisis in the lab of Söderpalm and Ericson. Isoflurane (Forene 100%) was used as the anesthetic drug and given in inhalation form using a vaporizer. When using this method general anesthesia is induced by placing the animal in a chamber that contains a constant flow of isoflurane (flow 600ml/min, concentration 4%), during 5 minutes. The animal was then transferred to a facemask and the flow and concentration of isoflurane was decreased to 400ml/min at 3.6% to maintain the general anesthesia during the procedure.

This method was used in trial 1 for the first animal, which did not survive the surgical procedure. It was believed that a probable cause for its death might have been a too heavy sedation. Since vagotomy is a longer procedure than the probe-insertion for microdyalisis it is probable that by keeping the flow and concentration of isoflurane at 400ml/min at 3.6% during a longer period of time may cause respiratory distress or severe hypotension. We figured that maybe by gradually decreasing the flow rate and

concentration of isoflurane during the procedure could increase the chances of survival. This hypothesis was tested during surgery for the next animal in trial 1. Induction was performed in the same way as described above. After transferring the animal to the facemask the flow rate and concentration was decreased to 400ml/min at 3.8%. Flow rate and concentration of isoflurane was then gradually decreased during different steps of the surgery. Using this method a schedule for anesthesia was established (see table 1) for all of the following procedures across the 4 experiments.

Flow rate (ml/min)	**Concentration (%)**	**Surgical step**
600	4	Induction
400	3.8	Moving to face mask
345	3.6	Performing vagotomy
340	3.4	Closing

Table 1. Schedule for anesthesia with Isoflurane (Forene 100%)

Operation

The surgical procedure for selective gastric vagotomy was based on the method described by Smith and Jerome in 1981 [19]. After anesthesia the animal was placed on a heating pad on the operation table to prevent hypothermia during surgery. The procedure began with a mid-line incision in the abdomen from the xyphhoid process down to the level of the umbilicus. The abdominal muscles were retracted with four clamps placed two on each side in order to gain exposure into the abdominal cavity. Wet sponges were used to move the liver laterally for better access to the ventricle. Care was taken to not harm the liver with any sharp objects. Wet sponges were also placed above any exposed parts of the intestines. With a hemostat the xyphoid process was bent cranially to make free way in to the ventricle. The ventricle was then pulled

outwards from the abdominal cavity with forceps and a 3-0 silk suture were placed in the major curvature. The ends of the sutures were then clamped with a hemostat and light traction of the ventricle was made possible by gently pulling the hemostat. This gave us better exposure of the esophagus as it entered the abdomen below the diaphragm. When reaching this stage of the operation a microscope was used to identify the fibers of the vagus nerve. There are two major vagal trunks located on the left (posterior) and the right (anterior) side of the esophagus just below the diaphragm (see fig 1). When following the anterior vagus nerve caudally one can identify the hepatic branch leaving to the liver. Below that level is the beginning of the right (anterior) gastric branch, which runs along the lesser curvature of the ventricle. The left gastric branch emerges at the level of the distal esophagogastric junction where the left vagus nerve divides into the coeliac and left gastric branch. The left gastric branch then runs posterior to the ventricle and can therefor be hard to identify (see fig 1). One way to locate the left gastric branch is to rotate the ventricle slightly to the right. After identification of what was thought to be the right and left gastric branches dissection of these nerve fibers were made using micro-dissection forceps. The fibers were dissected until they no longer were seen in the area. Care was taken to not harm the wall of the esophagus and the ventricle. Any minor bleeding was stopped with moist gauzes and the area was flushed with saline in order to clear the field of any debris. After finishing dissection the suture in the major curvature of the ventricle was removed and the ventricle was placed back in its original position. The wet sponges were also removed and the liver placed back in place. The abdominal muscle layers were then closed with interrupted 4-0 vicryl sutures. The skin was closed with interrupted 4-0 ethilon sutures. After closing the wound Rimadyl (Caprofen, 5mg/kg) was injected subcutaneously to manage the post-operative pain.

In trial 4 a new surgical approach was taken. In these animals a lower mid-line incision was made in the abdomen. After retracting the abdominal muscles the small and large intestines were lifted out and placed extra abdominally and covered with plastic, which was kept moist with sterile water. This allowed us to navigate more freely in the abdominal cavity and approach the ventricle and lower esophagus from underneath. This resulted in less risk of damaging the liver or diaphragm compared to the method described above. The remaining part of the operation was carried out as described above and after dissection of the vagus nerve branches the intestines were put back into the abdominal cavity in a careful manner. Initially the wound was closed with sutures. In trial 4 we experienced for the first time a problem with animals chewing through their sutures. Therefore the wound was secured again with wound-clips.

Sham operation was carried out in the same exact manner, except no dissection of the nerve fibers were made.

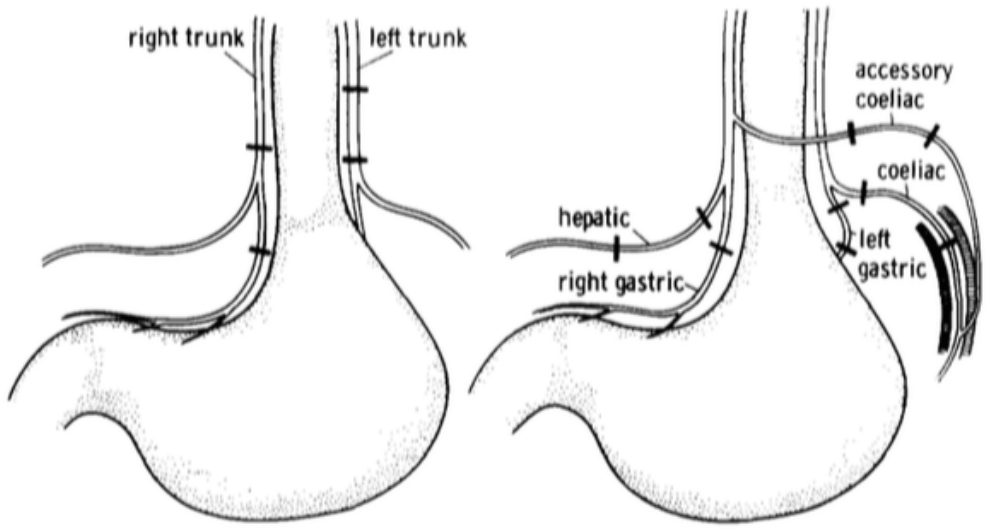

Fig 2. Shows a schematic picture of the abdominal branches of the vagus nerve.
Source: Jerome and Smith. Gastric Vagotomy inhibits drinking after hypertonic saline. Physiology & Behaviour 1981 14th Sep, Vol 28.

Post-operative care

After operation the rats were placed in single cages with access to food and water ad libtium. The animals as well as the bottles containing water and food were weighed after 4 p.m. everyday. The general state of the animals was closely observed and the wound was checked for infection or leakage. Rimadyl (5mg/kg) s.c was administrated daily the first 5 days for pain-management. The average follow-up time before termination for trial 1-3 was 10 days due to rapid weight loss and 21 days for trial 4.

Diet

Different types of diets were used during the study in order to find an alternative that was best suited to prevent rapid weight loss in the animals. In trail 1 the main aim was to have animals surviving the surgical procedure. Therefore regular solid food, in the form of pellets (from Beekay Feeds), was given both before and after surgery. It was quickly observed that food intake decreased post-operatively in these rats. Thus in trial 2 the pellets were replaced with a more soluble type of gel food (DietGel Recovery from Clear H20, 120kcal/cup), which was thought to pass easier through the gastrointestinal canal. The weight loss was less rapid with the gel food but still not satisfactory. Therefore a liquid diet consisting of nutrition drinks (Resource 2.0 from NestleHealthScience, 2kcal/ml) was used for trial 3 and 4. Because it was obvious that the animals had a significantly lower food intake after surgery a nutrition drink with a high calorie content/ml (2kcal/ml) was chosen, with the aim to stabilize the post-operative weight. All animals had access to the chosen diet during 3-5 days before the day of operation. This was to let the animals adapt to the special diet. The animals were weighed every day during this period. After surgery, all animals had access to the chosen diet and tap water ad libitum.

Water was given in clear plastic bottles with ball-valve sprouts. The bottles were filled with fresh water every other day. The pellets were placed on the cage as it was during the acclimation period. Gel food was also administrated by being placed on the cage. A fresh batch of gel food was given everyday and the old batch was replaced, this was to prevent the food from hardening. Administration of gel food in cups placed inside the cage was tested, but this alternative was abandoned due to the fact that the animals chewed through the plastic cups. The liquid diet was administrated in clear plastic bottles with ball-valve sprouts. The bottles were filled with fresh liquid diet every day in order to prevent the dairy in the diet from going sour.
The bottles with water and the liquid diet in trial 3-4, as well as the animals were weighed daily.

Verification

It is known that gastric vagotomy decreases motility and acid secretion in the ventricle. This leads to retention of solid food and distention of the ventricle in vagotomized animals [20]. Completeness of gastric vagotomy can therefore be verified by measuring ventricle to total body weight ratio after 12h food deprivation. This ratio needs to be >0.020 for successful verification [20]. The animals were switched to solid pellet food at the end of the study and then deprived of food for 12h before being terminated. The animals were weighed and then heavily anaesthetized with isoflurane during 5 minutes before termination was performed by decapitation. After termination the abdominal sutures were removed and the wound cut open. The ventricle was located and ligated at the esophagogastric junction and the proximal duodenum before taken out and being weighed. After weighing the ventricle it was cut open to check the consistency of the content. Solid content was another sign of

successful vagotomy due to the lack of acid breaking down the food. The intestines were also checked thoroughly for signs of perforation. The ratio was calculated by dividing the weight of the ventricle by the total bodyweight of the animal.

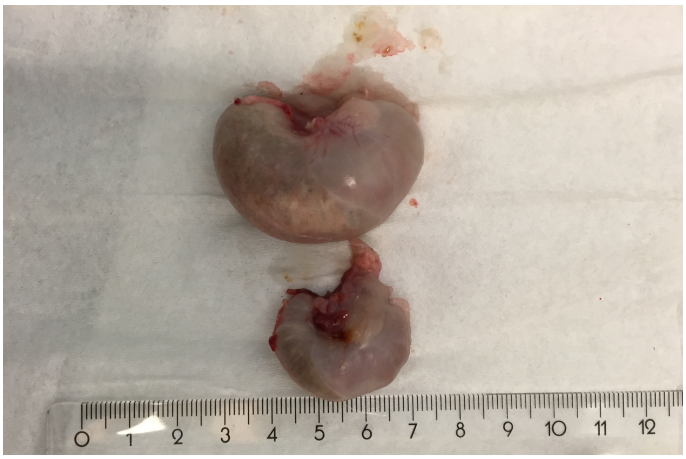

Fig 3. Shows a picture comparing the size of two ventricles during verification. The top ventricle belongs to a successfully vagotomized rat and the bottom ventricle belongs to a rat judged to have unsuccessful vagotomy.

Results

In the present study 16 rats were used in order to develop a successful method ligating the vagus nerve. The animals were grouped equally (4 animals/trial) into four different trials (as described below) where each trial was followed by an evaluation.

Trial 1

A total number of 4 animals were operated on in trial 1. Three of the animals survived surgery, while one animal died on the operating table. It is believed that the cause of death was an overdose of anesthesia. Animals in trial 1 were fed a solid diet consisting of pellets. Post-surgical weight loss was seen in all of the surviving animals, with rat 3 having the most rapid weight loss of 8.33 g/day and a total loss of 14.5% of the bodyweight in 6 days. Overall, animals in trial 1 experienced the most

rapid weight loss compared to the other trials. Due to this fact all animals had to be terminated within 8 days after surgery in accordance to ethical guidelines. After termination rat 2 and 4 were judged to have successful vagotomies (see method section for details regarding verification method). Except weight loss, no other post-operative complications, such as infections, were observed.

Day	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)
1	350	365	357
2	342	385	356
3	330	346	347
4	322	340	344
5	314	326	331
6	317	312	
7	323		
8	313		
Average weight change (g/day)	-4.62	-8.83	-5.20

Table 2. Shows the weight measured everyday for every animal in trial 1 (solid diet) until termination. Rat 1 died during surgery.

Trial 2

Another 4 animals were operated on in trial 2. Animals in trial 2 were fed a gel-based diet (described in the method section). This time all of the animals survived surgery, but rat 1 had to be terminated 4 days after surgery to do respiratory complications. Rat 1 presented with rhonchi and had lost 17% of his bodyweight in 4 days. Weight losses were seen in all animals in trial 2 but the weight loss was less rapid compared to trial 1. The animal with the greatest weight loss lost on average 3.72g/day compared to 8.33g/day in trial 1. All animals in trial 2 were terminated after 11 days since they showed no sign of gaining weight. After termination successful vagotomy was determined in all of the remaining 3 animals. The three animals that survived until the end of the experiment showed no other post-operative complications except weight loss.

Day	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)
1	283	285	299	284
2	270	276	303	274
3	249	266	290	265
4	236	255	275	258
5	-	245	269	260
6		249	268	263
7		253	269	259
8		256	265	260
9		259	263	261
10		266	259	262
11		269	258	263
Average weight change (g/day)		-1.45	-3.72	-1.90

Table 3. Shows the weight measured everyday for every animal in trial 2 (gel diet) until termination. Rat 1 had to be terminated day 4 do to respiratory complications.

Trial 3

In trial 3 malfunction of the heat pad lead to serious complications during surgery. Two of the animals died during surgery while two suffered from burn injuries. These two rats were treated with zinc-paste and anti-analgesic but had to be terminated after 10 days due to steady weight loss. Animals in trial 3 were fed a liquid diet. A big difference in the speed of weight loss was seen between the two surviving animals, where one lost 7.5 g/day compared to 2.6g/day. After termination none of the animals were judged to have successful vagotomies. Due to the severe complications during surgery, results from trial 3 are determined to be unreliable. Therefore we did another try with liquid diet in trial 4.

Day	Rat 1 (g)	Rat 4 (g)
1	346	335
2	350	336
3	332	312
4	323	303
5	319	297
6	315	289
7	319	290
8	326	290
9	323	278
10	320	260
Average weight change (g/day)	-2.60	-7.50

Table 4. Shows the weight measured everyday for every animal in trial 3 (liquid diet) until termination. Rat 2 and 3 died during surgery.

Trial 4

As mentioned above another try with liquid diet was made in trial 4. This time the heat pad was also carefully controlled to prevent any burn injuries. Unfortunately, the first animal died during surgery due to damage inflicted on the diaphragm. An accidental cut was made in the diaphragm resulting in cardiac arrest. After this a different surgical approach was taken as described in the method above. Using this method all of the three remaining animals survived surgery. We decided to perform a sham-operation on the last animal (Rat 4). Unfortunately, this animal had to be terminated the day after surgery since it had chewed through its sutures and ruptured the mid-line incision. In order to prevent this from happening to our last two animals, we decided to secure their wounds with wound-clips. These two animals started to show a positive weight change after 5 days post-surgery and kept on gaining weight throughout the whole experiment. Thus both animals reached the goal of surviving 3 weeks post-surgery and had a final bodyweight that exceeded their weight pre-surgery, gaining 10.1% and 6.9% of their bodyweight. After termination, rat 3 was

determined to have a successful vagotomy while rat 2 was unsuccessful. Thus trial 4 resulted in 1 animal achieving a stable weight gain with a successful vagotomy.

Day	Rat 2 (g)	Rat 3 (g)
1	297	290
2	276	276
3	267	269
4	264	260
5	265	267
6	264	274
7	264	272
8	273	271
9	276	275
10	277	279
12	278	277
13	279	274
14	281	290
15	293	298
16	291	284
17	300	291
18	310	300
19	318	308
20	327	315
Average weight change (g/day)	+1.50	+1.25

Table 5. Shows the weight measured everyday for every animal in trial 4 (liquid diet) until termination. Rat 1 died during surgery and Rat 4 had to be terminated the day after operation due to it eating through its sutures.

Animal	Weight of ventricle (g)	Bodyweight (g) at termination	Ventricle: bodyweight ratio	Successful Vagotomy (ratio > 0.020)
1:2	13	301	0.043	Yes
1:3	4	304	0.013	No
1:4	7	328	0.021	Yes
2:2	8	272	0.029	Yes
2:3	14	260	0.054	Yes
2:4	6	263	0.023	Yes
3:1	4	319	0.013	No
3:4	5	260	0.019	No
4:2	6	307	0.020	No
4:3	16	291	0.055	Yes

Table 6. Shows which animals (1:1 = Trial 1: Rat 1) were successfully vagotomized.

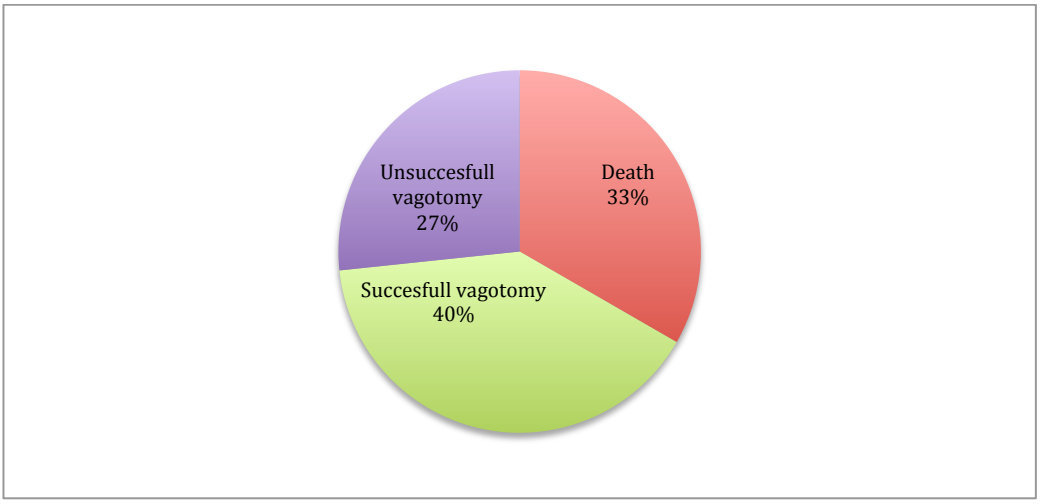

Fig 4. Shows that 40% of all animals were successfully vagotomized.

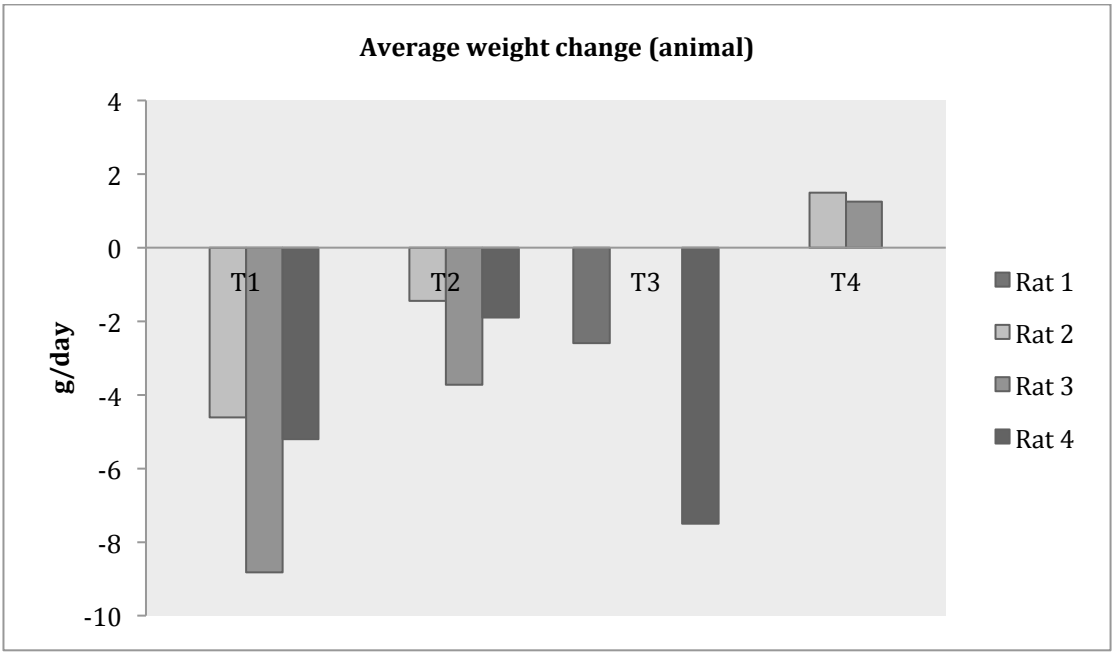

Fig 5. Shows the average change in weight for every animal across the 4 trials (T1 = solid food, T2 = gel food, T3 = liquid diet, T4 = liquid diet).

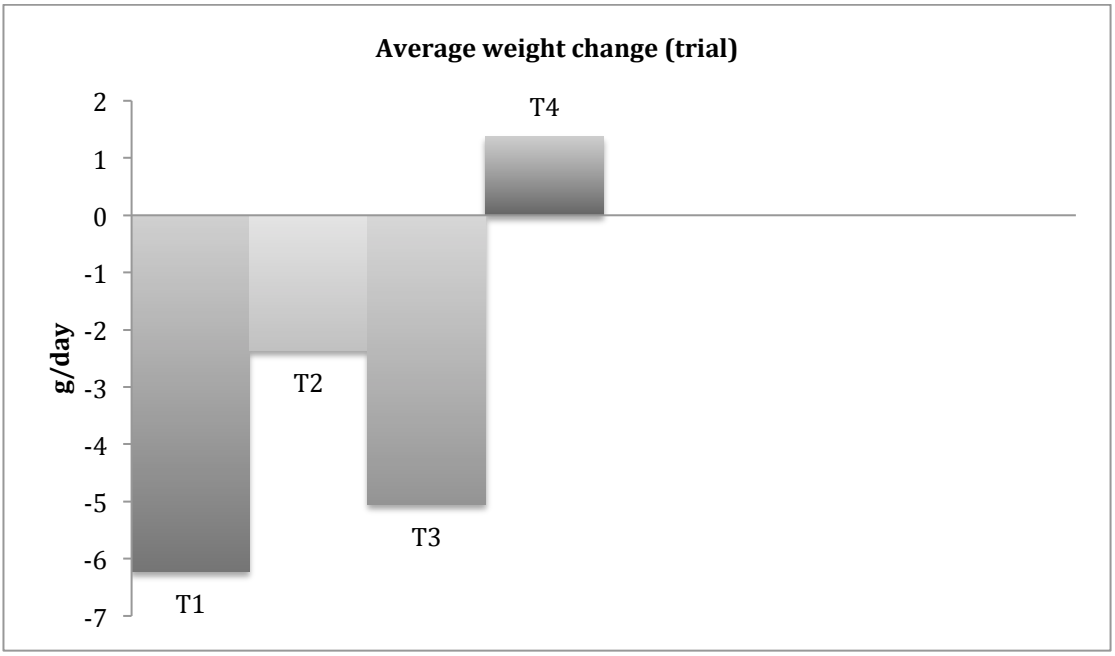

Fig 6. Shows the average change of weight in the animals for the respective trial (T1 = solid food, T2 = gel food, T3 = liquid diet, T4 = liquid diet). This shows that animals on solid food had the most rapid weight loss while animals on liquid diet it trial 4 were the only ones with a weight gain.

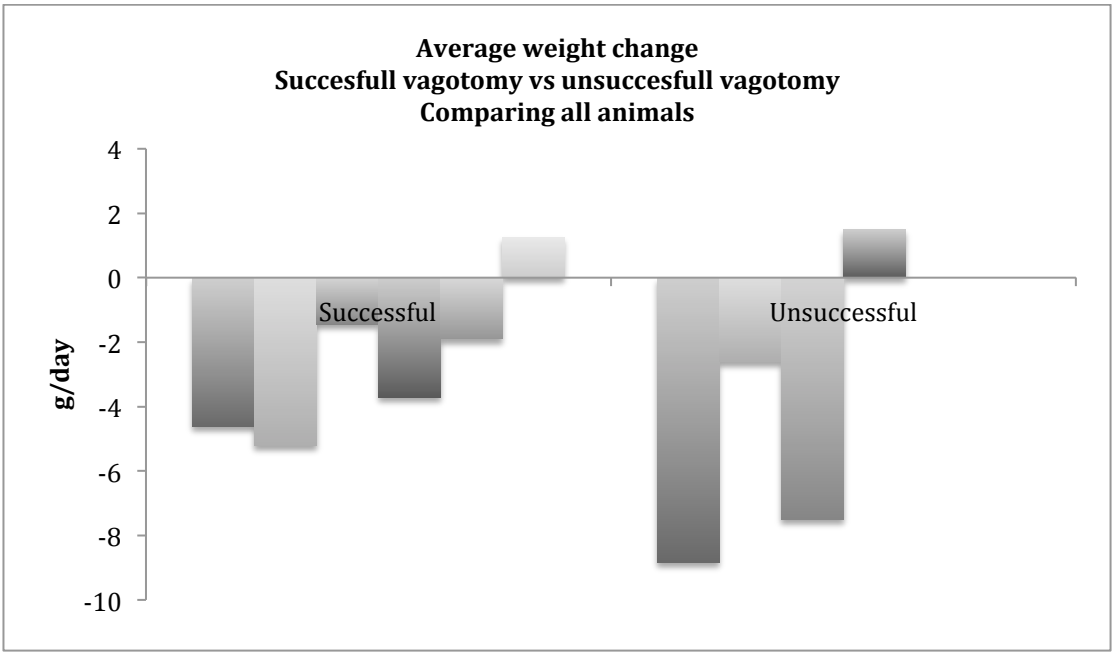

Fig 7. Shows the difference in the average weight change between animals with successful vagotomy compared to unsuccessful vagotomy.

Discussion

From our results we can see that out of the 16 animals that underwent surgery only 1 animal managed to achieve a stable weight gain with a successful gastric vagotomy. A majority of the animals did survive the surgical procedure and most of these also had a successful vagotomy. It was in the post-surgical phase that we faced the biggest challenge with trying to maintain the weight of the operated animals. The best results to do so were obtained with the liquid diet in trial 4. This diet was also used in trial 3 without any sign of weight gain, but we think that this is due to the burn damage that was inflicted on the rats.

The fact that subdiaphragmatic vagotomy causes weight loss in the rat has previously been described in several studies. It has been shown that post-operative syndromes after vagotomy include hypophagia, hypodipsia and loss of body weight if rats are remained on a solid diet. These symptoms can be counteracted if rats are instead maintained on a liquid diet after surgery [21]. This is in line with the results from our study where the only animals showing weight gain were the ones fed with a liquid diet. A weight loss following abdominal vagotomy has also been observed in humans, where a loss of 10-30% of the preoperative weight has been reported [22]. This weight loss is believed to be caused by the loss of both efferent and afferent function of the abdominal vagus nerve after vagotomy. It is known that the vagus nerve is involved in the motility of the ventricle where it controls the gastric contractions and the following gastric emptying. Thus, manipulating this nerve control can cause gastroparesis resulting in gastric retention of solid food. To avoid this, pyloroplasty was often performed in combination with abdominal vagotomy in order to create drainage of the gastric contents [22]. Unfortunately, it has also been documented that

pyloroplasty can cause complications such as dumping syndrome where food is passed to quickly to the small intestine, exacerbating weight-loss [23]. Also previous experiences show that it is difficult to successfully perform pyloroplasty in the rat without leakage and subsequent abdominal infections. Because of this we wanted to avoid performing pyloroplasty on the animals in our study. Instead by using a liquid diet we managed to achieve a weight gain without a pyloroplasty and thus we think that this drainage procedure can be avoided as long as we keep the animals on this specific diet. Furthermore, some studies indicate that the weight loss after vagotomy is not primarily caused by gastric retention, but by decreased food intake [24]. Since the vagus nerve is predominantly an afferent nerve it has been shown that it mediates sensory information from the gut to the brain using both mechanoreceptors and chemical signaling, such as via hormones like cholecystokinin (CCK-8) [25]. It seems like ligation of the afferent vagus nerves abolishes this sensory information resulting in decreased sensation of hunger and food intake [26]. Knowing that vagotomized rats present with a decreased food intake, we decided to prevent further weight loss by choosing a liquid diet with a high calorie content. Thus even if the animals drank less after surgery compared with intake pre-surgery, they would hopefully still have a calorie intake sufficient to produce weight gain. This strategy seemed to work since both animals in trial 4 showed a weight gain, where one of them also had a successful vagotomy. Having said this, it is also important to highlight the fact that weight loss were seen both in animals with and without successful vagotomies (see fig.7). There may be several reasons for this occurrence. Only animals with complete dissection of all fibers of the vagus nerve are judged to be successfully vagotomized with the verification method we used (with 95%-confidence). Therefore animals judged to have unsuccessful vagotomies could have

had parts of the vagus nerve fibers dissected, enough to present with a weight loss but not enough for a successful vagotomy. Furthermore, although care was taken to not inflict damage to the esophagus during surgery, the closeness of the gastric vagus nerve branches puts the esophagus at risk. Thus if any damages to the esophagus were made these could have caused dysphagia in the animal, resulting in a weight loss. However, liquid diet should facilitate food intake even though dysphagia is present, which is in line with the fact that liquid diet was the only diet that produced a post-operative weight gain in our study. Other possible reasons for weight loss in non-vagotomized rats could include the ordinary conditions after any surgery such as nausea and pain. Unfortunately, our sham-rat in trial 4 did not survive; otherwise we would have had more information about to which extent nausea and pain effects food intake. What can be said is that we had careful daily observation of the operated animals and saw no signs of vomiting in the cage. Pain is on the other hand a very possible cause to decreased food intake. It can therefor be discussed if our pain-management was sufficient or if we need a more continuous administration alternatively stronger drugs. Although, since we were using Carprofen, a NSAID, care must be taken to not prolong administration more than necessary in order to prevent gastric ulcers in an already sensitive ventricle after the vagotomy. The other option is to use morphine, but in our case this is not appropriate mainly because of two reasons. Firstly it is known that morphine causes decreased bowel motility, which results in obstipation, which in turn contributes to the retention of food that we want to avoid. Secondly, since our aim is to use these vagotomized animals in a future ethanol-consumption study we can´t take any risks of having an analgesic like morphine, which effects central opioid receptors.

Another important point to address is the accuracy of the verification method used to determine successful vagotomy. In past studies several different methods have been presented to verify the success of gastric vagotomy in the rat. One of the more widely used verification methods is the anatomic verification of vagotomy. When using this method sutures must be placed where the two vagus nerve branches are ligated during surgery. After termination of the animals these sutures are again located using a microscope. If no nerve fibers are seen to connect the two nerve ends the vagotomy is judged to be complete [27]. Although this anatomical verification is frequently used in studies we decided to use another method measuring post-fast stomach to bodyweight ratio. The reason for this is that the stomach-bodyweight ratio method is much more practical to use and has less technical challenges compared to the anatomic verification method. The post-fast method has also been shown to have a great accuracy to determine successful vagotomy in a study where success of vagotomy was judged with both post-fast stomach-bodyweight ratio and electrophysiological verification, which is the most reliable verification method. This study showed that electric transmission in the vagus nerve was impaired in rats with a stomach-bodyweight ratio exceeding 0.020 and concluded that this ratio can be used with a 95%-confident to determine successful vagotomy [20]. With this information we find the post-fast method used in our study to be an adequate verification method at this stage of the study where we are still developing the procedure. Using the post-fast method, 40% of the operated animals in our study were judged to be successfully vagotomized. Surely a greater success rate must be achieved before we can feel confident enough to use this present method in a future drinking study of a larger scale. We believe that in order to increase the success rate of the operation, simply more surgical practice is needed by the operator. Also the new surgical approach,

described in the method above, were we temporarily put the intestines extra-abdominally to gain easier access to the nerves, may result in more successful vagotomies. We feel that this approach provides less risk to damage vital organs such as the liver and diaphragm and also gives better visualization of the vagus nerve branches. However more trials must be conducted using this method in order to evaluate this theory.

As mentioned above vagotomy induces difficulties in the digestive system, which results in decreased food intake and weight loss. Since our aim is to later use these vagotomized rats in a voluntary alcohol consumption study measuring ethanol intake after intermittent administration of nicotine, we must assure that these digestive complications do not affect ethanol intake. Ethanol is known for it's high calorie content and does in that aspect share similarities with food. This may produce the same behavioral effect to ethanol as to food in the vagotomized rat, in other words a decreased intake. We therefore believe that we must achieve a steady post-surgical food intake in the rat, preferably comparable to the intake of sham-rats, before introducing the animals to ethanol. This will allow the influence of calorie-dependent ethanol intake to be minimal since animals already show a positive behavior to food intake with a subsequent weight gain. We are hopeful that the liquid diet used in experiment 4 will allow us to achieve this behavior of steady food intake in the vagotomized rat since it shows promising results regarding weight gain. Unfortunately, we lost our only sham-operated rat in experiment 4. Even though data from only on sham-rat is not enough, it would still have given us important direction about whether this diet is able to produce the same weight gain in both rats who have underwent manipulation of the vagus nerve and those who haven´t.

In summary, we have managed to describe a method for gastric vagotomy that includes a regimen for anesthesia, a new surgical approach, and a post-surgical management plan and verification method. It is clear that the post-surgical weight loss is the greatest challenge when performing gastric vagotomy, but that the weight of the animals can be successfully maintained using a liquid diet with high calorie content. Surely more practice is needed to increase the accuracy rate of successful vagotomies, but we believe that we have identified the fundamental of a method that can be used in future drinking studies. Our plan is to proceed with more trials in order to further develop the skills for the surgical procedure, evaluate pain-management and diets and gain more data about the behavior of vagotomized rats compared to sham-operated rats. When we have achieved the success rate we want, we hope to use this method to further investigate if manipulation of the vagus nerve truly affects ethanol intake in the nicotine pre-treated rat during an ethanol-consumption study.

Populärvetenskaplig sammanfattning

Nikotin-inducerad eskalation av etanolintag – en effekt som förmedlas via vagusnerven?

Alkoholberoende och missbruk är en kronisk återfallssjukdom med vittgående medicinska och sociala konsekvenser. Nyligen publicerades en artikel (Lancet (2012) 380: 2224–60) som fann att alkohol är den tredje största bidragande orsaken till för tidig död i ett världsperspektiv (efter högt blodtryck och tobaksrökning). Om vi ökar förståelsen för hur man blir beroende av alkohol kan vi försöka utveckla nya läkemedel mot sjukdomen och på så sätt minska ett enormt lidande.

Denna studie fokuserar sig på det nära samband som har visat sig existera mellan alkoholberoende och nikotinintag. Ungefär 70 % av alla alkoholister är rökare och det är tio gånger vanligare att en rökare är alkoholist än en icke-rökare. I en djurmodell har man tidigare visat att nikotinbehandling till råtta driver upp dess frivilliga alkoholkonsumtion. Vi har även funnit att ökningen av alkoholintaget inte primärt beror på en central (i hjärnan) mekanism utan inkluderar sannolikt en perifer (ute i kroppen) funktion. Vi tror alltså att nikotin påverkar nikotinreceptorer ute i kroppen och att det sedan sker någon koppling mellan de perifera receptorerna och de centrala vilket får en effekt på alkoholintaget. Vi har skäl att tro att en sådan koppling kan ske via en ökad autonomisk signaltransmission och förmedlas via en nerv som heter Vagus. Vår hypotes är därmed att om man bryter vagusnervens influens kommer nikotinbehandling inte att driva upp det frivilliga alkoholintaget hos råtta. Syftet med denna studie var därför att utveckla en kirurgisk metod för att klippa av delar av vagusnerven hos råtta och på så sätt bryta dess signalöverföring. Om vi lyckas med detta kan vi använda metoden i framtida alkoholkonsumtionsstudier för att närmare

undersöka vilken roll vagusnerven spelar i kopplingen mellan nikotin och alkoholintag.

Totalt opererades 16 råttor under denna studiens gång och vi lyckades klippa av vagusnerven hos 6 av dessa. Dock möttes vi av en stor utmaning i form av snabb viktnedgång hos djuren efter operation. Vi behövde därför använda oss av olika dieter för att motverka viktnedgången, och till slut fann vi att en kaloririk näringsdryck var det bästa alternativet för att stabilisera vikten. Av de 16 djur som deltog i studien var det bara 1 djur som genomgick en lyckad manipulering av nerven samt kunde upprätthålla en stabil viktuppgång. I denna studie lyckades vi beskriva en metod för att klippa av en del av vagusnerven, men det krävs fortfarande mer kirurgisk träning för att uppnå den träffsäkerhet som krävs för att använda sig av metoden i en större alkoholkonsumtionsstudie. Vår slutsats är att med mer träning och en post-operativ diet bestående av kaloririk näringsdryck hoppas vi att på att kunna uppnå en tillräcklig bra metod för manipulering av vagusnerven som kan användas i senare alkoholkonsumtionsstudier.

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