Astrocytes as a novel target against chronic pain disorder

What is the role of astrocytes in neuropathy, allodynia, and hyperalgesia

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Abstract

The human nervous system is of interest in many fields of research. A lot is known about the human nervous system. Different neurons carry particular signals from and to the brain for processing. Like nociceptive neurons, causing a painful sensation. When activated these neurons signal from the peripheral nervous system towards the spinal cord simultaneously creating a reflex and sending the signal to the brain. After injury, these signals can turn malignant creating nonstop signaling, even after healing, inducing a pain sensation that does not diminish thus creating chronic pain. Most research from the past about chronic pain had been on neurons, but there is a large group of cells from the nervous system that has not been thoroughly studied called glial cells. Glial cells are about a factor ten to fifty more common in the central nervous system. In this thesis, the focus will lie on astrocytes as a novel target to counter chronic pain. The activation of astrocytes is a prerequisite to the induction of allodynia and hyperalgesia. Furthermore, the protein that is responsible for this is GFAP and its upregulation during and after injury because if the GFAP protein is disabled there is no long term allodynia. It is shown that the activation of the σ1 receptor, as well as TNF-α, induced activation of the Erk2 and JNK MAPK pathway respectively which in turn activated the upregulation of the GFAP protein, astrogliosis and in turn allodynia and hyperalgesia.

List of Abbreviations

5-HT	5-Hydroxy Tryptamine (Serotonin)
aa	Amino acid
ACh	Acetyl Choline
CaM	Calmodulin
CCI	Chronic Constriction Injury
CNS	Central nervous system
COX2	Cyclooxygenase 2
ERK	Extracellular signal-Regulated Kinase
GFAP	Glial Fibrillary Acidic Protein
HNS	Human Nervous System
MAP	Mitogen-Activated Protein
N-methyl-D-aspartate	NMDA
PAG	Periaqueductal grey
PGE2	Prostaglandin E2 (dinoprostone)
PNS	Peripheral nervous system
RVM	Rostral Ventromedial Medulla
SER	Serotonin

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Introduction

The Human Nervous System (HNS) is of interest in many fields of research. A lot is known about the human nervous system. The human nervous system actually consists of two systems, the Central Nervous System(CNS) and the Peripheral Nervous System(PNS). The brain and spinal cord together create the CNS, the PNS consists of cranial nerves, ganglia outside of the CNS and spinal nerves. These systems combined, control the body's motion and process all the information received through neurons.[1] Neurons, the wiring of the HNS, are electrically excitable and use molecules called neurotransmitters to transfer information between other neurons. These neurons are unidirectional which means that there are two kinds of neurons, ascending (from the periphery to the brain) and descending (from the brain to the periphery) neurons. Neurons exclusively use glucose as an energy source and are unable to efficiently turn fat into energy. [2] Although when the blood-glucose levels are low the neurons still function properly, this is because neurons are supported by glial cells which can transfer glucose, broken down from glycogen to the neurons. Glial cells nourish, regulate, and support neurons. There are multiple cells categorized as glial cells, these are Oligodendroglia, Schwann cells, Microglia, and Astrocytes.[3]

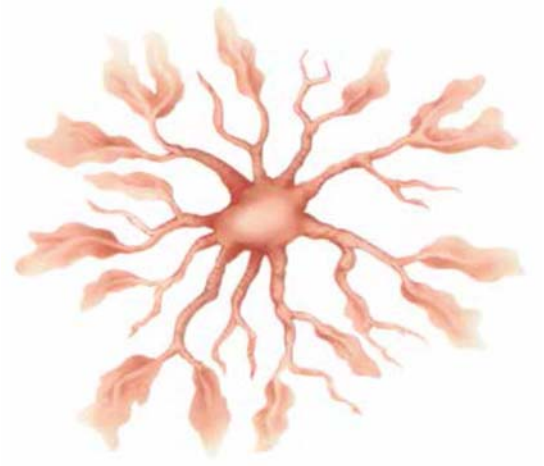

Figure 1 Illustration of astrocyte [1]**"Astro from the Latin word of star"**

In this thesis, the focus lies on astrocytes. These cells get their name from their shape, under the microscope, the cells look like stars (figure 1). Astrocytes fill the space between neurons, are 10 to 50 times more abundant, and have a big range of supporting functions in the CNS. The most important functions are metabolic and structural support. Astrocytes are able to perform glycogenesis and store glycogen. When neurons get deprived of oxygen or glucose, glial cells can 'feed' the neurons.[4] Another essential function is regulating the chemical composition outside of the neurons, the extracellular space. Ions in the extracellular space are regulated by astrocytes to modulate the synaptic transmission between neurons.[5] The role of astrocytes can be seen in the sensitization of pain.

Pain is a reaction of the body to noxious mechanic, acidic or thermal stimuli. This stimulus can be pain from outside the body, nociceptive pain, pain from inside the body, inflammatory pain, and through damages in the nervous system which is called neuropathic pain.[6] Pain from outside the body is first perceived by nociceptive neurons which contain receptors for temperature, acid, and pressure called nociceptors. The triggering of nociceptors in the peripheral nervous system creates a signal that is sent through the neuron to the thalamus and sensory cortex. There are two main types of nociceptive neurons unmyelinated C fibers and myelinated Aδ fibers, where c fibers cause a burning delocalized sensation and Aδ fibers a very localized sensation.[7]

When nociceptors are activated (**2-3**), noxious stimuli travel from the Aδ- and c fibers through the spinal cord (**4-5**) to the thalamus (**6**) where the signal is perceived as pain (figure 2). After passing through the thalamus, the signal is sent towards the cerebral cortex (**7**) where the location of the pain is processed. [6] After activation through noxious stimuli, nociceptors sensitize which effectively increasing the sensitivity to stimuli and increases the perception of pain at a lower threshold, this is

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called hyperalgesia.[8] When the noxious stimulus is removed the nociceptor undergoes homologous desensitization by means of the Ca^{2+} binding protein Calmodulin (CaM) which binds to a 35 amino acid (aa) segment in the C end of nociceptors. [9] The signal towards the brain can be perceived based on neuronal modulation.

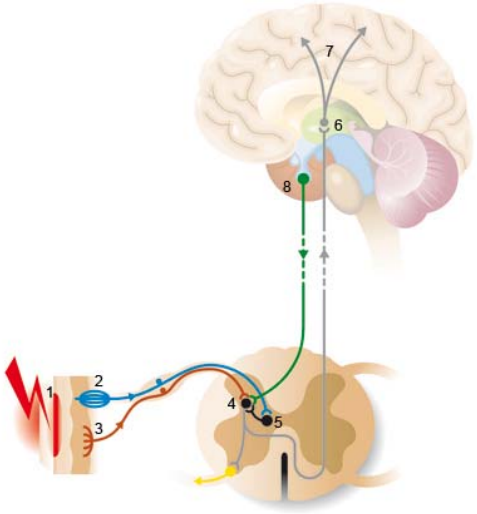

Figure 2: [1] A noxious stimuli hits the body (2-3) transduction of signal through (2 Aσ / 3 C) fiber (4) signal transmission towards thalamus (5) inter neuron sends reflex signal (6) pain perception by thalamus (7) localization of pain by cerebral cortex (8) modulatory signal towards spinal cord

When the pain signal is active different mechanisms can modulate the signal to induce minor or full analgesia. Pain modulation can be due to several factors, for example, the descending pain modulation pathway from the medulla to the spinal cord that causes a shift in concentration of K^+ in the neurons or endogenous opioids.[6] The periaqueductal gray (PAG) is the coordinating area for descending pain modulation. The PAG is located around the cerebral aqueduct within the tegmentum of the midbrain.[10] On activation, the descending analgesic neuron releases endogenous opioid peptides that inhibit ascending presynaptic release of neurotransmitters as well as postsynaptic excitation in the spinal cord and dorsal horn.[11] If nociceptive neuron cannot signal in the spinal cord, ascendingly to the brain, pain cannot be perceived. When these modulatory signals fail or the nervous system gets damaged pain can be perceived even without noxious stimuli triggering chronic pain.

Chronic pain is a pain that negatively impacts the quality of life and is characterized by a pain that lingers for more than 6-12 months, between 3 and 6 months it is characterized as subacute pain. Approximately 10.1% to 55.2% in various countries have or had chronic pain symptoms.[12] Chronic pain can originate from the body, in the brain or in the spinal cord and is often difficult to treat.[13] There are various conditions that can cause chronic pain for example Diabetes, persistent postsurgical pain, lower back pain, and neck pain.[14] The risk that an individual acquires chronic pain can be due to genetics (genotype, gender, and epigenetics), living environment and personality. Most common chronic pain is continuing nociceptive signaling. If the nociceptive signal persists for a period of 3-6 months, the peripheral and/or central sensory nervous system will be altered. The main reason behind this is that nociceptive signaling does not react to descending pain modulatory circuits, or undergo receptor desensitization, which is called “chronification”. [11] Chronic pain can be divided into two main categories, nociceptive pain caused by inflamed or damaged tissue and neuropathic pain caused by a broken or damaged nervous system.[15]

A lot of research has been done on the development of medication that inhibits chronic pain. Opioids, for example, are the go-to medication for most medical professionals. The opioids will stop the pain signal at the spinal cord level, where it stops the pre and postsynaptic signaling of nociceptive fibers. [6], [16], [17] Alternatives for opioids include NSAIDs, local anesthetics, *N*-methyl-D-aspartate (NMDA) antagonists and cannabinoids. But for a long time, there was no research on cells other than neurons, which has changed over the last couple of years. A shift is seen in the focus of the research towards glial cells. Can astrocytes be targeted to relieve chronic pain symptoms or to diminish the cause of chronic pain? And if so, what is the role of astrocytes and how do they get activated?

Astrocytes help neuronal signaling

Astrocytes, the star-shaped glial cells in the human nervous system, have numerous functions. In 2008 the lab of Mriganka Sur found that astrocytes react to Ca^{2+} signaling in the eye the same way neurons do, only a bit slower and that the spatial orientation between neurons and astrocytes are highly precise. [18] After the activation of calcium receptors the astrocyte releases glutamate which can activate various receptors on the neurons. [19] In the CNS, astrocytes react to ischemia and low oxygen levels by converting glycogen into lactate to provide the neuron with energy. Besides ischemia, astrocytes will activate during injury to fulfill multiple roles.

Astrocyte activity during injury

After injury astrocytes have been found to get activated in the CNS. This was tested by surgically creating a spinal injury in mice at the L5 ligament, astrocytes have been found to always react strongly ipsilateral of the lesion site in the dorsal horn within 7 days. [20] The reaction mentioned is the upregulation of Glial Fibrillary Acidic Protein (GFAP) a marker that is related to astrogliosis, an abnormal increase in astrocytes.[21] In a paper by Guo et al. researchers hypothesized that neuron-to-glia signaling plays a crucial role in neuronal hyperexcitability.[22] Lidocaine, which is a local anesthetic, was injected in mice approximately 5mm closer to the periphery relative to the site of inflammation in the primary afferent fiber. This was performed not to stress the immune cells in the CNS at the exact point of inflammation. 10 minutes after injection with lidocaine, the rats were injected with complete Freund's adjuvant (CFA) to induce inflammation. The rats injected with lidocaine did not show an increase in GFAP or IL-1β receptor, while the rats without local anesthesia showed a doubling in GFAP expression (figure 3a) and a threefold increase in IL-1β receptors (figure 3b). This showed that afferent or ascending signaling is important in the activation of astrocytes and astrogliosis. Further tests suggested that IL-1β receptor signaling from astrocytes directly controls NMDA receptor phosphorylation on neurons.

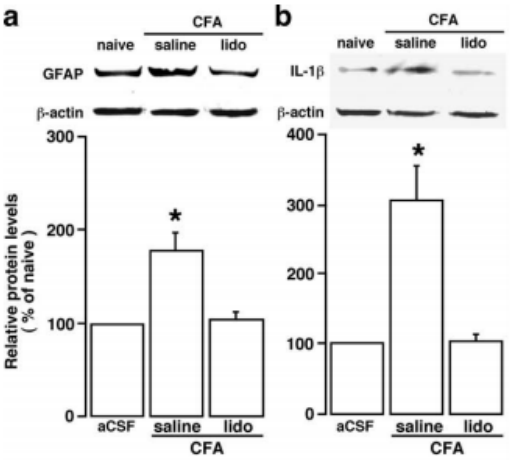

Figure 3: relative protein levels; A) GFAP concentration **B)** IL-1β concentration

After peripheral nerve injury or inflammation glial cells have been found to play a role in hyperalgesia too. Sweitzer et al. released a paper in which cytokine expression and glial activation were observed in rats after peripheral nerve damage.[23] Rats were injected with formalin or zymosan to induce inflammation. The rats injected with formalin showed increased allodynia, which is hyperalgesia through non noxious stimuli, at the site of injection for 1 hour while rats injected with zymosan showed increased allodynia across the whole paw for 7 days. Immunohistochemistry of the L5 spinal nerve showed a baseline activation of astrocytes even though there was an increase in cytokine IL-1β. This concludes that only the upregulation of cytokines alone is not responsible for prolonged hyperalgesia, the activation of astrocytes and upregulation of GFAP is a prerequisite for chronic hyperalgesia.

Astrocytes aid pain sensitization

In 2008 the group from Guo et al. released a paper showing that glial-neuronal interaction inhibits the descending pain pathway.[24] To study if hyperalgesia and allodynia occurred after induced chronic constriction injury (CCI), in the infraorbital nerve of the rostral ventromedial medulla (RVM),

mice were injected with Fluorocitrate, propentofylline or minocycline. Where Fluorocitrate stops the tricarboxylic acid cycle in astrocytes by blocking aconitase, Propentofylline a phosphodiesterase disrupts microglial and astrocytic activation by inactivating cAMP, and minocycline protects neurons and inhibits inflammation.[25],[26],[27] The rats injected with propentofylline, 3 and 14 days after CCI, showed significant attenuation to mechanical hyperalgesia and allodynia for approximately 4 hours. An injection on the 14th day after surgery with Fluorocitrate attenuated mechanical hyperalgesia and allodynia fully for 6 hours like propentofylline. Strangely an injection on the 3rd day after surgery with Fluorocitrate did not show any signification change in hyperalgesia or allodynia. On the other hand, Minocycline only showed a decrease in hyperalgesia and allodynia only at the 3rd day after surgery, and no reaction on the 14th day after surgery. Showing that an injection with the right drug can reduce chronic pain locally.

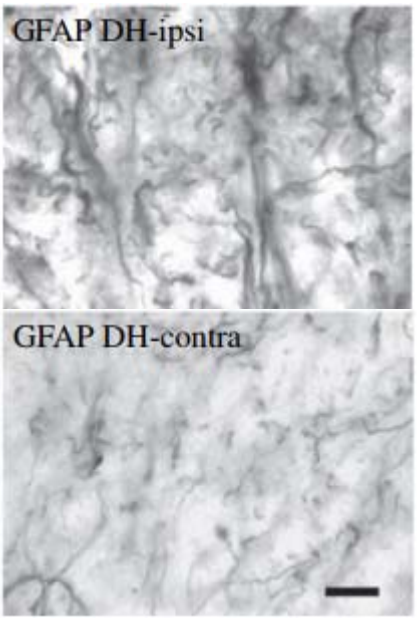

Figure 4 GFAP stained astrocytes in the dorsal horn. top ipsilateral of injury, bottom contralateral of injury

The group of Zhang et al. found that although astrocytes took a longer time to react to injury they stayed active for far longer than all other mechanisms.[28] In this research, the left common sciatic nerve was encapsulated in polyethylene tubing to induce neuropathic pain. The withdrawal threshold from rats due to mechanical stimulation was measured over a period of 150 days. The withdrawal threshold of the mice decreased to its maximum at 20 days showing no significant increase in threshold until the end of the experiment. Astrocytes became activated around day 7 after surgery and had altered shapes (figure 4). The upregulation of GFAP did not decrease until the end of the period, the astrocytes did not go back to resting state. Providing evidence that chronic pain and astrocytes are strongly related. Further tests on the GFAP protein and its expression was done by the University of California.[29] In this research, mice underwent surgery where the spinal nerve of rats was tight ligated between the L5 spinal nerve and the dorsal root ganglia. The withdrawal rate was determined to show that hyperalgesia was induced, where the maximal withdrawal threshold was in 20 days and after that decreased to normal levels within 80 days. The same experiment was performed on GFAP knock-out mice. The same pain threshold was observed until 7 days after surgery, then the pain threshold returned to normal within 40 days. This showed that GFAP did not play a role in the onset of hyperalgesia but instead the maintenance of. The experiment was performed again on normal mice, but this time antisense RNA for GFAP was introduced into the spinal cord 3 and 20 days post-surgery. The withdrawal rate of the paw was increased for 2 days after the injection on the 3rd-day post-surgery, the injection 20 days post-surgery did not show any difference in withdrawal rate. Which is more proof that GFAP expression in astrocytes plays a role in allodynia maintenance, not onset, and that medication should be provided as soon as possible to stop expression of GFAP.

Astrocytes are activated by the MAP kinase pathway

The Mitogen-activated protein (MAP)- Extracellular signal-Regulated (ERK) and -c-Jun N-Terminal (JNK) kinase pathway are well-studied cell signaling pathways and activates many downward phosphorylations on various protein to induce transcription. [30] It has been found that spinal cord injuries induce MAPK activity in glial and neuronal cells, in astrocytes this is observed largely in late-phase injury. [30],[31],[32] In more recent research it was found that a partial sciatic nerve ligation not only induces GFAP transcription but also showed increases in phosphorylated ERK and JNK proteins in astrocytes. [33]

A study from Moon et al. in 2013 reported that an intrathecal injection with a σ1 receptor, a chaperone protein, agonist induced the increased activation of p38-MAPK.[34], [35] But when the mice were pretreated with a σ1 receptor antagonist the p38-MAPK did not show increased activation. Furthermore the research showed that the mechanical allodynia in mice was decreased by injecting a σ1 receptor antagonist.[35] This is in line with the 2009 study of de la Puente, where mice were used with a σ1 receptor knock-out.[36] In the research of de la Puente, it was found that mice lacking the σ1 receptors showed significant attenuation to mechanical allodynia, in line with previous research. [29] Proving that the activation of p38-MAPK is a vital step for inducing sensitization, hyperalgesia, and allodynia. In 2014 the team of Moon et al. found further evidence that the σ1 receptor activated astrocytes via the p38-MAPK pathway and induced mechanical allodynia in mice.[37] It was found that the σ1 receptor significantly increased in numbers in astrocytes and neurons after CCI, which peaked at 3 days and was back to normal by 7 days post-surgery. But mice injected with a σ1 receptor antagonist showed that the p38-MAPK activity decreased in astrocytes but not in neurons. Furthermore, when mice were injected with a p38-MAPK inhibitor the concentration of the GFAP protein and mechanical allodynia decreased.

Proinflammatory signaling molecules cause hyperalgesia

There are various extracellular molecules that astrocytes react to, some of these can trigger or maintain chronic pain. Tumor Necrosis Factor α (TNF-α) is a well-studied molecule that plays an important role in many signaling pathways. In an early study of 1996, it was found that an endoneurial injection of TNF-α induced thermal hyperalgesia and mechanical allodynia for a period of 3 days in mice, where the mice showed the same behavior as a CCI operated mouse.[38] A study from 2000 showed that astrocytes, in a Petri dish, react to TNF-α by upregulating GFAP, further observations showed that the MAPK/ERK pathway was also activated.[39] Later in the experiment, it was shown that adding a TNF-α antagonist attenuated Erk2 and GFAP expression. Later in 2001, it was hypothesized that an arthritis medicine called Etanercept, which competitively inhibits TNF-α, could possibly reduce chronic pain after CCI.[40], [41] In this research mice underwent CCI surgery and were injected with etanercept 50µg or 87.5µg a day where the latter produced significant attenuation on day 4 and 7. Although a further increase in etanercept did not increase the withdrawal threshold, this research proved that TNF-α is playing an important role as a signal molecule. In a study from 2006 mice were surgically induced with allodynia by transecting the lumbar 5 ventral root.[42] It was found that TNF-α was significantly increased from 1-day post-surgery, peaked at day 3 and 7 and was returned to baseline after 21 days. After injecting a TNF-α synthesis inhibitor, thalidomide, intraperitoneally the paw withdrawal threshold was increased significantly between 7 and 13 days post-surgery. This research also showed that starting the injections of thalidomide 7 days post-surgery did not show any change in the paw withdrawal threshold. Showing evidence that TNF-α is an important factor for the onset of allodynia and hyperalgesia. Gao et al showed in a paper of 2010 that TNF-α activated astrocytes can induce allodynia in mice.[43] In this experiment, cultured astrocytes were incubated with TNF-α for 15 minutes and injected intrathecally between the L5 and L6 in mice. The paw withdrawal threshold was greatly decreased showing the development of mechanical allodynia after 3 hours of injection. It was also shown that inhibiting JNK before incubating the astrocytes with TNF-α induced no allodynia. This evidence illustrates that the activation of the JNK/MAPK pathway by TNF-α is the only element necessary to induce allodynia.

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Conclusion / Discussion

Pain after injury, surgery or inflammation can be paralyzing, even more, when the pain does not diminish. The mechanism behind the chronification of pain due to the sensitization of pain fibers in the central nervous system is not yet widely understood. Research, highlighted in this essay, suggests that glial cells, largely astrocytes, play a key role in allodynia and hyperalgesia. The upregulation of glial fibrillary acidic protein in astrocytes is observed in all reviewed studies about the cellular mechanisms in chronic/neuropathic pain. The direct pain a subject experiences after injury or surgery is normal and does not show a significant correlation with the activation of astrocytes but the excitability of nociceptors near the wound. The problems of chronic pain start with the activation of astrocytes after healing of the wound.[28]

In a nutshell, it was found that astrocytes play an imperative role in the onset of allodynia and hyperalgesia. Furthermore, the protein that is responsible for this is GFAP and its upregulation during or after injury because, if the GFAP is disabled there is no long term allodynia. Later it was described that the GFAP upregulation is due to the activation of the Erk2 and JNK MAPK pathways which can be activated through various receptors like the σ1 receptor or the TNF-α receptor.

There are several pathways found to be involved in the onset of allodynia and hyperalgesia. It was shown that the activation of the σ1 receptor on astrocytes induced phosphorylation/activation of the p38-MAPK pathway which in turn activated the upregulation of the GFAP protein. The activation of astrocytes in injury was shown to be within seven days and stayed activated for 150 days, this is when most research observed in this theses were discontinued. Interestingly the study about the intrathecal injection of various substrates that blocks astrocytes activity was only run for 20 days. [24] This research showed that the intrathecal injection of propentofylline, which disrupts the activation of glial cells, increased the withdrawal threshold of mice the 3rd and 20th day after injury for 6 hours. This shows that disabling astrocytes locally can actually abolish hyperalgesia and allodynia. It would be interesting in future research to increase the length of the experiment to 150 days, like the other experiments, and see if the reaction of mice is equal to the 3rd and 20th day of previous research. If so it would be alluring to research a pump, molecule or another way that can slowly or periodically release propentofylline, if there are no complications to regular sensations. [26]

The σ1 receptor activates the Erk2 MAPK pathway and in turn, upregulates the GFAP protein.[44] Whereas the cytokine TNF-α activates the JNK MAPK pathway and upregulates the GFAP protein. We have seen that the sole activation of astrocytes by TNF-α was enough to induce allodynia and that the timescale when medicine should be administered is crucial. In further research, it would be interesting to see what the roles are of other glial cells and Schwann cells. This due to the fact that Schwann cells can upregulate TNF-α and in turn activate astrocytes.[45]

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