ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DO PORTO
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"Descending pain modulation in a Kaolin-induced hydrocephalus rat model"

Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do Grau de Mestre em Bioquímica em Saúde, no ramo de Bioquímica Clínica e Metabólica realizada sob a orientação científica da Professora Doutora Isaura Tavares e orientação institucional da Professora Doutora Cristina Prudêncio.

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Abstract

Pain transmission at the spinal cord is modulated by descending actions that arise from supraspinal areas which collectively form the endogenous pain control system. Two key areas involved of the endogenous pain control system have a circunventricular location, namely the periaqueductal grey (PAG) and the locus coeruleus (LC). The PAG plays a crucial role in descending pain modulation as it conveys the input from higher brain centers to the spinal cord. As to the LC, it is involved in descending pain inhibition by direct noradrenergic projections to the spinal cord.

In the context of neurological defects, several diseases may affect the structure and function of the brain. Hydrocephalus is a congenital or acquired disease characterized by an enlargement of the ventricles which leads to a distortion of the adjacent tissues, including the PAG and LC. Usually, patients suffering from hydrocephalus present dysfunctions in learning and memory and also motor deficits. It remains to be evaluated if lesions of the periventricular brain areas involved in pain control during hydrocephalus may affect descending pain control and, herein, affect pain responses.

The studies included in the present thesis used an experimental model of hydrocephalus (the rat injected in the cisterna magna with kaolin) to study descending modulation of pain, focusing on the two circumventricular regions referred above (the PAG and the LC). In order to evaluate the effects of kaolin injection into the cisterna magna, we measured the degree of ventricular dilatation in sections encompassing the PAG by standard cytoarquitectonic stanings (thionin staining). For the LC, immunodetection of the noradrenaline-synthetizing enzyme tyrosine hydroxylase (TH) was performed, due to the noradrenergic nature of the LC neurons. In general, rats with kaolin-induced hydrocephalus presented a higher dilatation of the 4^{th} ventricle, along with a tendency to a higher area of the PAG. Due to the validated role of detection the c-*fos* protooncogene as a marker of neuronal activation, we also studied neuronal activation in the several subnuclei which compose the PAG, namely the dorsomedial, dorsolateral, lateral and ventrolateral (VLPAG) parts. A decrease in the numbers of neurons immunoreactive for Fos protein (the product of activation of the c-*fos* protooncogene) was detected in rats injected with kaolin,

whereas the remaining PAG subnuclei did not present changes in Fos-immunoreactive nuclei. Increases in the levels of TH in the LC, namely at the rostral parts of the nucleus, were detected in hydrocephalic animals. The following pain-related parameters were measured, namely 1) pain behavioural responses in a validated pain inflammatory test (the formalin test) and 2) the nociceptive activation of spinal cord neurons. A decrease in behavioral responses was detected in rats with kaolin-induced hydrocephalus was detected, namely in the second phase of the test (inflammatory phase). This is the phase of the formalin test in which the motor behaviour is less important, which is important since a semi-quantitative analysis of the motor performance of rats injected with kaolin indicates that these animals may present some motor impairments.

Collectively, the results of the behavioral studies indicate that rats with kaolin-induced hydrocephalus exhibit hypoalgesia. A decrease in Fos expression was detected at the superficial dorsal layers of the spinal cord in rats with kaolin-induced hydrocephalus, further indicating that hydrocephalus decreases nociceptive responses. It remains to be ascertained if this is due to alterations in the PAG and LC in the rats with kaolin-induced hydrocephalus, which may affect descending pain modulation. It remains to be evaluated what are the mechanisms underlying the increased pain inhibition at the spinal dorsal horn in the hydrocephalus rats. Regarding the VLPAG, the decrease in neuronal activity may impair descending modulation. Since the LC has higher levels of TH in rats with kaolin-induced hydrocephalus, which also appears to increase the noradrenergic innervation in the spinal dorsal horn, it is possible that an increase in the release of noradrenaline at the spinal cord accounts for pain inhibition. Our studies also determine the need to study in detail patients with hydrocephalus namely in what concerns their thresholds to pain and to perform imaging studies focused on the structure and function of pain control areas in the brain.

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Abreviations

ARs: Adrenoreceptors

CSF: Cerebrospinal fluid

IASP: International Association for the Study of Pain

IP: Intraperitoneal

IR: Immunoreactive

LC: Locus coeruleus

PAG: Periaqueductal grey

RVM: Rostral ventromedial medulla

TH: Tyrosine Hydroxylase

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Introduction

1. Pain

1.1. Pain definition

According to the International Association for the Study of Pain (IASP), pain is defined as a sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries (Loeser and Treede, 2008; Barrot, 2012).
Acute pain serves as a warning to potential internal or external to the body dangerous situations, and it is important for survival. On the contrary chronic pain, which is consided as the pain lasting more than 3-6 months after injury and persists beyond normal tissue healing time, has no biological advantage, being difficult to treat (Fornasari, D, 2012). Regarding treatment, no new effective analgesic has been produced in the last decade. Non-steroidal anti-inflammatory agents and opioids are the most usual treatments, but opioids are associated with adverse side-effects such as dependence and tolerance. The lack of effective treatment with controlled side effects reflects how poorly the molecular pathophysiology underlying cronic pain is understood (Tsantoulas, 2014).

There are two different types of chronic pain: neuropathic and inflamatory pain. Neuropathic pain is defined as spontaneous pain and hypersensitivity to pain associated with damage to or pathological changes in the peripheral nervous system (Fornasari, D, 2012). Inflammatory pain is caused by tissue damage, as occurs after trauma, surgery or during chronic inflammatory diseases. In these conditions, damaged cells and inflammatory cells recruited to the site of damage release substances that activate, and sensitize, peripheral nociceptors. (Fornasari, D, 2012; Grace, P.M., 2014).

1.2. Nociceptive Transmission

After transmission of potentially painful information (i.e. nociceptive) from the periphery to the spinal cord by primary sensory neurons (nociceptors), several events are triggered in order to control and modulate the information transmitted to higher brain centres. (Fig. 1. Heinricher et al., 2009). Nociceptors are excited only when stimulus intensitie reaches the noxious range, suggesting that they possess biophysical and molecular properties that

enable them to recognize and react to potentially harmful stimuli. There are two major classes of nociceptors: Aδ- and C-fibers. Aδ-fibers have medium-diameter, medium to large cell bodies and are thinly myelinated, conducing action potentials quickly; heavily myelinated Aβ-fibers have a larger diameter; C-fibers are characterized as small-diameter, unmyelinated fibers, small cell bodies and they conduct action potentials slowly (Fornasari, 2012; Basbaum, 2009).

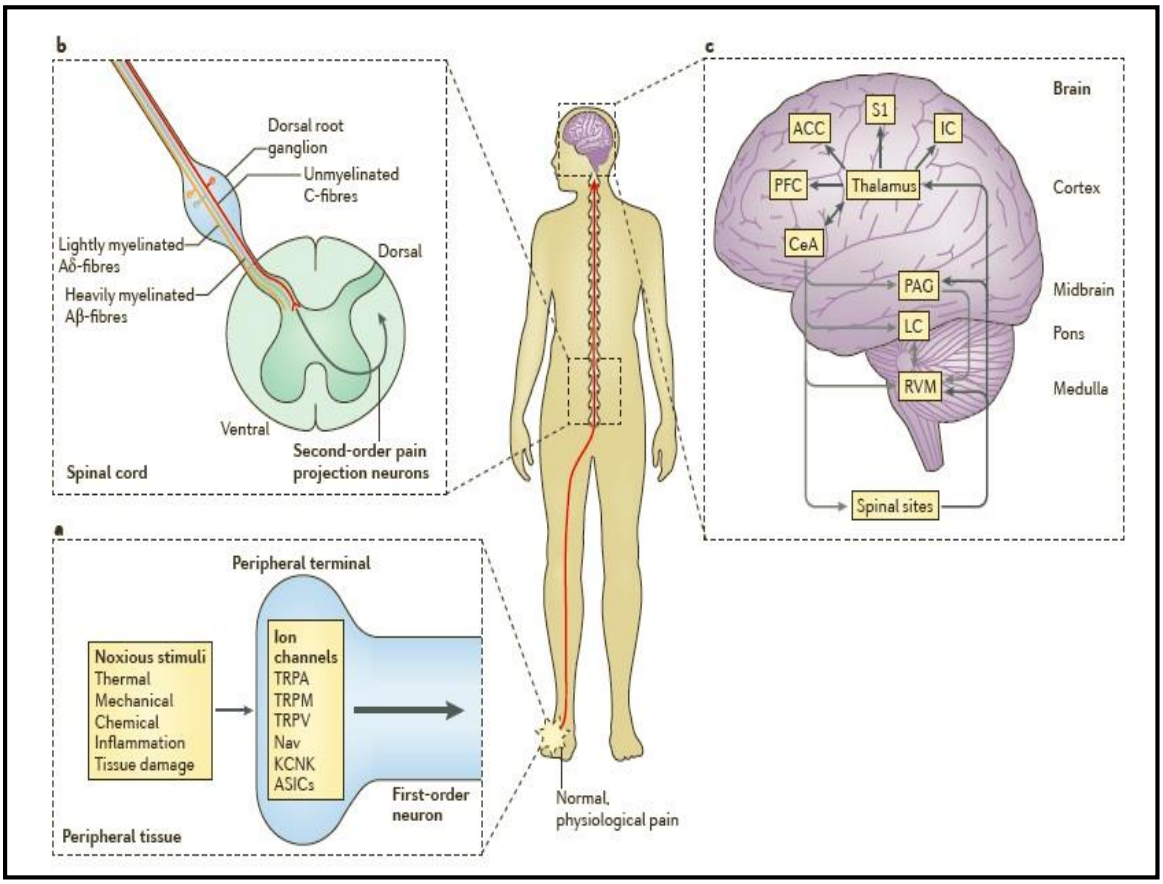

Figure 1. Transmission of nociceptive information. Nociceptive information is conveyed by primary afferent neurons (a) from the periphery to the spinal cord and then reaches the brainstem through ascending pathways (b) constituted by second order neurons. In the brain the nociceptive information is evaluated and no single brain region is essential for pain. Pain perception and response results from the activation of a widespread brain areas (c) Adapted from Grace, P. M. 2014.

Primary afferent neurons have three distinct roles in nociception: transduction, conduction and transmission. Transduction is the detection and the passage of the stimulus into electrical activity in the outlying terminals. This process is mediated by non-selective cation channels causing membrane depolarization (Fig. 1). When potential action generated is sufficient to achieve the

threshold for the activation of voltage gated sodium channels, causing the transmission of stimuli to central terminals of nociceptors in the spinal cord – conduction (Fornasari, D. 2012). Transmission is the transfer of synaptic input from one neuron to another. Action potentials generated in primary afferents induce the release of neurotransmitters that have the potential to excite second-order neurons, which constitute the ascending pathways, in the dorsal horn of the spinal cord or hindbrain (Basbaum, A.I 2009; Fornasari, D.2012; Grace, P.M., 2014).

Second-order nociceptive projection neurons project to supra-spinal sites, which in turn project to cortical and subcortical regions across third-order neurons, allowing an appropriate response, conveyed by descending pathways to the spinal dorsal horn (Fig.1, Grace, P.M., 2014). No single brain region is essential for pain, but rather pain results from the activation of a distributed group of structures. Rather, pain results from activation of a distributed group of structures, some are more associated with the sensory-discriminative properties, such as the somatosensory cortex, and others with the emotional aspects, such as the anterior cingulate gyrus and insular cortex (Basbaum, A. I 2009; Ossipov, M. H. 2010; Grace, P.M., 2014).

Imaging studies demonstrated activation of prefrontal cortical areas, regions not generally associated with pain processing, but the contribution of the activation of these areas to pain perception is not totally clear (Basbaum, A. I 2009).

1.3. Endogenous pain control system

The concept of a pain matrix is not intended to suggest a rigid regulatory pathway but a collection of brain regions that modulate pain transmission at the spinal cord. The endogenous pain control system was initially accounted to be involved in pain inhibition. Recently it was discovered, that it is also involved in pain facilitation and the balance between inhibition and facilitation is dynamic, and can be altered in different behavioral, emotional and pathological states. (Almeida et al., 2006; Heinricher et al., 2009; Ossipov et al., 2010).

The most well characterized pain modulatory areas belonging to the endogenous pain control system are located in the brainstem. The PAG is the central area of that system and play a crucial role in descending pain modulation from the brain. There are several brain

areas involved in pain modulation that project to the PAG and, in this way, find a gateway of arrival to the spinal cord (Heinricher et al., 2009; Ossipov et al., 2010).

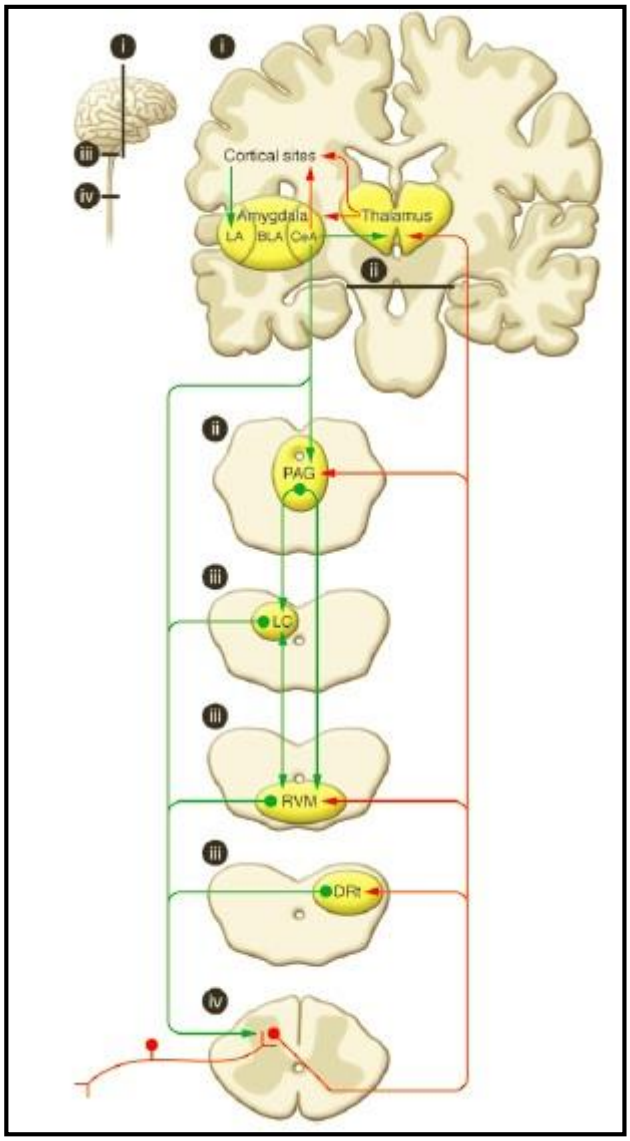

Figure 2. Schematic representation of the pain modulatory circuitry. Primary afferent neurons convey nociceptive inputs to the spinal dorsal horn. Ascending projections (labelled in red) from the dorsal horn targeting the thalamus, the DRt, the RVM and the PAG. The thalamus is connected to some cortical sites and to the amygdala. Descending pain modulation is mediated through projections (labelled in green) from these cortical areas to the PAG, which communicates with the RVM and the LC, and send descending projections to the spinal dorsal horn. Adapted from Ossipov et al, 2010.

The PAG-rostral ventromedial medulla (RVM)-dorsal horn circuity has been extensively described as important role players in pain modulation. The PAG is heavily interconnected

with the hypothalamus and limbic forebrain structures including the amygdala. The PAG projects to the RVM which in turn sends its output to the spinal dorsal horn (Fig. 3, Heinricher et al., 2009). It exerts both descending inhibition and facilitation of pain. This dual effect is correlated with the co-existence at the RVM of neurons involved in descending antinociceptive actions (OFF-cells), along with cells responsible for descending pronociceptive effects (ON-cells). (Almeida et al., 2006; Silva, M. et al., 2013) Imbalance in the activity of pronociceptive ON- and antinociceptive OFF-cells was shown to occur in traumatic neuropathic pain, with increase in "ON-cell" firing and the reverse for "OFF-cell", which facilitates nociceptive transmission at the spinal cord. Descending facilitatory influences from the RVM are critical for the development and maintenance of exaggerated pain behaviors produced by noxious and non-noxious stimuli during neuropathic pain (Heinricher et al., 2009; Ossipov et al., 2010; Silva, M. et al., 2013).

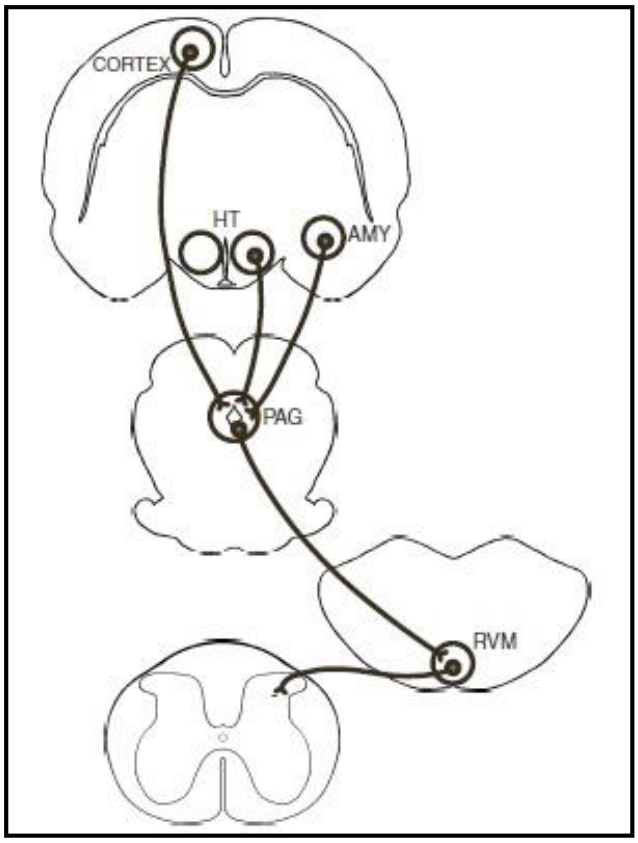

Figure 3. Schematic PAG-RVM system. The PAG is directly connected with the hypothalamus and limbic forebrain structures, including the amygdala, and also receives direct spinomesencephalic input. This area is also connected with several brainstem areas, including the RVM, and largely exerts modulatory effects on the nociceptive transmission to the spinal cord, using the RVM as a relay. Adapted from Pertovaara, A. and Almeida, A. 2006.

1.4. Noradrenergic Modulation

Several studies demonstrated a strong contribution of noradrenaline in antinociception associated with descending inhibition (Hammond, DL., et al 1985; Cui, M., et al 1999; Ossipov et al., 2010) Noradrenaline plays a key role in the inhibition of nociceptive transmission at the spinal cord level. While the PAG contain noradrenergic neurons, this region communicate with noradrenergic sites important to pain modulation, classified as A1-A7 (Fig. 4) The A1 cell group is located at the level of the area postrema, A2 is distributed throughout the dorsal vagal complex, A3 is in the medullary reticular formation, and A4 surrounds the fourth ventricle. The A5 cell group is in the ventrolateral pons, A6 or the LC is dorsally in the pons and A7 is in the lateral part of the pons, close to the lateral lemniscus. (Pertovaara, A., 2006; Ossipov et al, 2010). These noradrenergic cell groups are connected with a number of other nuclei influencing pain-related behavior, and all of them receive projections from the midbrain periaqueductal gray. (Bajic and Proudfit, 1999) Contrary to the spinal cord, the effects of noradrenaline in the brain are complex, depending on the pain control area and the adrenoreceptors (ARs) involved. Supraspinally, noradrenaline can either facilitate or inhibit pain depending on the site of release, the type of adrenoceptor activated, and the intensity and duration of the pain stimulus (Pertovaara, A 2006; Martins, I. et al, 2013). From all the above mentioned noradrenergic cell groups, only LC, A5 and A7 were shown to account to direct projections to spinal cord in several animal studies.

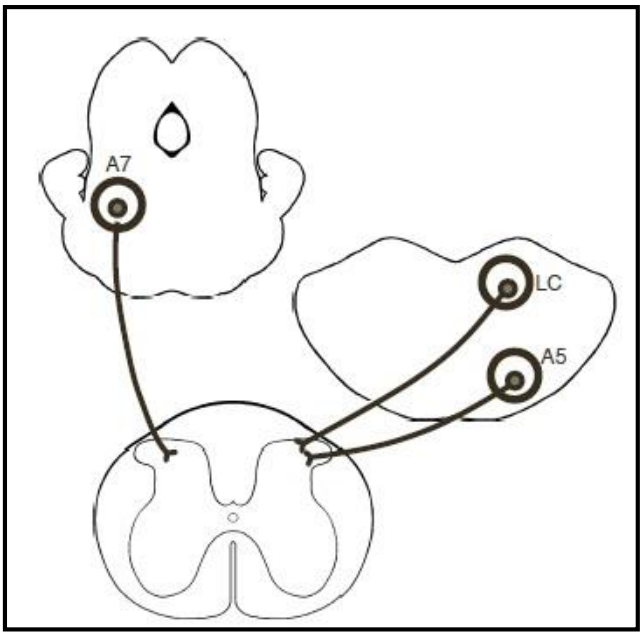

Figure 4. Noradrenergic descending pain inhibitory pathways originating in the catecholaminergic nuclei of the brainstem. The locus coeruleus, A5 and A7 cell groups are connected with other pain control centers and all of them receive projections from the PAG. Pertovaara, A. and Almeida, A. 2006.

Intrinsic noradrenaline in the spinal cord is released from descending axons originating in noradrenergic nuclei of the brainstem. Stimulation of these noradrenergic structures or some non-adrenergic structures in the brainstem induce spinal release of norepinephrine (Hentall et al., 2003). Antinociception induced by stimulation of A5, A6 and A7 cells group is attenuated by intrathecal administration of alpha-2-adrenoceptor antagonists.

The activation of descending noradrenergic pathways by peripheral noxious stimulation forms a negative feedback system that suppresses ascending pain signals at the spinal level (Pertovaara, A. 2006).

There is abundant evidence indicating that descending noradrenergic pathways originating in the pons contribute to pain regulation, particularly to feedback inhibition of pain. At the spinal level, the descending noradrenergic inhibition of pain-related responses is predominantly mediated by α2 adrenoceptors. Activation of α2-adrenergic receptors has been shown to inhibit nociceptive transmission at the level of the spinal cord through presynaptic activity, inhibiting release of excitatory neurotransmitters from primary afferent terminals, as well as through postsynaptic sites (Ossipov, M. H., 2010, Song Z., 2013).

1.5. The noradrenergic Locus coeruleus

The locus coeruleus or the A6 cell group in the pons is the major site of noradrenergic cell bodies in the brain. (Fig.5) The LC has a significant role in both the processing and modulation of pain. The LC is an important relay nucleus in the pain-processing system: it is connected with other pain control centers and receives projections from the PAG. Additionally, the LC receives projections from the central nucleus of the amygdala, preoptic area, paraventricular nucleus of the hypothalamus and lateral hypothalamus (Pertovaara, A. and Almeida A., 2006; Szabadi, E. 2013). and sends many projections into different parts of the brain including hypothalamus, thalamic relay nuclei, amygdala, basal telencephalon, the cortex, forebrain, brainstem, cerebellum and the spinal cord (Stone, EA., 2011).

Emerging evidence suggests a contribution of the LC to pain facilitation. Following plastic changes induced by injury or inflammation, the contribution of the noradrenergic system in pain control is increased (Brightwell, J. J. and Taylor, B.K., 2009; Song Z., 2013). Noradrenergic LC lesions inhibited the development of allodynia and hyperalgesia and inactivation of the LC reduced established neuropathic pain. Noradrenergic neurons in the LC participate in the development and/or maintenance of allodynia and hyperalgesia in the setting of peripheral nerve injury (Brightwell, J. J. and Taylor, B.K., 2009).

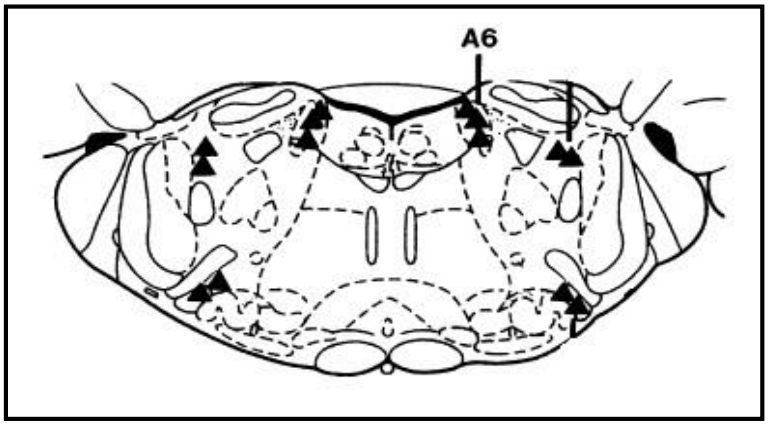

Figure 5. Noradrenergic nuclei A6 (the locus coeruleus), in the pons. The descending axons of these two pontine nuclei are the main sources of norepinephrine in the spinal cord. Adapted from Pertovaara, A. 2006.

2. Hydrocephalus

2.1. Hydrocephalus definition

Hydrocephalus constitutes an important issue in clinical neurology and neurosurgery. Not only the symptomatology, but also the morphological and histopathological changes in hydrocephalus have been widely investigated in humans and in experimental animals.

Hydrocephalus can be either congenital or acquired. Acquired hydrocephalus is one of the most common disturbances of the mature central nervous system (Shinoda M. and Olson L., 1997). During brain development, susceptibility to develop hydrocephalus is high, with a prevalence of 0.5 - 1 per 1000 births (Olopade F. et al, 2012, Lattke, M. et al 2012).

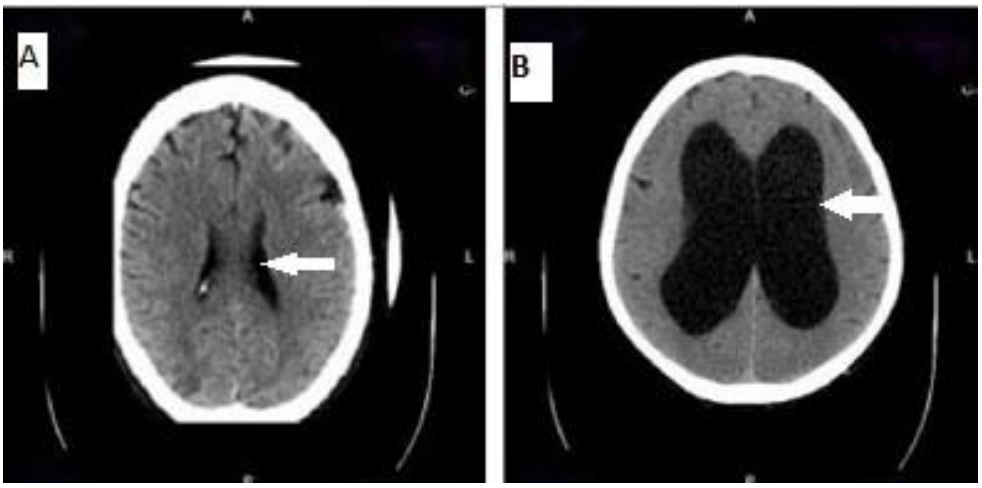

Figure 6. Axial plans demonstrative of normal brain (A) and Hydrocephalus brain.

Hydrocephalus is a common medical heterogeneous neuropathological disorder characterized by abnormalities in the secretion, circulation and resorption of cerebrospinal fluid (CSF), resulting in ventricular dilatation (Hwang Y S et al 2009; Xu, H. et al, 2012 a, b; Olopade F. et al, 2012). This compresses the periventricular tissue leading to a mechanical distortion and changes in brain metabolism and neurochemical function. Ventricular dilatation has some adverse effects that include gliosis, inflammatory responses, damage to neurons and cellular pathways, destruction of periventricular axons, demyelination, impaired cerebral blood flow, and altered clearance of proteins and toxins.

However, the mechanisms underlying these deficits are not fully understood. The neuropathological consequences of this increased intracranial pressure are lethal in 20–50% of patients if left untreated.

2.2. Cerebrospinal fluid

CSF is produced in the brain by modified ependymal cells in the choroid plexus (approximately 50-70%) and the remainder is formed around blood vessels and along ventricular walls (Del Bigio, M.R. 2010; Skjolding, A. D., 2010; Chiewvit, S., et al. 2014). It circulates from the lateral ventricles to the foramina of Monro (Interventricular foramina) and exit lateral ventricle to third ventricle pass through aqueduct of Sylvius (Cerebral aqueduct) to fourth ventricle and eventually exit the cranial vault via foramen of Magendie (Median aperture) and foramina of Luschka (Lateral apertures), subarachnoid space over brain and spinal cord (Chiewvit, S., et al. 2014; Krishnamurthy, S., 2014).

Absorption of CSF is via arachnoid villi that are associated with venous sinuses as well as lymphatics that are associated with cranial and spinal nerves (Del Bigio, M.R. 2010).

CSF has multiple functions, including cleansing of potentially noxious byproducts of brain metabolism, a route for molecular communication and trophic factor transport, and protection of brain by absorbing from vascular and extraneous shock waves.

2.3. Pathogenesis of brain damage

Hydrocephalus can be associated with specific functional and neuropsychological abnormalities, many of which can be attributed to dysfunction of periventricular brain regions including the projections to the spinal cord (Del Bigio MR, 1993; Olopade F. et al, 2012). The pathogenesis of the brain damage is multifactorial. At the simplest level, ventricle enlargment cause damage by stretching periventricular axons, inflammatory responses, damage to neurons and cellular pathways, demyelination impaired cerebral blood flow and altered clearance of proteins. However, more complex processes interact with this phenomenon (Xu, H. et al 2012).

Ventricular enlargement is initially tolerated as the local shape changes from a narrow to a more rounded one and the corpus callosum becomes horizontal. With continuous ventricular enlargement the corpus callosum is elevated and local tissue stretching becomes problematic.

The severity of damage is affected by the age of onset, the rate of ventricle enlargement, the size of the ventricles, the intracranial pressure (which in turn dictates the cerebral perfusion pressure), coexisting pathological changes, in particular cerebrovascular disease in older individuals (Graff-Radford and Godersky, 1987; Hofmann et al., 1995; Del Bigio, M.R. 2010).

Brain function can be evaluated by the integrity, connectivity, and function of neurons. Hydrocephalic brains has abnormalities in sensory and motor-evoked potentials exhibit changes in conduction along several pathways. Several studies both in humans and animals reported learning and memory dysfunction which may be reversible after shunting surgery (Shim et al., 2003; Vachha et al.,2006). Regarding subset of sensory modality, the pain perception, no studies were even directed to patients with hydrocephalus.

Concerning motor function, physical disabilities have reported, including gait instability as well as arm and hand dysfunctions, deficits in balance and posture (Hwang, Y. S. 2009; Del Bigio, M.R. 2010).

2.4. Experimental Hydrocephalus

Animal studies seem to reflect the human situation quite well. Pathophysiology of hydrocephalus has been studied since the late 19th century (Del Bigio, M.R. 1993). Dandy and Blackfan in 1913 (Dandy WE, Blackfan KD, 1913) were the first to describe a model of experimental hydrocephalus. In their studies, dilatation of the third and lateral ventricles in dogs was caused by plugging the aqueduct with cotton. In this model hydrocephalus was induced by inflammatory obstruction of the subarachnoid spaces surrounding the brain stem (Del Bigio, M.R. 1993). Throughout time several models were being used to study hydrocephalus such as obstruction of cerebral venous outflow, inoculation of viruses into

the cerebrum, selective breeding of genetic mutants, and exposure to teratogenic or toxic agents (Hochwald GM, 1985).

Besides the method used the animal's age at the time of induction is another determining factor in the pathophysiology since hydrocephalus can disturb brain development during the phase of cell proliferation (Mashayekhi et al., 2002; Owen-Lynch et al., 2003; Khan et al., 2006). In later stages of maturation, hydrocephalus can delay myelination, which may partially recover after surgery (Chumas et al., 1994; Del Bigio et al., 1994; Del Bigio et al., 1997). These findings are supported by human imaging (Hanlo et al., 1997) and autopsy studies.

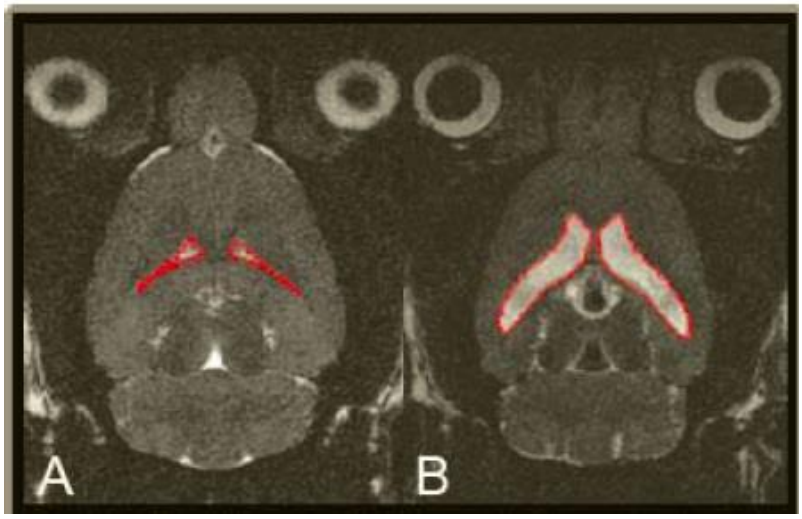

Figure 7. MRI imaging of the rat brain. Control (A) and Kaolin injection (B) Adapted from Skjolding, A. D. 2010

The model of kaolin-induced hydrocephalus was described for the first time in 1932 (Dixon and Heller, 1932). Injection of kaolin (aluminium silicate) into cisterna magna causes an inflammatory reaction in the meninges in the area of the fourth ventricle. As a consequence of the obstruction the connection between the ventricular system and the intracranial and subarachnoidal space is interrupted and obstruct the flow of CSF leading to ventriculomegaly (Fig. 7 Del Bigio, M. R. 1993; Kondiziella, D. et al 2002; Olopade, F.E. et al 2012). This model is commonly used for hydrocephalus research because it causes ventricular enlargement and can be considered analogous to the human situation (Del Bigio, M. R. 1993).

2.5. Neurotransmitter Changes in Hydrocephalus: Catecholaminergic System

Neurotransmitters play an important role in the function of each neuron and there is a relationship between neurotransmitters and neurological disorders. Changes in the content of neurotransmitters in the brain are a consequence of experimental hydrocephalus (Miyake, H. et al 1992).

In human hydrocephalus the major metabolite of neurotransmitter Dopamine, the homovanilic acid is elevated in ventricular CSF (Miyake, H. et al 1992). Nakayama et al reported functional changes in dopamine receptors in humans with chronic hydrocephalus suggesting decrease of postsynaptic dopamine (D2) receptor in the dorsal putamen and these findings can be correlated with the severity of gait instability (Nakayama et al, 2007). In fact these changes in the dopaminergic system may explain the parkinsonian symptoms often exhibited by patients with chronic hydrocephalus (Berger et al., 1985; Curran and Lang, 1994; Krauss et al., 1997; Racette et al., 2004).

In experimental models of hydrocephalus a decrease in levels of noradrenaline has been described in several brain areas such as frontal cortex, hippocampus, and in the cerebellum. Histochemistry studies revealed accumulation of noradrenaline in the cell body of the LC and subcoeruleus neurons (Ehara, K. et al 1991). In contrast, noradrenaline levels of the nerve terminals were reduced in the frontal cortex and the periventricular area of the hypothalamus (Chovanes, G. I., 1988, Ehara, K. et al 1991). This increase may indicate damage of axonal transport or in Locus Coeruleus´s projections (Chovanes, G. I., 1988).

Aims and methodology

The PAG, located at the mesencephalon and surrounding the aqueduct plays a crucial role in descending pain modulation as it conveys the input from higher brain centers to the spinal cord. For its crucial role as a gateway for descending pain modulation, it is expected that pathological changes that affect the PAG may have repercussions in pain responses (Heinricher et al., 2009). Based on the crucial role of the PAG, it comes with no surprise that structural or functional dysfunctions of the PAG may affect pain modulation from the brain in the clinical situations in which the PAG is lesioned.

Another area involved in pain modulation and located around the fourth ventricle is the noradrenergic LC. The LC is an important relay nucleus in the pain-processing system, connected with other pain control centers and receiving projections from the PAG (Pertovaara, A. and Almeida A., 2006). Due to its localization it is expected that structural changes in the fourth ventricle have an impact on its structure and functioning.

Since hydrocephalus is characterized by the enlargement of the ventricles, leading to a distortion of the adjacent tissues, (Hwang Y S et al 2009; Xu, H. et al, 2012 a, b; Olopade F. et al, 2012) including the PAG and LC, the aim of this work was to study neuronal dysfunctions at noradrenergic modulation in an experimental model of hydrocephalus (the Kaolin-induced rat model) and establish if those changes account for alterations in behavioural pain responses and changes in nociceptive activation of spinal neurons.

For chemical stimuli, a validated inflammatory pain model was used (formalin test) by injecting 5% formalin in the hind paw and analysing the licking, biting or shaking the injected paw. Additionally, the expression of the proto-oncogene c-Fos was evaluated, after chemical stimulation in the formalin test, a marker of neuronal activation, in the spinal dorsal horn (Barrot, M. 2012).

The second aim of this thesis consisted on studying the involvement of the noradrenergic LC in descending pain modulation in a Kaolin-induced Hydrocephalus by the evaluation of the expression of TH, which is the rate-limitng enzyme of noradrenaline synthesis.

Materials and Methods

Pathogen-free adult male Wistar rats (Charles River colony, France) were pair-housed in standard Plexiglas cages with free access to food and water. After stereotaxic injections, the animals were housed individually. The colony room was maintained at 22 ± 2°C on a standard 12/12h light/dark cycle. All experiments were conducted during the light phase. Upon arrival, rats were allowed five days of acclimation before any procedure. All procedures were performed in accordance with the European Community Council Directive (2010/63/EU) and the ethical guidelines for pain investigation (Zimmermann, 1983).

1. Surgical induction

Wistar rats weighing 285-300g were deeply anesthetized by an intraperitoneal (i.p.) injection of a mixture of ketamine hydrochloride (0.06 g/kg) and medetomidine (0.25 mg/kg). The rats were placed on a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA) by positioning the head of the rats in the incisor bar and then by inserting the earbars into each ear canal. Once each earbar was inserted verified by a blink reflex usually induced by the contact of the earbar with the ear canal it was immediately placed into the holder and fixed. The region of the head and neck was shaved and then cleaned with Betadine® solution. Posteriorly the neck was incised and the atlanto-occipital membrane was exposed. A sterile kaolin suspension, (0,05 ml 20%) (Skjolding, A. D. 2010) was injected into the cisterna magna with a 27-gauge needle. (Fig 8. Olopade, F. E., 2012) The suspension was injected at the slow rate and at the completion of the injection the needle was left in place for a few seconds, to avoid reflow, before being slowly removed (n=22). Control rats underwent the same procedure but received sterile saline injection instead Kaolin (n=18). After surgery, the animals were individually housed and monitored daily to evaluate body weight and the development of hydrocephalus seen as increased head circumference, affected gait and dull general appearance.

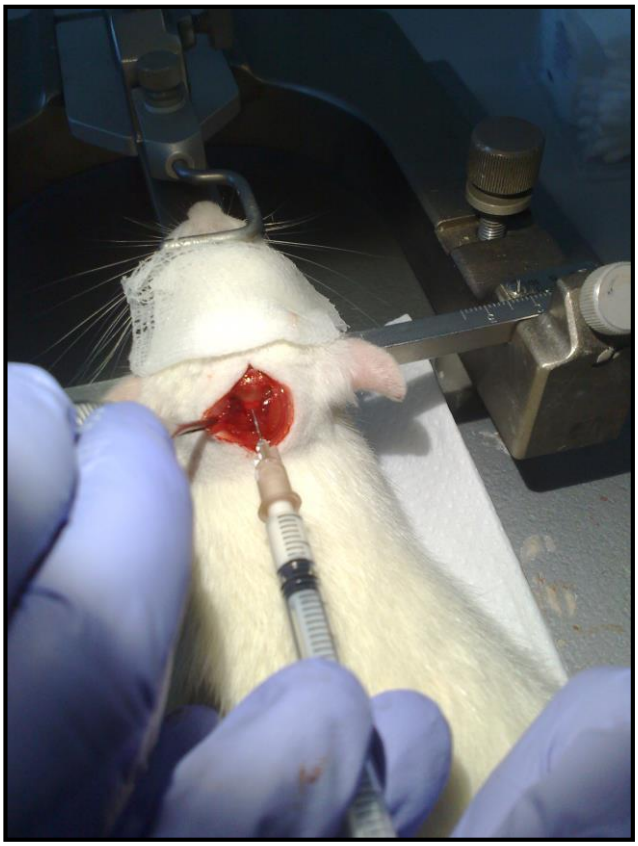

Figure 8. Surgical induction of hydrocephalus. Kaolin injection into cisterna magna of the rat placed on a stereotaxic frame.

2. Behavioral evaluation

2.1. Formalin test

The formalin test was performed as previously described (Tjølsen et al., 1992) after an habituation period of one week during which the animals were handled in the behavioral test room for 30 min every day. The criteria for adequate habituation were that animals did not freeze or defecate when placed in the test chamber (Abbott et al., 1995).

Three weeks after Kaolin/ saline injections, the animals were placed on a clear Plexiglas chamber and were subcutaneously injected into the dorsal surface of the left hindpaw with 50 μl of formalin (5%) using a 27-gauge needle (Tjølsen et al., 1992). Painfull behaviour induced by formalin injection was recorded and analyzed in 5 min epochs for 60 min, as previously described (Tjølsen, A. et al., 1992, Martins, I. et al., 2011), using the computer programme ODRec (Observational Data Recorder).

We evaluated the following behavioural categories: (i) time spent in focused pain-related activity: motor activity directed towards the injected paw, including biting, licking and shaking of the injected paw; (ii) time spent in non-focused pain-related activity: motor activity not directed towards the injected paw, but modified to protect the paw during movement (Almeida, A. et al., 1999). Pain behaviour was evaluated giving scores to each behaviour. A reduction in the weight put on the injected paw was given a score of 1, total elevation of the paw a score of 2, and licking, biting and shaking of the paw a score of 3. A mean score was calculated, with each score being weighted according to the time spent in each behavioural category (weighted pain score) (Tjølsen, A. et al., 1992).

Formalin injections produces a biphasic behavioural reaction with an initial phase within the first minutes post injection, followed by a quiescent period of around 10 min and a second phase lasting 20–40 min. The first phase is related to the direct stimulation of nociceptors, the second phase involves both inflammatory mechanisms and central sensitization within the dorsal horn (Tjølsen, A. et al., 1992, Barrot, M. 2012).

The statistical analysis was performed by using a two-way ANOVA followed by Sidak's post-hoc test using the computer programme GraphPad Prism 6.

2.2. Motor function

As described previously hydrocephalic animals present disturbances in gait. With the purpose of assessing the motor function of the animals a battery of reflexes and motor assessment were measured: placing/stepping reflexes, withdrawal reflex, toe spread reflex, observation of posture and ambulation abilities and a variation of the tail suspension. The rats performance was scored as (-) if there was no reflex, (+) if there was a modest reflex and (++) in a normal reflex (von Euler, M. et al 1996, Potes, C. et al 2006). In order to not disturb the realization of the formalin test, the motor function evaluation was performed on the day before of the formalin test.

Toe spread reflex was studied by lifting the rat with legs hanging free and observing the spread of the toes (von Euler, M. et al 1996).

Placing reflex was evaluated when the rat was held in one hand, supported by the trunk with legs hanging free. The dorsal and lateral aspects of each foot were rubbed against the table edge. The evaluation was performed by the speed and accuracy in placing the foot on the table (von Euler, M. et al 1996).

Withdrawal reflexes were evaluated as to both speed and force with wich the hindlimb was withdrawn when stimulated by extension, pain or pressure. This was done by pulling the hindlimb backwards with two fingers and pressing the foot between the testers thumb and index finger respectively (von Euler, M. et al 1996).

Posture and ambulation abilities were assessed by observing the rat's spontaneous activity (von Euler, M. et al 1996).

Tail suspension test were performed by lifting the rat's tail in order to force the hindlimbs to loose contact with the floor, compelling the rat to walk forward (Potes, C. et al 2006).

3. Vascular perfusion and material processing for immunohistochemical analysis

Two hours after formalin injection, the animals were anaesthetized by i.p. injection with an overdose of sodium pentobarbital (65mg/ kg of body weight). The animals were then placed in the supine position and the abdomen and thorax were opened to expose the heart. A catheter was then introduced into the ascending aorta and perfused with 200 ml of calcium-free Tyrode's solution, followed by 1 L of fixative solution containing 4% paraformaldehyde in 0.1 M PB, pH 7.2. After perfusion, the brain and the spinal cord were removed, immersed in fixative for a post-fixation period of 4 h followed by 30% sucrose in 0.1 M PB, pH 7.2 overnight at 4°C. The material was cut in a freezing microtome at 40 μm in coronal orientation. The brain and the spinal cord was serially cut and collected in 4 sets and stored in a cryoprotector solution at -20°C. One set of brain sections was used to determine the effectiveness of hydrocephalus induction and the degree of ventricular dilatation. The second set of brainstem sections was used for immunohistochemical analysis of TH expression. One set of spinal L4 sections was used to evaluate Fos expression.

4. Histological verification

To verify the effectiveness of induction of hydrocephalus, as well as the structural changes in the PAG, we proceeded to the measurement of the PAG and aqueduct areas in mesencephalic sections stained with thionin.

5. Immunohistochemical analysis of Fos expression

The spinal cord and brainstem sections were carefully washed with PBS 0.1 M and treated with 1% hydrogen peroxidase to inhibit the activity of endogenous peroxidase. The sections were then incubated with blocking solution (10% normal swine serum in 0.3% Triton-X 25% in phosphate buffer with 0.1 M glycine) before incubation with the primary antibody, a polyclonal anti-Fos antibody raised in rabbit (Oncogene, UK), diluted at 1:20000 in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 2% normal swine serum, for 48 hours at 4°C. After washing with PBS-T the sections were incubated for 1h with a swine biotinylated anti-rabbit serum (Dako, Danemark) diluted in PBS-T containing 2% normal swine serum. Sections were washed again and incubated for 1h in PBS-T containing the avidin-biotin complex (1:200; ABC; Vector Laboratories, U.S.A). After washing in 0.1 M Tris-HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3´-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, U.S.A.) and 0.025% H2O2 in the same buffer. The sections were mounted on gelatine-coated slides, cleared in xylol and cover slipped with Eukitt (Sigma, U.S.A. Fos-immunoreactive (IR) neurons was counted in the spinal dorsal horn (laminae I–III and laminae IV-VI). Photomicrographs of spinal cord sections were taken using a Zeiss® light microscope with a high-resolution digital camera for later counting the number of immunoreactive neurons using the cell counter plugin from computer programme Fiji.

Five sections were randomly taken from each rat and the numbers of Fos-immunoreactive (IR) neurons was counted in the PAG using a Nikon® light microscope with a high-resolution digital camera.

The statistical analysis was performed by using an unpaired t-Test for comparisons between the control and Kaolin groups using the computer programme GraphPad Prism 6.

6. Immunohistochemical analysis of TH expression

Brainstem sections were carefully washed with PBS 0.1 M and treated with 1% hydrogen peroxidase to inhibit the activity of endogenous peroxidase. The sections were then incubated with blocking solution (10% normal swine serum in 0.3% Triton-X 25% in phosphate buffer with 0.1 M glycine) before incubation with the primary antibody, a polyclonal anti-TH antibody raised in mouse (Affiniti, UK), diluted at 1:6000 in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 2% normal swine serum, for 24 hours at 4°C. After washing with PBS-T the sections were incubated for 1h with a rabbit biotinylated anti-mouse serum (Dako, Danemark) diluted in PBS-T containing 2% normal swine serum. Sections were washed again and incubated for 1h in PBS-T containing the avidin-biotin complex (1:200; ABC; Vector Laboratories, U.S.A). After washing in 0.1 M Tris-HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3´-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, U.S.A.) and 0.025% H2O2 in the same buffer. The sections were mounted on gelatine-coated slides, cleared in xylol and cover slipped with Eukitt (Sigma, U.S.A.). For the immunohistochemical analysis of TH expression in the spinal L4 sections the procedure was the same but with the anti-TH antibody diluted at 1:4000. Bilateral measurements at the spinal cord were performed.

Sections encompassing the rostro-caudal extent of the Locus Coeruleus were taken from each animal, photomicrographs of both left and right LC LC and spinal L4 sections were taken using a Zeiss® light microscope with a high-resolution digital camera.

The statistical analysis was performed by using an unpaired t-Test for comparisons between the control and Kaolin groups using the computer programme GraphPad Prism 6.

Results

1. Physical Observations

1.1 General conditions of the animals

The weight of the animals was taken as a measure of their well-being, so animals were weighted daily after surgery during the whole period of behavioural assessment. The statistical analysis was performed by using a two-way ANOVA followed by Bonferroni's post-hoc test using the computer programme GraphPad Prism 6.

Animals injected with saline gained 11.2 ± 4.1 g during the first week. Additionally, all animals showed normal exploratory activity and did not exhibit any visible behavioural abnormality until the end of the experiments.

Animals injected with Kaolin lost 15.2 ± 16.1 g during the first week. Weight growth of hydrocephalus animals was significantly slower (*$p<0.05$) than that of control group during the first week, as described by Olopade, F. E. et al, 2012. In the days after Kaolin injection, animals showed clinical evidence of hydrocephalus: general reduction in activity, reduced coordination, an abnormal posture, hind legs weakness, nasal and/or orbital secretion. These signs disappeared spontaneously within a few days. (Hwang, Y. S. et al 2009, Xu, H., et al 2012, Olopade, F. E. et al, 2012) Some animals of the hydrocephalic group developed an enlarged, domeshaped head usually detectable within one week of kaolin injection. They also developed varying degrees of unsteady gait (Olopade, F. E. et al, 2012). After the first week animals of the hydrocephalic group recover and gain weight normally as control animals as shown in the graph of Figure 9.

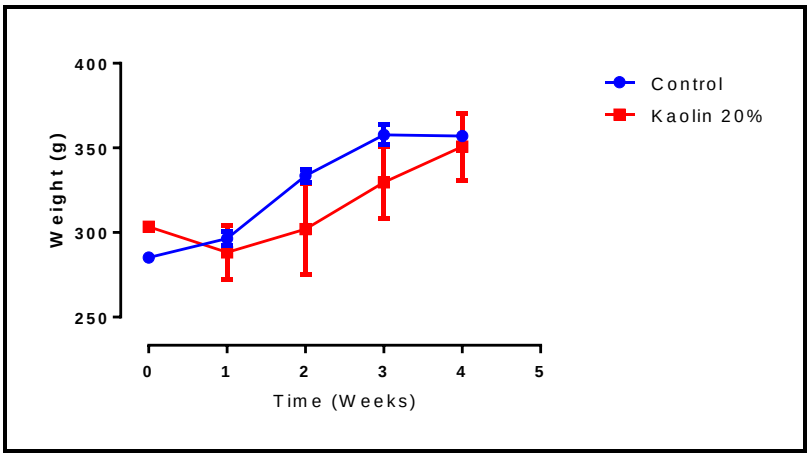

***Figure 9**. Animals' weight evolution after Kaolin/saline injection. Data as means ± SEM (saline – blue line n= 4; Kaolin – red line n=5) *p<0.05.*

1.2 Motor function

With the purpose of evaluating the motor function of hydrocephalic rats several semiquantitative tests were performed.

The results from motor function tests evaluated in the 21 animals are displayed in Table 1. Hydrocephalic animals, in general, show motor changes since most of them scored a modest or no reflex. Regarding motor assessment they show some difficulty in walking when forced to walk without the hindlimbs in the tail suspension test and show less exploratory activity comparing to the controls.

Table 1: Scores from the motor assessment tests performed in rats injected with saline (blue) and with kaolin (red). The reflex tests were scored as (-) if there is no reflex, (+) if there is a modest reflex, (++) normal reflex. The other evaluation tests were scored as (-) if they could not accomplish the test, (+) if they accomplished successfully the test and (±) if it passed the test with some difficulty. (numbers in blue- control animals, numbers in red – hydrocephalic animals).

	Placing stepping reflexe	Withdrawal reflexe	Toe spread reflexe	Posture ambulation	Tail susppension
1 – 22313	++	++	++	++	++
2 – 22314	++	++	++	++	++
4 – 22315	++	++	++	++	++
11 – 22322	++	++	++	++	++
12 – 22323	++	++	++	++	++
13 – 22324	++	++	++	++	++
4 – 22628	++	++	++	++	++
6 – 22629	++	++	++	++	++
7 – 22630	++	++	++	++	++
10 – 22633	++	++	++	++	++
5 – 22316	+	+	++	++	++
6 – 22317	+	-	+	+	±
7 – 22318	+	-	+	++	++
8 – 22319	+	+	+	+	+
9 – 22320	+	+	++	++	+
10 – 22321	+	+	+	+	+
1 – 22625	+	+	+	++	+
2 – 22626	++	++	+	++	+
3 – 22627	+	+	+	++	+
8 – 22631	-	-	-	±	±
9 – 22632	++	+	+	++	++

1.2 Validation of the model

With the aim of verifying the characteristic ventricular dilatation of hydrocephalus and to validate the model used, we measured the area occupied by the aqueduct. Hydrocephalic animals show a large increase in the total area of the Aqueduct when compared with control animals (Figure 10).

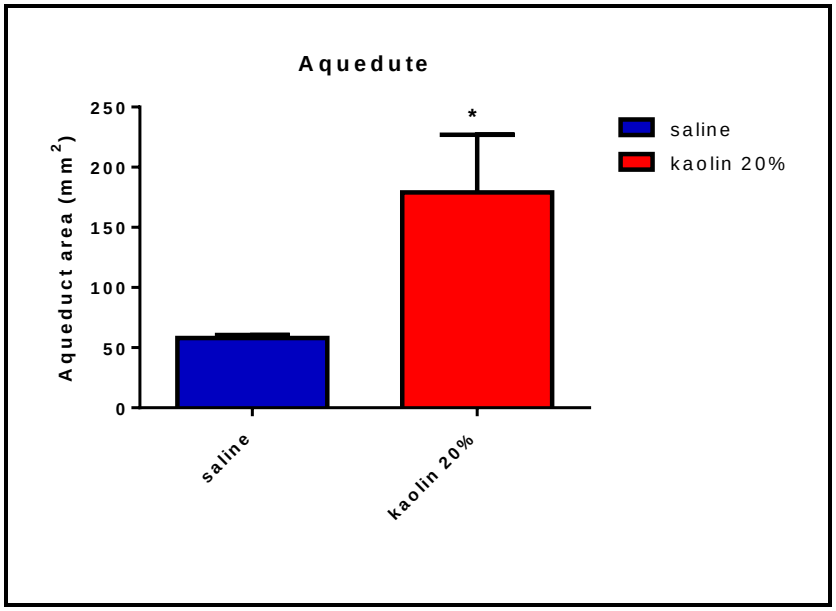

Figure 10. Area of the Aqueduct (mm^2). Hydrocephalic animals show a large increase of total area of the Aqueduct. Data as means ± SEM (Saline n=5; Kaolin 20% n =5) *p=0.0353.

1.3 Structural dysfunctions of the PAG

The evaluation of the area of the PAG was made in serial sections stained by the method of Thionin. Due to its periventricular localization and the enhancement of the ventricles, hydrocephalic animals show an increase in the total area of the PAG compared with control animals. (Figure 11)

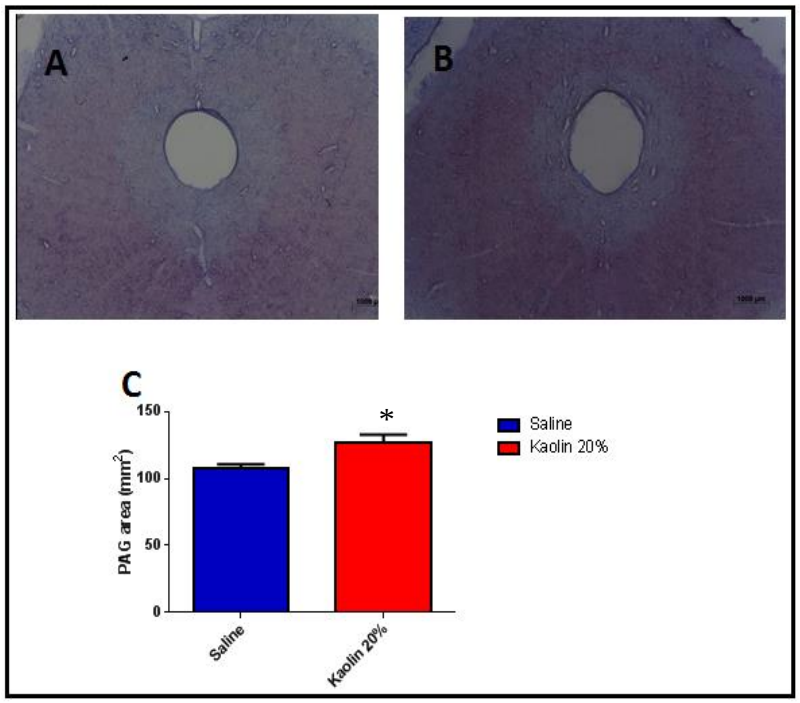

Figure 11. Area of the PAG (mm^2). Hydrocephalic animals show an increase of total area of the PAG. Data as means ± SEM (Saline n=5; Kaolin 20% n =7) *p=0.0241.

2. Behavioural effects

2.1 Formalin Test

Subcutaneous injection of formalin in both control and hydrocephalic animals produced a typical biphasic response consisting of licking and biting of the injected paw, and flinching of the paw and hindquarters, which are easily identifiable behaviours. Hydrocephalic animals shows a decrease in pain behaviours during the second phase as demonstrated in figure 12.

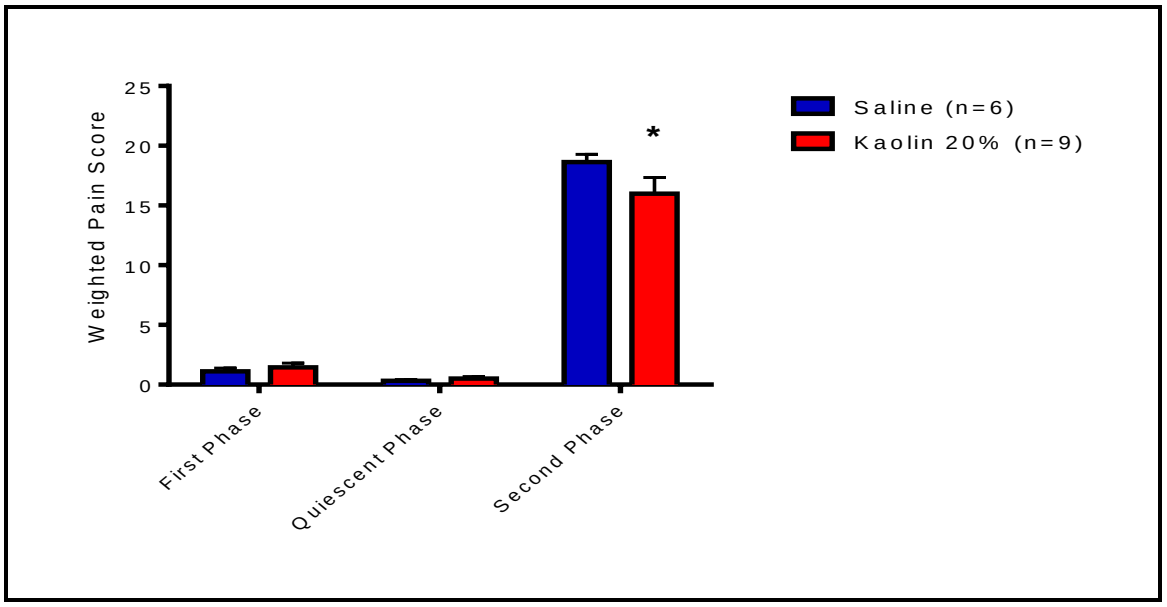

Figure 12: Formalin test results. Hydrocephalic animals show a decrease in pain behaviour's during second phase (saline n=6, Kaolin 20% n=9). Data are presented as mean ± SEM. (*$p < 0.05$ vs second phase saline treated group).

3. Fos expression

3.1 Spinal cord

The evaluation of c-Fos expression in the spinal dorsal horn by immunohistochemistry was performed using spinal sections of L4 segment (Saline n=10, Kaolin 20% n=10). Analysing dorsal horn (laminae I-VI) there was a decrease in the number of fos-IR cells in the group of animals with hydrocephalus ($p=0.0217$) (Fig.13). In a more detailed analysis, separating superficial laminae (I-III) of the deep laminae (IV-VI) the decrease of the number of fos-IR cells was only statistically significant for superficial laminae (Laminae (I-III) $p = 0.0032$ and not for the deep dorsal horn Laminae (IV-VI) $p=0.0694$) (Fig 14)

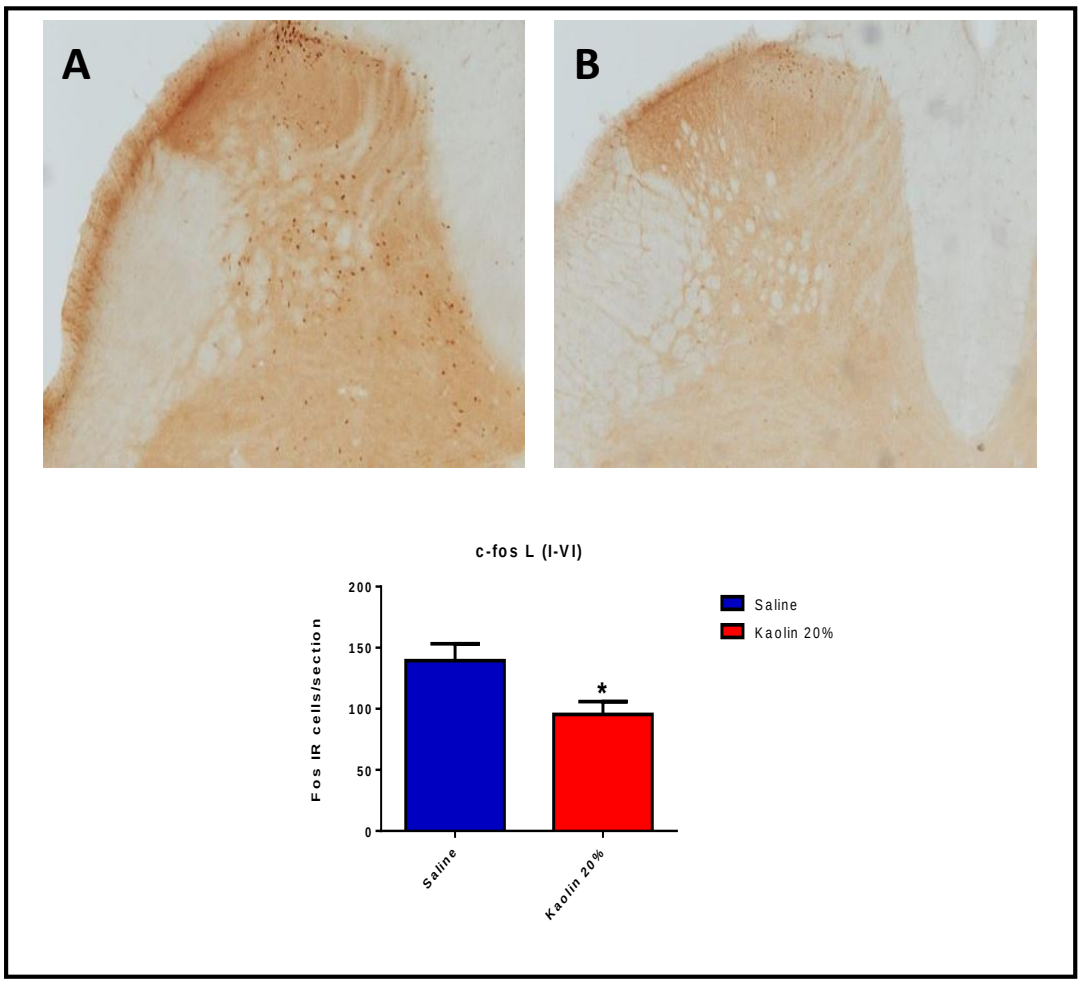

Figure 13: Representative photomicrographs of Fos-IR neurons in animals injected with Saline (A) and Kaolin 20% (B). Number of Fos-IR cells in dorsal horn. Data are presented as means ± SEM (Saline n=10; Kaolin 20% n = 10) p=0,0217*

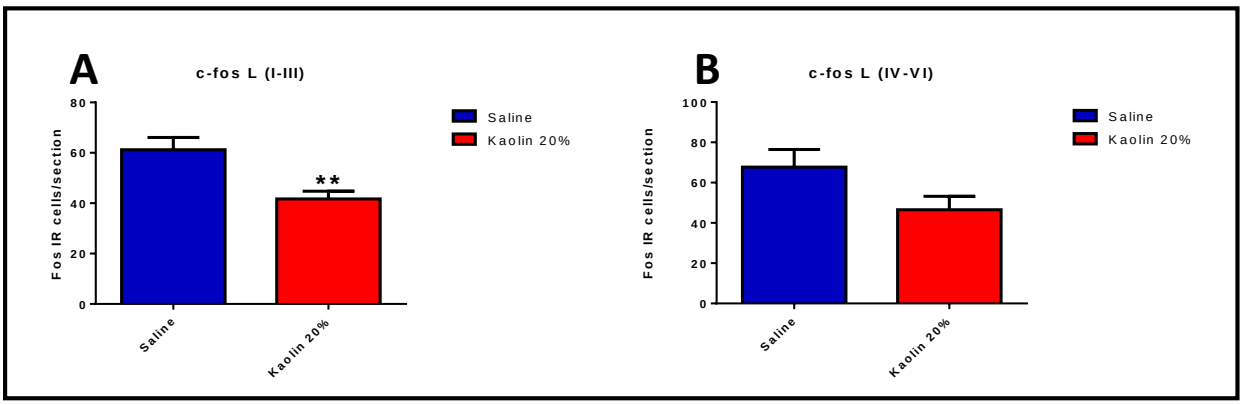

Figure 14: Number of Fos-IR cells in dorsal horn. Number of Fos-IR cells in laminae I-III (A) and laminae (IV-VI) Data as means ± SEM (Saline n=10; Kaolin 20% n = 10) **p=0.0032 (A), p=0.0694 (B).

3.2 PAG

The evaluation of FOS expression by immunohistochemistry was performed in sections containing the PAG (saline = 6, Kaolin 20% = 8). Analysing PAG there was a decrease in the number of fos-IR cells in the group of animals with hydrocephalus (*p=0,0106) (Fig.15).

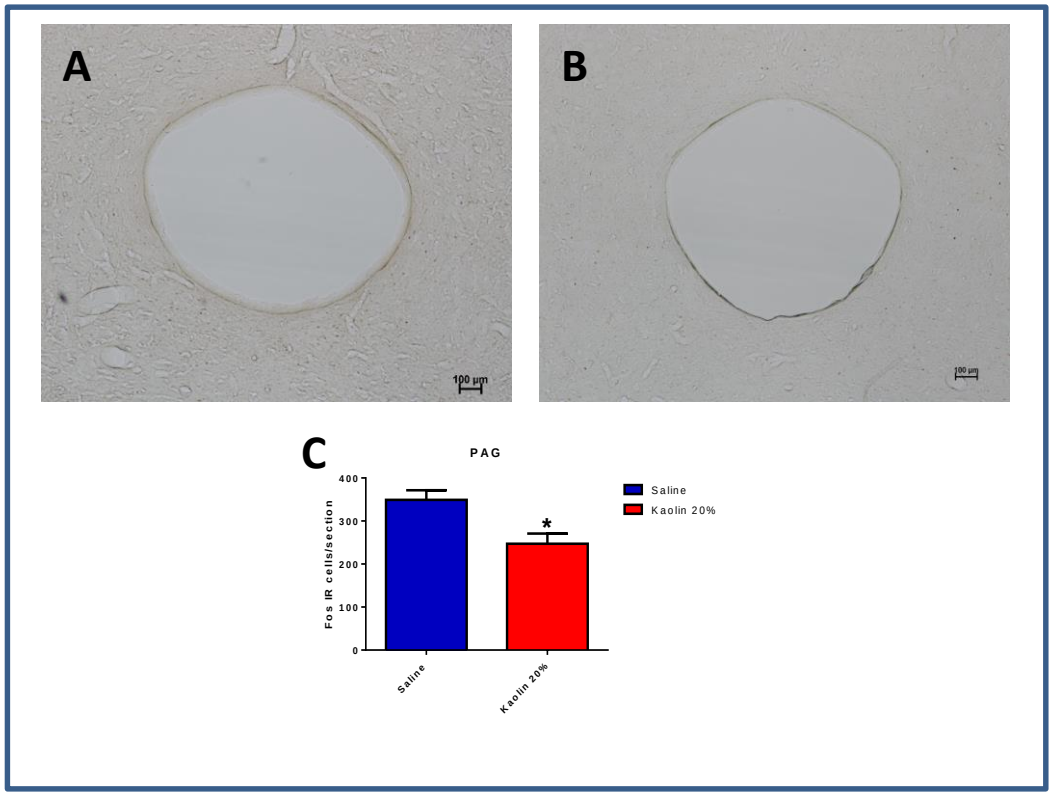

Figure 15: Number of Fos-IR cells in PAG. In A control group and in B Kaolin 20% group. C Number of Fos-IR cells in PAG. Data as means ± SEM (Saline n=6; Kaolin 20% n = 8) *p=0.0106.

After a global analysis, we analyzed the different portions of the PAG separately in order to verify if that reduction would be around or whether it would be in a specific portion. It was found that the decrease in the number of Fos-IR cells only reached statistical significance in VLPAG (****p<0,0001) (Fig 16).

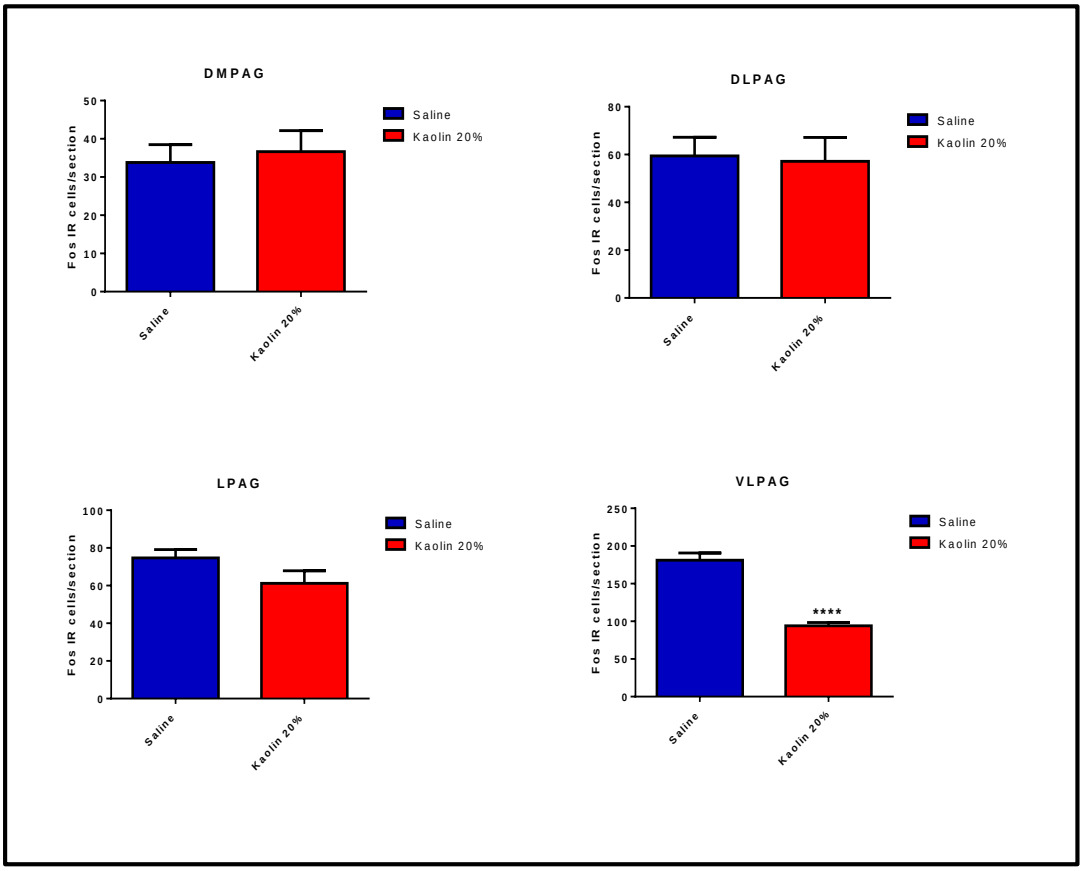

Figure 16: Number of Fos-IR cells in different portions of the PAG. (DMPAG p=0.7163, DLPAG p=0.8680, LPAG p=0.1471, VLPAG ****p<0.001) Data as means ± SEM (Saline n=6; Kaolin 20% n = 8).

4. TH expression

4.1 Locus Coeruleus

The evaluation of TH expression by immunohistochemistry was performed in sections containing the LC in all its extension. Rats with hydrocephalus have an increase of the expression of TH ($p < 0.0001$) (Fig 17).

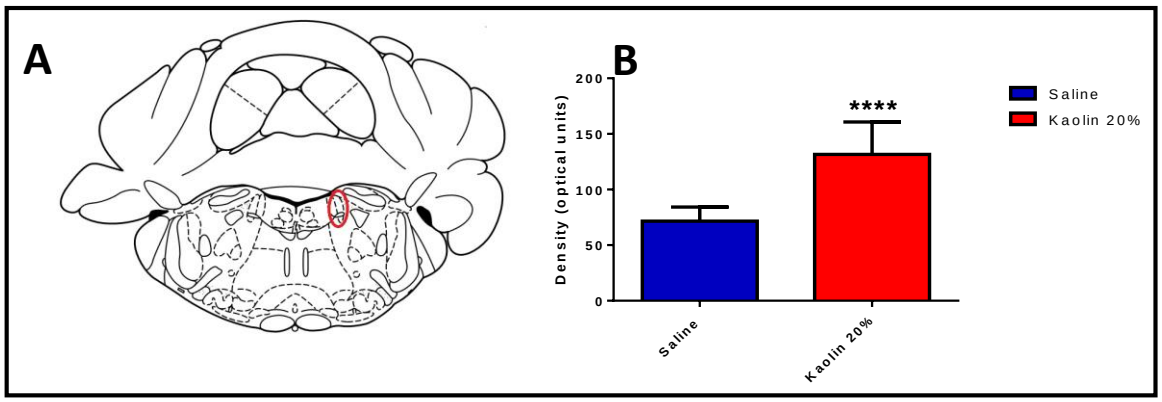

Figure 17: Schematic representation of the LC (A) Results of optical density of TH in LC (B) Data in B as means ± SEM (Saline n=6; Kaolin 20% n = 7) $p<0.0001$.

After this global analysis was performed another more detailed, dividing the LC into three parts: rostral (- 0.16 mm interaural and – 0.30mm interaural), intermediate (- 0.68 mm interaural and – 0.80 mm interaural) and caudal (- 1.04 mm interaural and – 1.30mm interaural) (Figure 18). As in the global analysis the expression of TH shows an increase in the group of animals with hydrocephalus, which was most evident in the rostral portion of of the LC (rostral p=0.1040, intermedial p=0.7310 and caudal p=0.4638).

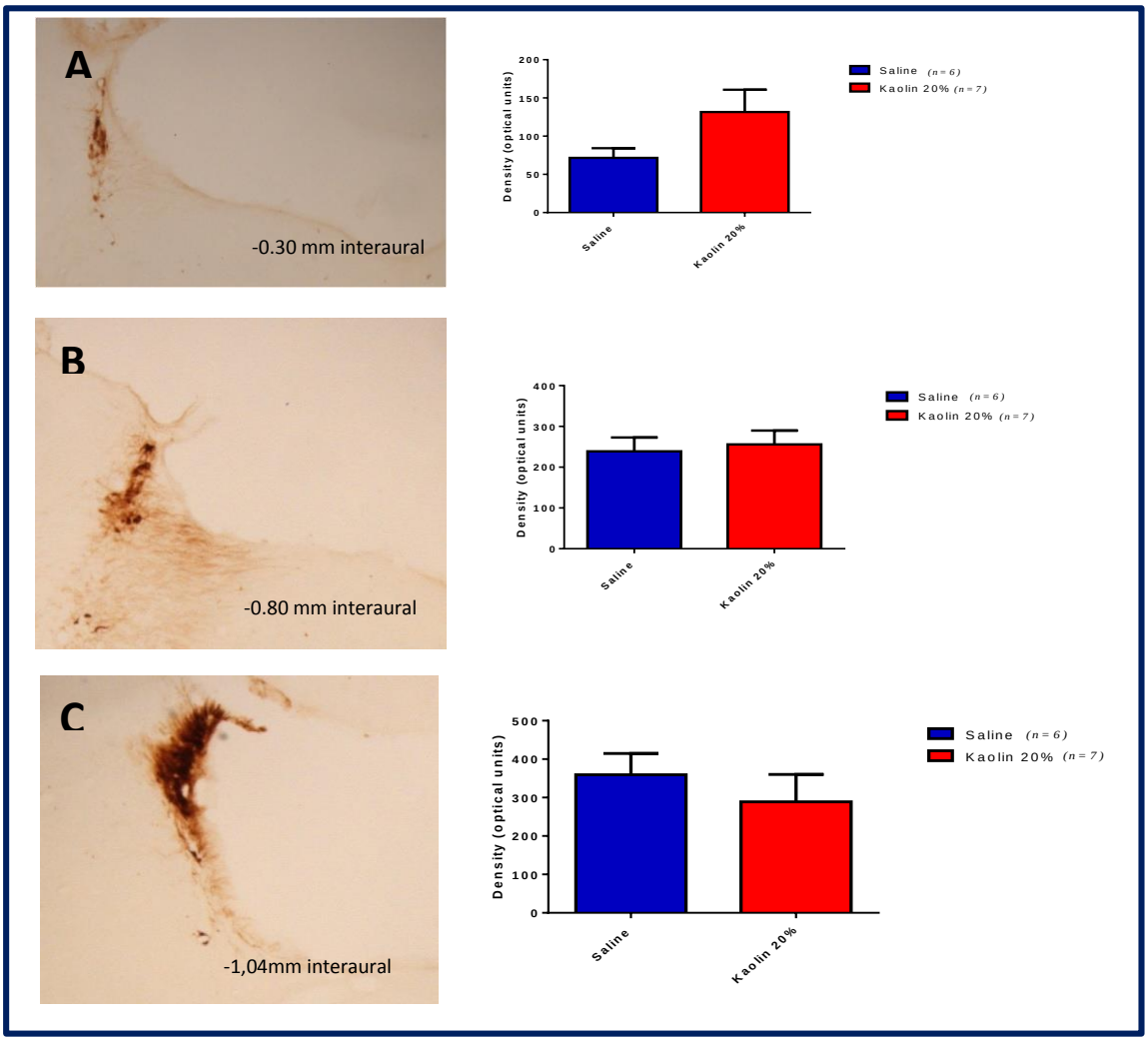

Figure 18:*Results of density of TH in LC. Rostral portion (A), Intermedial portion (B) and Caudal portions (C) Data as means ± SEM (Saline n=6; Kaolin 20% n =7) p=0.1040 (A), p=0.7310 (B), p=0.4638 (C).*

4.2 Spinal Cord

The evaluation of TH expression by immunohistochemistry in the Spinal Cord was performed in superficial laminae of L4 sections. Analysing dorsal horn there was an increase of the expression of TH (*p=0.0143) in hydrocephalic animals. (Fig. 19).

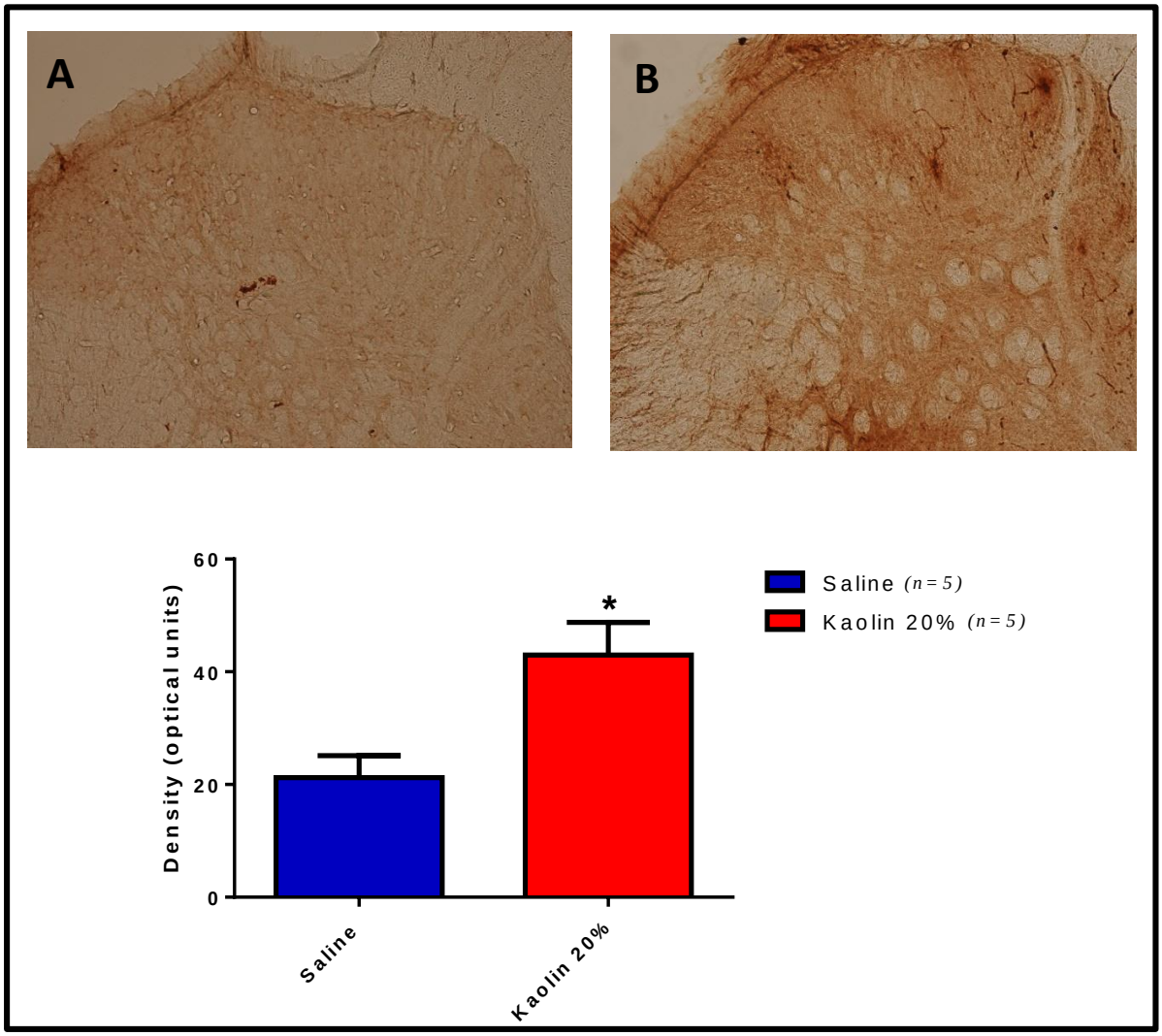

Figure 19: Results of density of TH in the spinal Cord, (A) Control group, (B) Kaolin Group. Data as means ± SEM (Saline n=5; Kaolin 20% n =5) *p=0.0143.

Discussion and Conclusions

The results gathered in the present thesis show, for the first time, that in an experimental model of hydrocephalus, there are impairments in descending pain modulation. It remains to ascertain the mechanisms which subserve the changes in the increased levels of noradrenaline at the locus coeruleus and the translational implications of the results obtained in the present study.

1. Animal model

In all animal research, the importance of selecting an animal model that mimics the disease is the always main question. In what concerns hydrocephalus, the animal model is a big challenge because brain damage observed in animals after a few weeks of hydrocephalus induction mimics lesions which in humans often take years to be observed (Del Bigio 2010). Hydrocephalus is mainly characterized by ventricular dilatation and there are several models which may be used to achieve that type of lesion. The most widely used method is the injection of kaolin in the cisterna magna, which causes an inflammatory process in the subarachnoid space leading to an obstruction of CSF circulation (Del Bigio, 1993). This model is easy and cheap to induce and does not compromise animal welfare in a significant manner. The latter was demonstrated by our current studies in which we monitored several parameters like weight gain and general motor behaviour and did not found differences in relation to animals injected with saline. In spite of some variations in the concentration and volume of kaolin injected and also on the post-injection time of sacrifice, the majority of studies using the kaolin-injected rat with hydrocephaly, also used 0.05ml of a Kaolin solution at 20%, in a manner similar to what we used in the present study.

As the development of hydrocephalus depends on the degree of inflammation caused there are always a lot of variability in the degree of ventricular dilatation. Because of the method used for the injection volume that actually enters in the cisterna magna may vary due to reflux while withdrawing the needle, as described by Okii, N (2007), leading to variation of the inflammatory states and, as a consequence, different degrees of ventricular dilatation. In our experimental group of rats with kaolin-induced hydrochephalus, we also

had a higher variation than control animals in structural parameters, such as ventricular dilatation and area of the PAG. The same was detected in what concerns the levels of TH in the LC. It remains to ascertain if the inflammatory process in the subarachnoid space was also variable.

2. Behavioral responses to pain

The present study is the first to evaluate pain responses in animal models of chronic hydrocephalus. In order to characterize the model of kaolin-induced hydrocephalus regarding to painful behaviours we elected a test (formalin inflammatory test) that has an acute (first phase) and an inflammatory phase (second phase). This test has also the advantage of allowing to correlate behavioural responses with levels of Fos in the spinal cord (detected by the immunoreaction for Fos protein). Since rats with kaolin-induced hydrocephalus present mild deficits in motor responses it could be argued that the lower scores obtained by these rats in the formalin test could be due to some motor impairment. However, if this was the case, hydrocephalic rats should also have a lower score in the first phase of the formalin test. Furthermore, the type of behavioural analysis performed in this study does not evaluate simple reflexes, like the paw jerks, but rather more complex behaviors which are more related to the nociceptive event itself. Further supporting that hydrocephalic rats present a decrease in nociceptive transmission, the studies of nociceptive activity of spinal cord neurons show that rats with kaolin-induced hydrocephalus present decreased numbers of Fos-immunoreactive neurons.

Regarding the formalin test, nociceptive stimulus caused by the injection of formalin allows assessing how the animal responds to moderate, continuous pain generated by injured tissue, unlike mechanical test that cause an response to an acute stimulus. Our results show that the hydrocephalic animals have a decrease in painful behaviours during the second phase of formalin test. The decrease in responses in the second phase of the formalin test in kaolin-induced hydrocephaly indicates that pain modulation from the brain is affect in hydrocephalic rats since supraspinal modulation affects mainly the second phase of the formalin test (Wheeler-Aceto and Cowan, 1991). Indeed, the second phase of the test is dependent on the prolonged changes in the CNS induced by enhanced spinal neuronal responsiveness. (Tjølsen et al., 1992, Porro and Cavazzuti, 1993). The increase of

the numbers of animals analysed in the formalin test, and the analysis of more complex behaviours typical of the second phase of the formalin test will be carried out in the near future with the objective to confirm this trend.

3. The spinal dorsal horn: nociceptive activation and noradrenergic innervation

Formalin induced activation of the c-fos protooncogene in spinal cord neurons is a validated method for studies of the nociceptive system and has the advantage of allowing a correlation between animal behaviour and the intensity of nociceptive activation (Pinto, M. et al 2007). In the present study, nociceptive activation of spinal cord neurons was studied after the performance of the formalin test. Since hydrocephalic animals exhibit a tendency to lower painful behaviour during the second phase of formalin test and this phase is necessary to maintain maximal levels of Fos expression (Abbadie et al., 1997), our behavioural results are consistent with those of c-fos.

In Kaolin-induced hydrocephalus model there is reduced activation of spinal cord neurons. Although the decrease was detected in all the dorsal horn it only reaching statistical significance in the superficial dorsal horn (laminae I-II). It is curious that the superficial dorsal horn presented the higher decreases in Fos expression. These layers contain a high contingent of neurons projecting to supraspinal brain areas, indicating that the nociceptive input arriving to the brain may be decreased during hydrocephalus.

The decrease in nociceptive activation can be due to the increased levels of noradrenaline at the spinal dorsal horn in rats with hydrocephalus. In fact, a higher number of fibers immunoreactive to TH was detected in hydrocephalic rats in comparison with controls. Inhibitory modulation by noradrenergic inhibition consists on blockade of the passage of nociceptive input from the spinal cord to supraspinal areas of the pain control system, and is mediated by spinal α2-adrenoreceptors. Noradrenaline was shown to be exclusively inhibitory by binding to α2-adrenoreceptors and inhibiting the release of excitatory neurotransmitters (Pertovaara, 2006). We will evaluate on the future if in kaolin-induced hydrocephalus there is an increase in noradrenaline levels of the spinal cord and if the function of α2-adrenoreceptors is preserved by performing intrathecal administration of the respective antagonists.

4. Recruitment of components of the supraspinal pain control system.

The present study shows changes in the PAG and LC in hydrocephalic animals. Regarding the PAG, a decreased neuronal activation was detected in a key subnucleus: the VLPAG. It should be noted that this is not likely to be related to the structural changes detected in the overall PAG since they affected only this specific subnucleus. The VLPAG is the most relevant area of the PAG in descending pain modulation and its relay in the RVM has been established for a long time (Yin J-B et al., 2014). It should be recalled that the PAG is involved in descending pain modulation by direct effects at the RVM, which exerts a bidirectional effect on pain modulation at the spinal cord (Heinricher et al., 2009). It is therefore likely that the decrease of neuronal activation at the VLPAG will affect RVM neurons and, herein, affect descending pain modulation. It is important to ascertain if the activity of the RVM is preserved in animals with hydrocephalus induced by kaolin injection.

Besides the VLPAG-RVM-spinal circuit, the PAG is also involved in descending pain control by inducing spinal antinociception associated with noradrenaline release into the spinal cord (Pertovaara, A., 2006). While the PAG does not contain noradrenergic neurons, it is connected with noradrenergic structures involved in pain modulation, such as the LC, A5 and A7 cells group. In the model of hydrocephalus, the increase in the PAG area due to the effects of ventricular dilatation may induce deregulation in the connections of the PAG to the aforementioned noradrenergic brainstem areas. In the case of the LC, it remains to be determined if the increase in noradrenaline levels represent an inhibitory modulation to compensate the injuries caused by ventricular dilatation in the PAG. It is also important to evaluate if the remaining noradrenergic brainstem areas, namely the A5 and A7 noradrenergic cell groups, which are not likely to be affected by ventricular dilatation, are affected in kaolin-injected hydrocephalic rats.

5. Conclusions and future perspectives

According to our results, in the kaolin-induced hydrocephalus model there are changes in the nociceptive system, which concur to the occurrence of analgesia. Changes in the noradrenergic modulation in hydrocephalic animals may be due to ventricular dilatation

present in hydrocephalus, since the periventricular structures are the first to be affected (Del Bigio, 2010).

Due to the absence of human studies and the results obtained in this thesis it would be of great importance to evaluate the descending pain modulation in human patients with chronic hydrocephalus. It is important to study in the threshold of pain responses in patients with hydrocephalus and to perform imaging studies of the brain of these patients to determine the structure and function of pain control areas in the brain.

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Apendix A: Composition of solutions

1. PHOSPHATE BUFFER SALINE (PBS) (1L)

Phosphate buffer (PB) 0,1M pH=7.2

$Na2H_2PO_4H_2O$ – 15,60g

$K2HPO_4$ – 17,4g

H_2O up to 1L

PBS:

PB 250 ml

H_2O up to 1L

NaCl - 9g

2. PHOSPHATE BUFFER SALINE WITH TRITON X-100 (PBS-T)

PBS - 996ml

Triton X-100 - 4ml

3. TYRODE'S SOLUTION (1L)

NaCl – 6,8 g

KCl – 0,40g

$MgCl_26H_2O$ – 0,32 g

$MgSO_47H_2O$ – 0,1 g

$NaH2PO_4H_2O$ – 0,17 g

Glucose 1 g

$NaHCO_3$ – 2,2 g

H_2O up to 1L

4. CRYOPROTECTOR SOLUTION (1L)

PB 0,1M pH=7.2 - 125 ml

H_2O - 375 ml

Sucrose - 300g

Ethylene glycol - 300ml

PB 0,1M pH=7.2 up to 1L

5. THIONIN STAINING

1. Solutions

Acid acetone: acetone / acetic acid (4 vol / 1 vol)

0.1% thionin in 10% formalin

2. Protocol

Incubate the slides in acid acetone for 5 min;

Rinse with distilled water;

Stain in formol-thionin for 1 minute;

Rinse with distilled water;

Dry at 37 ° C;

Dehydrate in xylene for 5 min;

Mount with Eukitt.