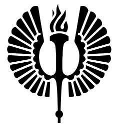

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Hippo pathway activity after a peripheral nerve injury

Institute of Biomedicine
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Master's thesis
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Transcriptional mechanisms underlying chronic neuropathic pain are still unclear. However, novel studies have suggested that the Hippo pathway and especially its compartments YAP and TAZ have a role in the pathology of neuropathic pain. Additionally, the active Wnt pathway is involved in the development of neuropathic pain after a nerve injury through its main effector β-catenin. The accumulation of YAP, TAZ and β-catenin into a nucleus of neurons and glia cells is suggested to activate proliferative genes. This would further cause hypersensitivity and pain chronification. The thesis project aimed to confirm the results of previous studies about the nuclear shuttle of YAP, TAZ and β-catenin in the peripheral and central nervous system.

The research was done by comparing hypersensitive chronic constriction injury rat model to controls. The protein expression levels of YAP, TAZ and β-catenin were analyzed by purifying cytoplasmic and nuclear proteins of spinal cord and dorsal root ganglia and conducting Western blot analysis. YAP and TAZ were overexpressed in the cytoplasm in the spinal cord and the dorsal root ganglia. Instead, β-catenin had cytoplasmic overexpression only in the dorsal root ganglia. The nuclear overexpression was not detected. Additionally, YAP was noticed to localize in the cytoplasm of healthy human spinal cord by help of immunohistochemistry technique.

The results suggest that YAP, TAZ and β-catenin are participating in the pathophysiology of neuropathic pain after a peripheral nerve injury in both peripheral and central nervous system. With help of the results, more specific analgesics for neuropathic pain can be developed.

Key words: neuropathic pain, hypersensitivity, YAP, TAZ, β-catenin

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1 Introduction

1.1 Chronic pain

Pain is uncomfortable sensations in the body that appear in various ways like burning, soreness, squeezing, dull ache or stinging. Pain can be classified based on either pain experience, underlying pathophysiology, or duration. Simplest, pain is divided to acute and chronic pain based on the duration. Acute pain has usually a well-defined location and a recognizable causality, and therefore it is quite simple to treat. Moreover, acute pain has an important purpose, for instance in warning of injuries through reflexes like withdrawal of a damaged limb. Furthermore, it encourages people to rest and heal. Instead, chronic pain is typically no longer beneficial for an individual. (Lussier et al., 2019)

Pain is defined to be chronic when it has lasted more than six months. Chronic pain usually extends the period of healing and is harder to localize than acute pain. It is a disease which starts to affect daily life such as sleeping and moving. Moreover, it has been commonly related to depression and other mental health issues. (Lussier et al., 2019) For an individual, chronic pain is an enormous burden. It decreases the quality of life because it causes absences from work, decreased productivity and early retirement. Therefore, it has an immense impact on the economy too. The biggest single reason for high costs is hospitalization (Breivik et al., 2013). In addition, psychosocial and emotional disorders associated with chronic pain further increase costs. Although exact assessments of total costs are not available. Effective treatment and prevention of pain would be valuable worldwide in so that pain patients would be able to live normally, and costs would decrease markedly too. (Smith and Torrance, 2012)

Chronic pain can be separated into subtypes such as nociceptive and neuropathic. Nociceptive chronic pain, referred also as inflammatory pain, is a result from a tissue damage. Instead, neuropathic chronic pain originates from a disease or a lesion of the nervous system. (Austin et al., 2012) Another common cause for neuropathic pain is diabetes (Schreiber, 2015). In addition to nociceptive and neuropathic, chronic pain can also be classified as nociplastic or dysfunctional pain that arises without any stimulation of nociceptors or a disease or a lesion of the nervous system (Vergne-Salle and Bertin, 2021). Based on the clinically relevant disorders, chronic pain can be further divided into musculoskeletal, cancer, postsurgical, visceral, or again neuropathic pain (Treede et al., 2015).

1.1.1 Epidemiology and etiology

Chronic pain is a global trouble as in 2014 it affected approximately 60 million people worldwide (Jackson T et al., 2014). In Europe, chronic pain affects approximately 20% of the population and most of them are women or elderly (van Hecke et al., 2013). However, incidence and prevalence of neuropathic pain are hard to estimate because of the lack of coherent diagnostic criteria. Hence diagnosis can depend on patients' home country. Nevertheless, the prevalence of neuropathic pain among European citizens is estimated to be one to eight percent (Haanpää, 2007). Probably the prevalence has increased during the last years because of the ageing population, diabetes, and lifestyle changes. (Colloca et al., 2017) Still the accurate information is lacking. (Smith and Torrance, 2012)

There are multiple factors that increase or decrease the probability of chronic pain formation. The risk factors associated with chronic pain are classified as demographic, lifestyle, clinical or other factors. Demographic risk factors that cannot be influenced are age, gender, ethnicity, and socio-economic background. Instead, smoking, alcohol use, physical activity, nutrition, and vitamin D intake are up to an individual. Clinical factors such as multi-morbidity and mortality, mental health, surgical and medical interventions, weight, sleep disorders, and genetics are affecting the risk for development of chronic pain too. Moreover, attitudes and beliefs about pain, history of violent injury, abuse, or interpersonal violence are related to the development of chronic pain. Even though some factors have a higher impact on incidence than others, chronic pain arises from a combination of factors and events. Discovering these factors may help in the prevention and treatment of chronic pain. (Mills et al., 2019)

1.2 Neuropathic pain

Neuropathic pain arises from lesions or diseases of the peripheral or central somatosensory component of the nervous system. The system is responsible for touch, pressure, temperature, position, movement, and vibration through thermoreceptors, mechanoreceptors, chemoreceptors, pruriceptors and nociceptors in the skin, joints, muscles, fascia. The signal detected by these receptors is forwarded into the spinal cord and into the brain for processing. During neuropathic pain, lesions or diseases of the nervous system cause altered or disordered transmission of these sensory signals into the central nervous system (CNS). (Colloca et al., 2017) This is shown as different clinical manifestations such as spontaneous pain (burning and tingling), dysesthesia and paranesthesia (abnormal nerve sensations), allodynia (pain resulting from normally non-painful stimulus) and hyperalgesia (increased sensitivity to pain). (Austin et al., 2012) Usually patients complain most

about cold and mechanical irritations. However, the symptoms vary among patients due to complex pathophysiology of neuropathic pain. The diagnosis of neuropathic pain requires the identification of location of pain, dysfunctions of sense of touch, and etiology. The clarification of etiology is not always necessary for the diagnosis because it can be uncertain which incidence or event has caused the nerve damage. (Haanpää, 2007)

Neuropathic pain can be divided anatomically into central and peripheral neuropathic pain. Peripheral neuropathic pain may occur with diabetes, HIV infections and chemotherapy. Additionally, pain is peripheral when it is associated with polyneuropathies and ruptured disc. Instead, central neuropathic pain is preceded by disease or injury in the CNS. These are for example neurodegenerative diseases (e.g., Parkinson disease), spinal cord injury and demyelinating diseases (e.g., multiple sclerosis). Typically, central neuropathic pain is less common and harder to treat than peripheral. (Colloca et al., 2017) Yet, the separation into peripheral and central may not be clinically useful because peripheral neuropathic pain causes changes in the CNS too (Haanpää, 2007). Although, the separation is useful for drug development.

1.2.1 Current treatments and unmet medical need

The treatment of chronic pain depends on the type of pain, other diseases and their treatment, and patient's psychosocial situation. First-line treatments for neuropathic pain involve SNRI (serotonin-norepinephrine reuptake inhibitor; duloxetine and venlafaxine) drugs, antiepileptics (gabapentinoids; gabapentin and pregabalin) and tricyclic antidepressants (amitriptyline, nortriptyline). SNRI drugs are used mainly for painful diabetic neuropathy. Gabapentinoids are effective on diabetic neuropathy too as well as on postherpetic neuralgia. Moreover, pregabalin is noticed to be the best one for central neuropathic pain. Instead, tricyclic antidepressants are proved to ease different symptoms of both peripheral and central neuropathic pain and usually they are first ones to be prescribed to neuropathic pain patients. (Haanpää, 2007; Suomalaisen Lääkäriseuran Duodecimin, 2015; Fornasari, 2017)

However, there are several other medications used for neuropathic pain if the first-line treatments do not ease the symptoms enough. Capsaicin and lidocaine creams or plasters can help to cope with peripheral neuropathic pain. Also, weak opioid tramadol can be used if other medications do not work. Some patients have an advantage of using even strong opioids although there is variable information about their effectiveness. (Haanpää, 2007; Suomalaisen Lääkäriseuran Duodecimin, 2015; Fornasari, 2017) Some sources have claimed that opioids are ineffective particularly for neuropathic pain (Busse et al., 2018). Especially in the United States of America and in Canada opioid crisis is a notable issue. Opioids have been prescribed to patients too easily which has led to opioid-

related overdose deaths and newborns' withdrawal symptoms. (Busse et al., 2018) Therefore, use of opioids for neuropathic pain should be avoided if possible.

Because there are multiple mechanisms underlying neuropathic pain, one drug will not probably ease the disease or its symptoms good enough. Therefore, drug combination treatments are frequently used. However, the treatment is started using only one drug at the time. On top of medications, patients need encouragement, mental support, physiotherapy, stimulation therapies or even acupuncture. Combining different treatments usually offers the best outcome, although drug treatment is usually the most valuable one for patients. Nonetheless, drugs ease the symptoms only partly. When analyzing drug effectiveness, drug response is concerned already to be good when pain is halved. (Haanpää, 2007)

Although there are a lot of treatment options for neuropathic pain, there are still a lack of accurate and effective drugs. Current drugs have various adverse effects, and they ease pain poorly. Moreover, placebo responses in clinical trials have arised to be an issue (Finnerup et al., 2015). Therefore, more accurate treatment options based on pathophysiological mechanisms of pain are needed. To develop more specific analgesics, the underlying mechanisms of neuropathic pain must be first clarified.

1.2.2 Pathophysiology of neuropathic pain

Although the mechanisms of pain are not completely defined, some of the pathophysiology is already known. Briefly described, after a peripheral nerve injury, sensory neurons start to transfer altered signals to second-order neurons which results in an imbalance between excitatory and inhibitory signaling systems. The loss of inhibitory signals leads to a hyperexcitability state which is contributed to chronic pain when altered signals are constantly transferred from the periphery to the CNS. In peripheral neuropathic pain, the sensitized sensory nerve fibers are myelinated Aβ and Aδ or unmyelinated C fibers. The alterations happen in sodium, calcium and potassium ion channels within the affected nerve fibers which leads to increased excitability, signal transduction and neurotransmitter release. Due to the increased excitability, second-order nociceptive neurons in the spinal cord start to transfer sensory information into the brain generating central sensitization so peripheral neuropathic pain affects the CNS too. (Colloca et al., 2017)

So, the alteration and sensitization of second-order nociceptive neurons arises originally from an increased number of neuropeptides and excitatory amino acids. One of the postsynaptic changes in second-order neurons is phosphorylation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which leads to excess of signaling. (Colloca

et al., 2017) In addition, the excess of glutamate and peptide stimulation can lead to degradation of inhibitory transduction neurons of which GABAergic neurons (gamma-aminobutyric acid) are the most significant. This leads to an imbalance between inhibition and excitation which could be one of the most remarkable reasons for pain chronification. After the inhibitory neuronal damage, more synapses are formed also in places where they did not exist earlier which could explain allodynia. (Haanpää, 2007) Additionally, changes in non-neuronal cells in the spinal cord like microglia and astrocytes are connected to hyperexcitability (Colloca et al., 2017). They release chemokines, cytokines and neurotrophins that promote central sensitization (Shi et al., 2012).

Compared to peripheral neuropathic pain, the pathology of central neuropathic pain originates from a specific injury or disease in the CNS. The development of central neuropathic pain requires an injury of spinothalamic tract which is the sensory pathway from the dorsal horn of spinal cord to the thalamus. Moreover, there is sensitization in the CNS too which has been related to decreased expression of the inhibitory receptors and signals. (Haanpää, 2007) However, the pathophysiology varies among peripheral and central neuropathic pain patients due to various etiologies. Even the same causality for neuropathic pain can cause different clinical symptoms. Therefore, discovering the pathophysiology for each patient individually could provide better and more efficient treatments. (Haanpää, 2007)

1.3 Neuropathic pain models

Different *in vitro* and *in vivo* models are used to understand the molecular mechanisms of neuropathic pain. *In vitro* models bring basic information about mechanisms of pain. Because most models are derived straight from humans, they function as a connection between animal models and human patients. DRG (dorsal root ganglion), which is part of the peripheral nervous system, is widely used *in vitro* studies because it contains a lot of sensory neurons, and it is proven to be involved in the development of neuropathic pain. (Krames, 2014) The availability of DRG neurons of human donors (hDRG) is improved during the years. Before they were received only from fetuses but nowadays DRG neurons can be isolated during spinal surgeries and from healthy post-mortem donors too. They are used to assess the transnationality of rodent studies to humans. Along with hDRG, the use of stem cell-derived sensory neurons (SC-SNs) has expanded too. They are derived from human embryonic stem cells (hESCs) or from induced pluripotent stem cells (iPSCs). Compared to hDRG neurons, SC-SNs enable to have a greater supply of neurons. However, DRG isolated from rodents remain the most used *in vitro* model in pain research because they are more convenient and cheaper to get. (Chrysostomidou et al., 2021)

Rats remain the most used species *in vivo* pain studies too. There are several neuropathic pain animal models available. They are for instance chronic constriction injury (CCI), spinal nerve ligation (SNL), spared nerve injury (SNI) and partial sciatic nerve ligation (PSNL). In some studies, mechanical and thermal stimulation models and oxaliplatin-induced models are used too. (Sousa et al., 2016) The choice of a model depends on the wanted phenotype because models represent different neuropathic pain subtypes. In CCI operation, animals are influenced to a peripheral nerve injury which will lead to chronic pain and hypersensitivity for at least a month. Injured animals experience sensory dysfunction and behavior disabilities which can be measured. Eventually, the hypersensitivity fades away. Instead, SNL and SNI models can demonstrate neuropathic pain by showing the sprouting of primary sensory neurons. *In vivo* studies are convenient when studying the effectiveness of analgesics too. After behavioral analyses, the study can be continued with post-mortem isolated tissues. Usually, tissues isolated from pain model animals are DRG, spinal cord and sciatic nerve. Spinal cord and DRG are preferred due to a greater amount of tissue (Austin et al., 2012) which are anatomically more accessible. (Krames, 2014)

1.4 Signaling pathways related to pain formation

Neuropathic pain is caused by variation of altered mechanisms over the body. Although alterations in transmitters and modulators are roughly known, transcriptional mechanisms underlying neuropathic pain remain still elusive. Nevertheless, they are known to be complex. Novel studies have suggested that the Hippo and Wnt signaling pathways are relevant for pain formation. Normally, the Hippo pathway regulates the size of cells and organs by controlling proliferation and increasing apoptosis. The pathway is activated for instance by high cell density which results in the inhibition of proliferative genes which can lead for instance to inhibition of mitosis. The Hippo pathway has been studied mostly on *Drosophila*, but it is conserved across phyla (Hilman and Gat, 2011). Afterwards it has been ensured to exist in rodents and humans too. Quite similarly, the Wnt pathway is needed for organogenesis, stem cell regeneration and cell survival (Krishnamurthy and Kurzrock, 2018), and for synapse formation and plasticity in adults. (Piccolo et al., 2014; Pocaterra et al., 2020) The pathway is highly important during embryogenesis but may be activated during diseases as well (Komiya and Habas, 2008). Other pathways and transcriptional factors that are connected to chronic pain formation are for instance CREB, cFos and DREAM (Yang et al., 2021; Cheng et al., 2002) but the study will be concentrating on the Hippo and Wnt pathways.

1.4.1 Hippo signaling pathway

The Hippo pathway has different signaling components of which Yes-associated protein 1 (YAP) and WW domain-containing transcription regulator 1 (TAZ or WWTR1) might be the most important ones considering chronic pain. YAP and TAZ are required for growth of embryonic tissues, wound healing, and organ regeneration. Though they are usually inactive or degraded in the cytoplasm because these processes are needed only occasionally. The active Hippo pathway is responsible for the inactivation of YAP/TAZ. The signaling cascade starts with MST1/2 (Mammalian Ste20-like) kinases binding to their regulatory protein SAV1 (Salvador family WW domain containing protein 1) to form an active enzyme that phosphorylates and activates LATS1/2 (Large tumor suppressor) kinases in the cytoplasm. Simultaneously, MST1/2 phosphorylates the regulatory subunits of LATS1/2 called MOB1A/B (Mps one binder) forming an active complex LATS1/2-MOB1A/B. This complex further phosphorylates YAP/TAZ resulting in their inactivation and retention in the cytoplasm. Without their phosphorylation, YAP/TAZ would translocate into the nucleus. (Piccolo et al., 2014) (Figure 1)

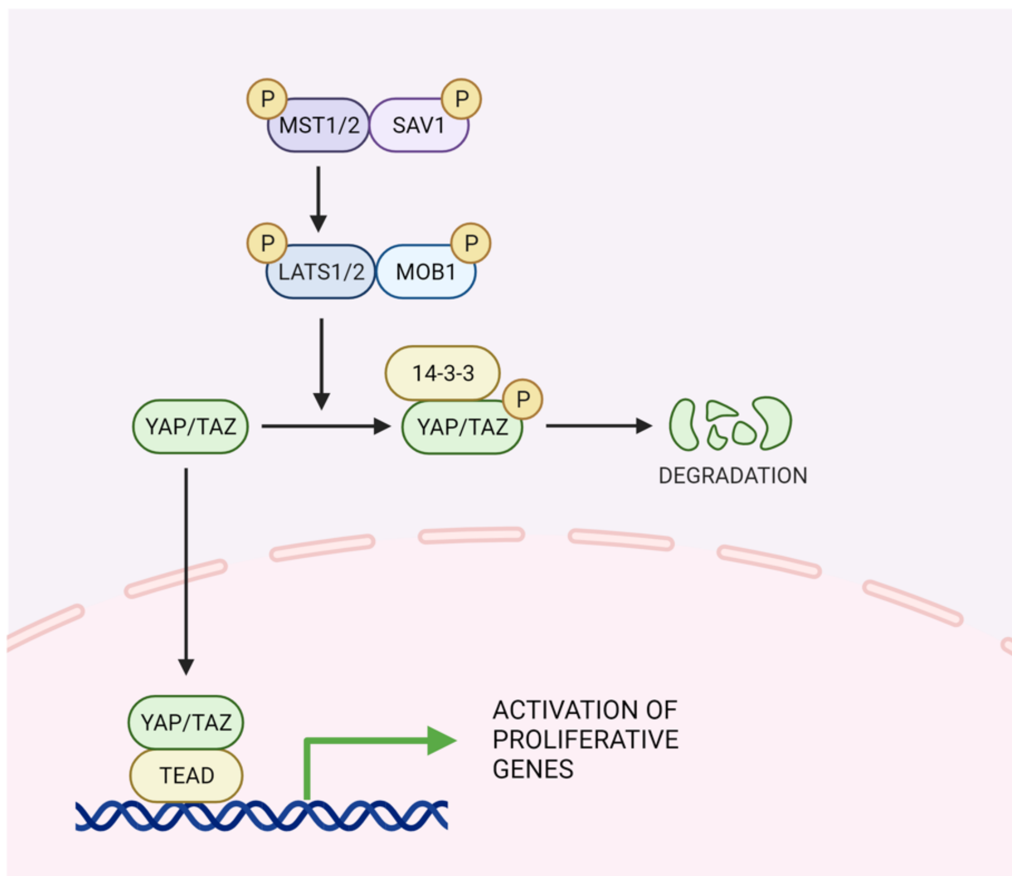

Figure 1. The Hippo signaling pathway. Active Hippo signaling pathway results in phosphorylation of YAP/TAZ, and hence their retention or degradation in cytoplasm. Otherwise, YAP/TAZ are shuttled into nucleus where they bind to transcription factor TEAD.

When the Hippo kinases phosphorylate Ser127 and Ser381 residues on YAP and Ser89 and Ser311 residues on TAZ, these transcription regulators are kept inactive (Piccolo et al., 2014). Afterwards, 14-3-3 family proteins can bind to phosphorylated Ser127 on YAP which promotes the retention in the cytoplasm. Furthermore, phosphorylation of Ser381 on YAP and Ser311 on TAZ allows additional phosphorylation by CK1 (casein kinase 1) resulting in a phosphodegron which is recognized by β-TrCP (β-transducin repeat containing E3 ubiquitin protein ligase). This will end up in polyubiquitylation of YAP/TAZ via E3 ubiquitin ligases and in proteasome degradation. It is known that TAZ is quite easily degraded in the cytoplasm which can be explained by formation of a second phosphodegron on TAZ. Its residues Ser58 and Ser62 can be phosphorylated by glycogen synthase kinase 3α (GSK3) creating a binding site again for β-TrCP. This leads to TAZ degradation through the SCF/β-TrCP ubiquitylation pathway. (Reggiani et al., 2021) YAP is lacking this second phosphodegron and it is therefore more stable than TAZ. (Piccolo et al., 2014) In addition, YAP can be spliced into two alternatively isoforms which distinguish from each other by having either one or two WW domains (Kim et al., 2018).

YAP/TAZ are active only in regenerative or malignant conditions. Then the Hippo pathway is inactive which causes non-phosphorylation of YAP/TAZ. For instance, there is shown to be an increased level of active YAP/TAZ in different types of cancers. They sustain tumor growth, drug resistance and malignancy by activating genes that are likely to promote proliferation. Active YAP/TAZ translocate into nucleus where gene regulation occurs together with TEAD (Transcriptional enhancer associate domain) factors because they itself lack DNA-binding domain. (Piccolo et al., 2014) According to Kim et al. YAP with two WW domains activate TEAD-dependent transcription more strongly than TAZ or YAP with one WW domain. Mammals have four different TEAD (1–4) proteins which share identical DNA-binding domains. However, they might have distinct biological functions due to their tissue-specificity and developmental stage-specificity. (Kim and Gumbiner, 2019) Other DNA-binding transcriptional factors and regulators that interact together with YAP/TAZ are p73, ERBB4, EGR-1, TBX-5, SMADs and RUNXs but they may not be associated with chronic pain (Kim et al., 2018).

The Hippo pathway and YAP/TAZ are regulated by a network of factors and mechanisms including cell-cell adhesions, cell polarity, extracellular forces excreted by cell's microenvironment, metabolic pathways, and extracellular growth factors. For instance, it is known that a high stiffness of extracellular matrix (ECM) promotes YAP/TAZ activity. Instead, a compact and round cell and a soft ECM favor the cytoplasmic retention. The membrane-associated components Merlin and Scribble

both inhibit YAP/TAZ activation. Merlin, encoded by NF2 (neurofibromatosis type 2), inhibits YAP/TAZ after their phosphorylation by LATS1/2 kinase. Merlin also takes part in the export of YAP from the nucleus by forming Merlin-YAP complex (Dobrokhotov et al., 2018). Scribble is needed for activation of LATS1/2 kinase, and therefore it enhances the phosphorylation of YAP/TAZ. Furthermore, EMT (epithelial-mesenchymal transition) causes delocalization of Scribble and inactivation of the Hippo cascade which leads to increase in the YAP/TAZ dependent gene expression. (Piccolo et al., 2014) Moreover, other signaling pathways like Wnt can alter YAP/TAZ activity.

1.4.2 Wnt signaling pathway

Other important participating pathway to chronic pain formation is the Wnt pathway. Wnt protein, secreted by glycoproteins, binds to its extracellular receptors causing activation of multiple intra-cellular pathways. The binding to a Fz-LRP6 receptor (Frizzled low-density lipoprotein receptor) leads to the activation of the Wnt/β-catenin dependent pathway. This pathway is not part of the physiological pain processes, but it is suggested to be involved in the development of neuropathic pain after a nerve injury. The pathway's main effector β-catenin is controlled by a cytoplasmic destruction complex including Axin, glycogen synthase kinase-3 (GSK3), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and others which phosphorylate β-catenin in the absence of Wnt. More precisely, GSK-3 is responsible for phosphorylating β-catenin (Gordon and Nusse, 2006). Normally the absence of Wnt induces the phosphorylated β-catenin to stay in the complex along with phosphorylated YAP/TAZ. The whole complex stays in the cytoplasm which inhibits the translocation of β-catenin and YAP/TAZ into nucleus. (Piccolo et al., 2014) β-catenin can be further ubiquitylated by β-TrCp (Krishnamurthy and Kurzrock, 2018) when it will become vulnerable for degradation by proteasomes (Gordon and Nusse, 2006). (Figure 2)

Thus, the Wnt pathway stimulation causes release of YAP/TAZ from β-catenin destruction complex. Simultaneously the complex does not phosphorylate or ubiquitylate β-catenin anymore which leads to accumulation of unphosphorylated β-catenin in the cytoplasm. The accumulated β-catenin and YAP/TAZ proteins are then shuttled into the nucleus where they indirectly induce gene expression. (Xu et al., 2016) (Figure 2)

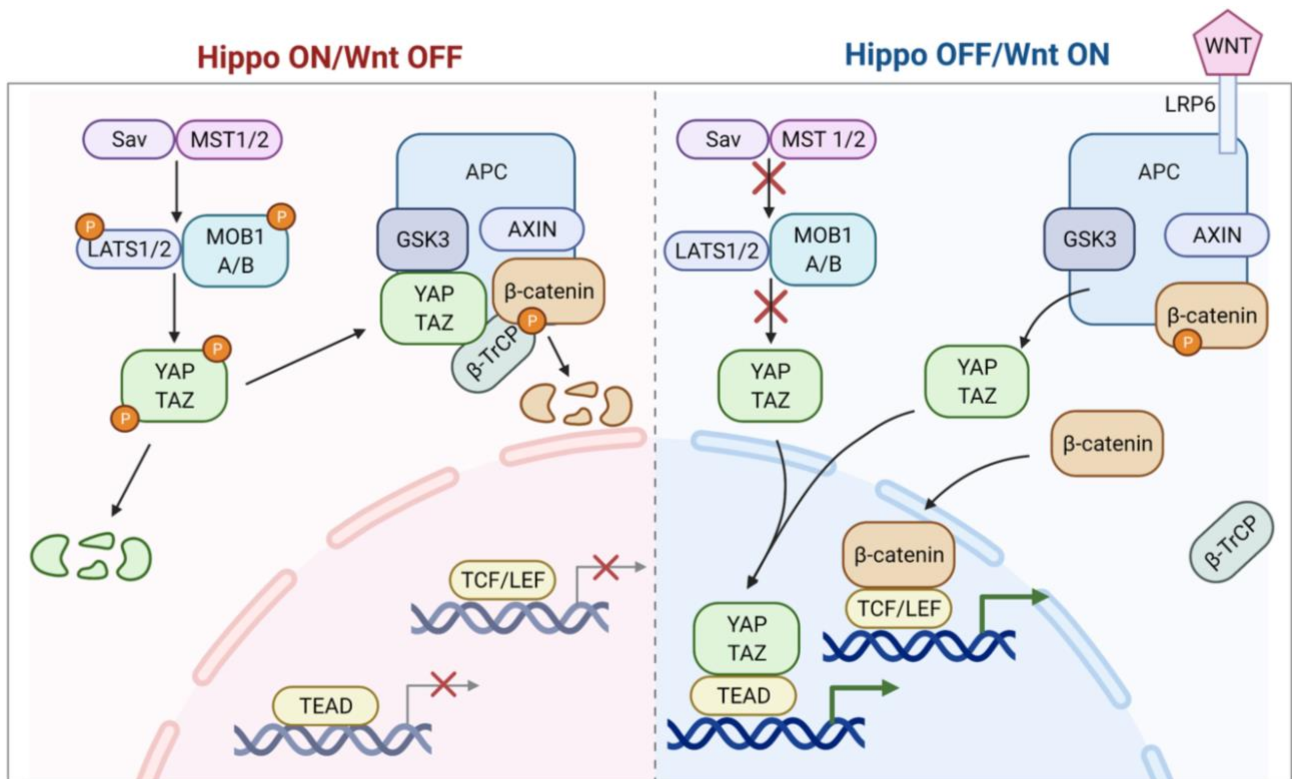

Figure 2. The Hippo and Wnt pathways regulate transcription regulators YAP, TAZ and β-catenin. Active Hippo pathway cause phosphorylation and hence inactivation of YAP and TAZ. Simultaneously, Wnt pathway is inactive, and β-catenin is bind by cytoplasmic destruction complex. Instead, inactive Hippo pathway and active Wnt pathway results in YAP-, TAZ- and β-catenin-induced gene expression in nucleus.

1.4.3 β-catenin- and YAP/TAZ-induced gene expression

In the nucleus, β-catenin functions together with TCF (T cell-specific factor) and LEF (lymphoid enhancer-binding factor) to activate the transcription of Wnt target genes. When entering nucleus, β-catenin replaces transcriptional repressor Grouche from TCF. This leads to an active transcriptional complex including histone acetylase GBP/p300 and SWI/SNF complex member Brg-1 which remold chromatin of TCF binding sites and allow gene activation. (Gordon and Nusse, 2006) Additionally, Legless and Pygopus coactivators are necessary for nuclear retention and gene activation ability of β-catenin (Komiya and Habas, 2008). Nuclear β-catenin cause for instance the expression of *c-Myc*, *cyclin D1* and *CDKN1A* (cyclin dependent kinase inhibitor 1A) genes. *c-Myc* is known to be a proto-oncogene whereas CDKN1A protein regulates cell cycle progression at G1 phase (Xu et al., 2016). The activation of these genes promote proliferation.

Whereas gene expressions caused by β-catenin are known, the expressed genes by YAP/TAZ remain partly elusive, especially genes related to chronic pain. Genes induced by YAP/TAZ are suggested to promote proliferation, cell growth, number of membrane channels, cytokines, and tissue repair. In the case of neuronal cells, proliferation means axonal

sprouting that can cause sensory hyperexcitability and pain chronification. (Krishnamurthy and Kurzrock, 2018; Piccolo et al., 2014) Some of the YAP/TAZ target genes are identified like *CYR61, CTGF, AREG, MYC, Gli2*, *VIM*, and *AXL* but these are characterized only with cancer and not pain. (Kim et al., 2018)

1.5 Transcriptional mechanisms of YAP, TAZ and β-catenin in neuropathic pain

Although the transcriptional mechanisms underlying chronic pain are still unclear, novel studies have suggested that YAP/TAZ and β-catenin are involved in the development and maintenance of chronic neuropathic pain. However, only a few studies have been concentrating on these transcription regulators and pain. Therefore, the results can vary significantly.

Earlier studies have clarified some of the connections between pain chronification and YAP/TAZ and β-catenin. The role of β-catenin has been studied in both the spinal cord and DRG. In the spinal cord, CCI surgery has caused a nuclear accumulation of β-catenin (Xu et al., 2016). Instead, according to Simonetti et al., β-catenin is not present in the DRG after a peripheral nerve injury. They concluded that β-catenin is not crucial in pain hypersensitivity at least not in the DRG. (Simonetti et al., 2014) In Xu et al. study, CCI surgery caused the nuclear accumulation of YAP/TAZ in the spinal cord. To be precise, YAP/TAZ were in the superficial layers of dorsal horn which are critical areas for neuropathic pain formation. CCI operation resulted also in the inhibition of kinases that phosphorylate YAP/TAZ. Contrary to the spinal cord, YAP/TAZ were not transported into the nucleus in the DRG after a peripheral nerve injury. In the same study it was confirmed that sham operation (placebo surgery) did not affect results. (Xu et al., 2016).

Another study had similar results considering the differences of transcriptional factors in the spinal cord and DRG. When it comes to Wnt and Fz receptors, CCI operation increased their expression in both tissues. But in the spinal cord, many Fz receptor subtypes like Fz1, Fz3, Fz4, Fz5, Fz8 and Fz10 were activated. Instead, only Fz8 was overexpressed in the DRG after CCI. The increased expression of Wnt and Fz receptors led to increase in phosphorylated and dephosphorylated β-catenin at least in the spinal cord. Hence, β-catenin is needed for chronic pain induction in the spinal cord. However, in the DRG, Wnt seems to function β-catenin independent way after a nerve injury. Furthermore, Shi et al. noticed β-catenin to express in lamina II in the spinal cord dorsal horn where interneurons, like second-order neurons, are located. These interneurons have a critical role in central sensitization as mentioned earlier. (Zhang et al., 2013) Zhang et al. noticed also that blocking Wnt signaling in the spinal cord inhibited the production and persistence of CCI-induced neuropathic pain because canonical Wnt signaling is important regulator of YAP/TAZ activity. Also *in vivo* knockdowns of

YAP/TAZ suppressed mechanical allodynia which represents hypersensitivity and chronic pain. (Xu et al., 2016)

Furthermore, *in vivo* studies have shown that YAP has a significant role in the induction and persistence of chronic pain. Hence in Xu et al. study, YAP had its nuclear concentration peak in the early phase of the chronic pain formation whereas TAZ and β-catenin seemed to stay longer in the nucleus. However, they all were considered to have a long-lasting accumulation into the nucleus of spinal cord neurons. Additionally, TAZ is known to shuttle into the nucleus of microglial cells as well. (Xu et al., 2016) On the contrary, YAP is not expressed in glial cells. Moreover, Li et al. showed that TAZ was mainly associated with spinal cord second-order neurons. The same study suggested that TAZ would be involved in initiating the process through a neuro-glial crosstalk, whereas YAP would have a role in sensitization of primary afferent neurons in the dorsal horn of spinal cord. (Li et al., 2013) In addition, Li et al. showed first time on rats that YAP/TAZ are important in hyperalgesia and allodynia processes. Moreover, Xu et al. demonstrated that enhancement of nuclear accumulation of YAP/TAZ causes pain in naïve animals.

There is enough evidence to believe that the nuclear accumulation of YAP, TAZ and β-catenin really originate from a peripheral nerve injury. Therefore, it has been suggested that by inhibiting Hippo pathway after a peripheral nerve injury, chronic pain could be avoided or eased. (Xu et al., 2016) As mentioned earlier, YAP/TAZ are present in several cancer types. Therefore, there are multiple drugs in the pipeline targeting on YAP/TAZ for treatment of cancer. (Piccolo et al., 2014) Additionally, YAP might have a role in the pathology of other nervous systems diseases including Huntington's disease and Alzheimer's disease (Jin et al., 2020). Moreover, there are cancer drugs targeting on Wnt/β-catenin signaling pathway (Liu et al., 2020). These drugs and agents could possibly be used in the treatment of chronic pain too. However, before concerning any treatment targeting YAP/TAZ and β-catenin, unsolved transcriptional mechanisms underlying pain must be clarified. It would be necessary to confirm do they really shuttle into the nucleus when Wnt signaling pathway is activated. The differences between spinal cord and DRG should be clarified too to understand the mechanisms in both peripheral and central nervous system. Although it has been suggested that DRG is not involved in the pathology of neuropathic pain through YAP/TAZ and β-catenin transcription regulators, it should be still verified because of the small number of conducted studies. Moreover, it still unclear whether all unphosphorylated YAP/TAZ and β-catenin translocate into nucleus or will some stay in the cytoplasm.

1.6 Purpose of the study

The increased incidence and poor efficacy and dose-limiting adverse effects of current treatments are enormous issues in the field of neuropathic pain. Hence, the unsolved transcriptional mechanisms of neuropathic pain will be studied in this thesis project. If there are obvious discrepancies during chronic pain compared to physiological conditions, the results of this study can be utilized in developing more specific analgesics. In addition, the results will help to plan further studies.

The aim of this thesis study is to clarify how transcriptional regulators YAP, TAZ and β-catenin are regulated after a peripheral nerve injury. Previous studies suggest that a peripheral nerve injury leads to a nuclear accumulation of YAP, TAZ and β-catenin which indirectly activates proliferative genes. This could cause an increase in hypersensitivity due to axonal sprouting and changes in the signal transduction mechanisms. It is proposed that the nuclear accumulation of these transcriptional regulators happen only in the dorsal horn of spinal cord, and therefore the location of YAP in the spinal cord tissue sample will be studied as well.

2 Results

2.1 Hypersensitivity of sham and CCI operated rats

Spinal cord and DRG tissue samples that were used in the project were isolated from naïve, sham and CCI operated rats. Spinal cord is a part of the central nervous system whereas DRG belong to the peripheral nervous system. To ensure that CCI operation had caused hypersensitivity and hence neuropathic pain, the up-down von Frey technique was used to study sham and CCI rats' mechanical allodynia threshold. In the technique different size of filaments were applied to the injured paw. In rodents, mechanical allodynia correlates with the degree of hypersensitivity. When hypersensitivities of sham and CCI animals were compared, there were significant differences after 6 ($p<0.0001$), 14 ($p<0.0001$) and 21 days ($p<0.001$) from surgery. (Figure 3) Tissue samples used in the study were isolated 6 and 21 days after CCI surgery.

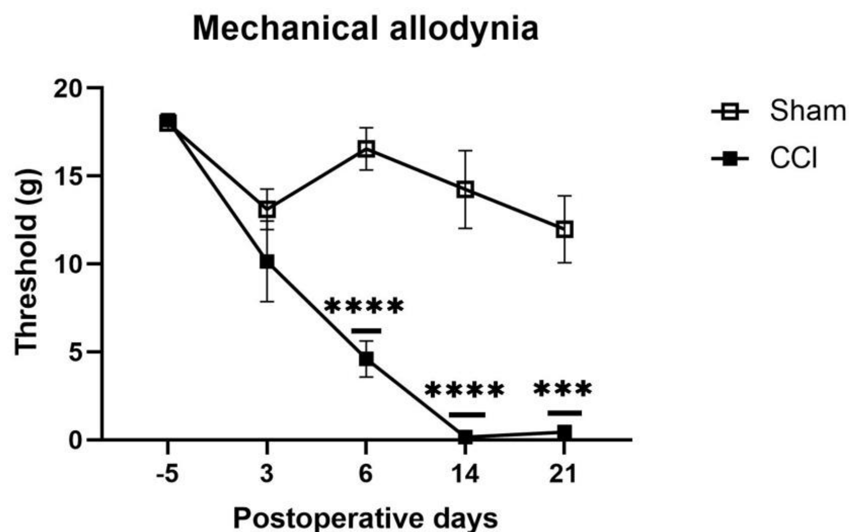

Figure 3. Mechanical allodynia in sham and CCI rats. Thresholds to von Frey filaments in sham and CCI rats. Two-way ANOVA, ***$p<0.001$ ****$p<0.0001$, N=8 in both groups, mean with SEM.

2.2 Characterization of protein expression in naïve animals

The study concentrated first on clarifying how transcriptional factors, associated with pain formation, are expressed in naïve animals. The spinal cord tissue samples from spinal segments L4–L6 were purified into cytoplasmic and nuclear protein fractions. The characterization was started with active β-catenin because its activity is more studied than the activity of YAP/TAZ. Afterwards, the characterization was continued with them. Along with the main proteins of interest, phosphorylated YAP/TAZ, DCC and TEAD proteins were studied too. Phosphorylated YAP/TAZ (p-YAP/p-TAZ)

are inactive forms of YAP/TAZ which should stay in the cytoplasm. DCC is known to locate only in the cytoplasm and cellular membranes, whereas TEAD is in the nucleus. Therefore, they were used to confirm that the protein purification into cytoplasmic and nuclear fractions was successful. In characterization of proteins, fluorescence imaging was used except with DCC and TEAD. They were detected with help of chemiluminescence.

In the spinal cord of naïve animals, β-catenin was more present in the cytoplasm than in the nucleus (p=0.0019). Similarly, YAP/TAZ were more abundant in the cytoplasm although the difference was not statistically significant (p=0.3280, p=0.5061). However, the trend can be seen from the results. Inactive YAP/TAZ had also a higher level of expression in the cytoplasm than in the nucleus (p=0.9980, p=0.9994). So, minor amounts of phosphorylated YAP/TAZ are seen in the nucleus too. (Figure 4A) DCC was found to be only in the cytoplasm. (Figure 4B) Instead, TEAD was located only in the nucleus. (Figure 4C)

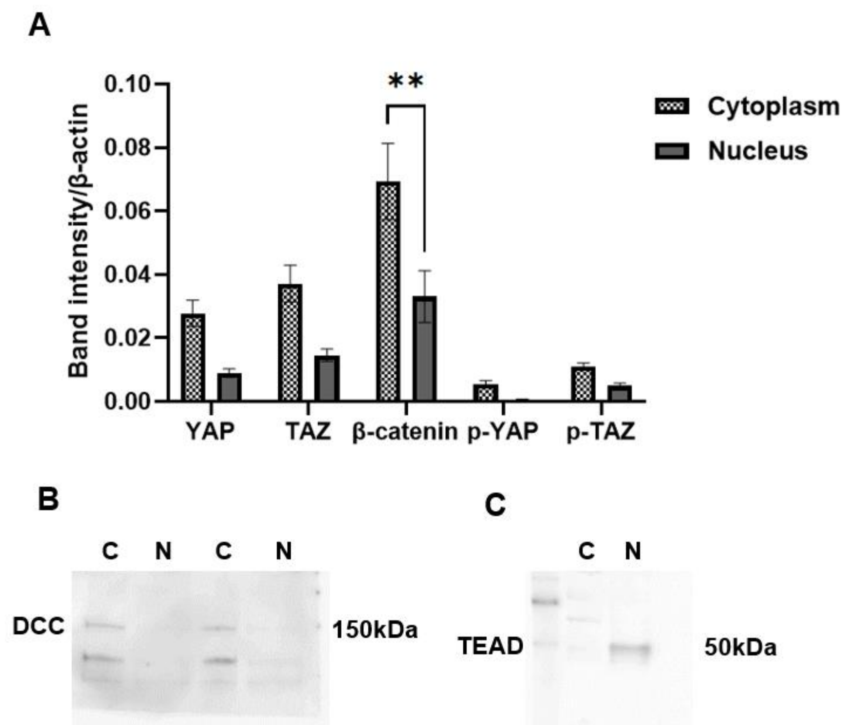

Figure 4. Cytoplasmic and nuclear protein expression in the spinal cord of naïve animals. A. YAP, TAZ, β-catenin and phosphorylated YAP/TAZ are expressed mostly in the cytoplasm in naïve animals when comparing their cytoplasmic and nuclear fluorescence signals. Cytoplasmic and nuclear YAP: n=16, n=16; TAZ: n=8, n=8; β-catenin: n=23, n=15; p-YAP: n=11, n=11; p-TAZ: n=4, n=4. Two-way ANOVA. YAP: p=0.3280; TAZ: p=0.5061; β-catenin: p=0.0019; p-YAP: p=0.9980; p-TAZ: p=0.9994. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 cytoplasm versus nucleus, mean with SEM. B–C. DCC and TEAD were imaged with chemiluminescence. B. DCC (150 kDa) is expressed only in cytoplasmic spinal cord protein samples. C. TEAD (50 kDa) is expressed only in nuclear spinal cord protein samples. C=Cytoplasm, N=Nucleus.

DRG tissue samples isolated from segments L4–L6 were not separated into cytoplasmic and nuclear fractions due to protein concentration issues. Hence similar characterization of protein expression in naïve animals was not conducted with DRG samples.

2.3 Changes in YAP, TAZ and β-catenin expression after a peripheral nerve injury

2.3.1 Chronic constriction injury causes overexpression of YAP and TAZ in spinal cord

The protein expression of YAP, TAZ and β-catenin were studied in naïve, sham and CCI spinal cord tissue samples using Western blot and fluorescence imaging. These three groups were further divided in two based on the time after the surgery. Naïve and sham groups were separated from each other as well because a variation was found. Finally, there were four animals in each group. The amount of protein loaded was 20 µg/ml, and β-actin (45 kDa) was used as a loading control. N represents number of animals and n represents number of repetitions.

After 6 days from a nerve injury, there were no statistically significant changes in YAP (70 kDa) expression in the cytoplasm ($p=0.9980$) nor in the nucleus ($p=0.3496$) when comparing CCI and sham groups. But after 21 days from CCI, the expression of YAP was significantly increased in the cytoplasm when compared to sham ($p=0.0002$). Instead, a similar difference was not detected in the nucleus ($p=0.0966$). At the same time, there was statistical difference between naïve and sham groups in nuclear samples in both day groups ($p=0.0008$, $p=0.0239$). (Figure 5)

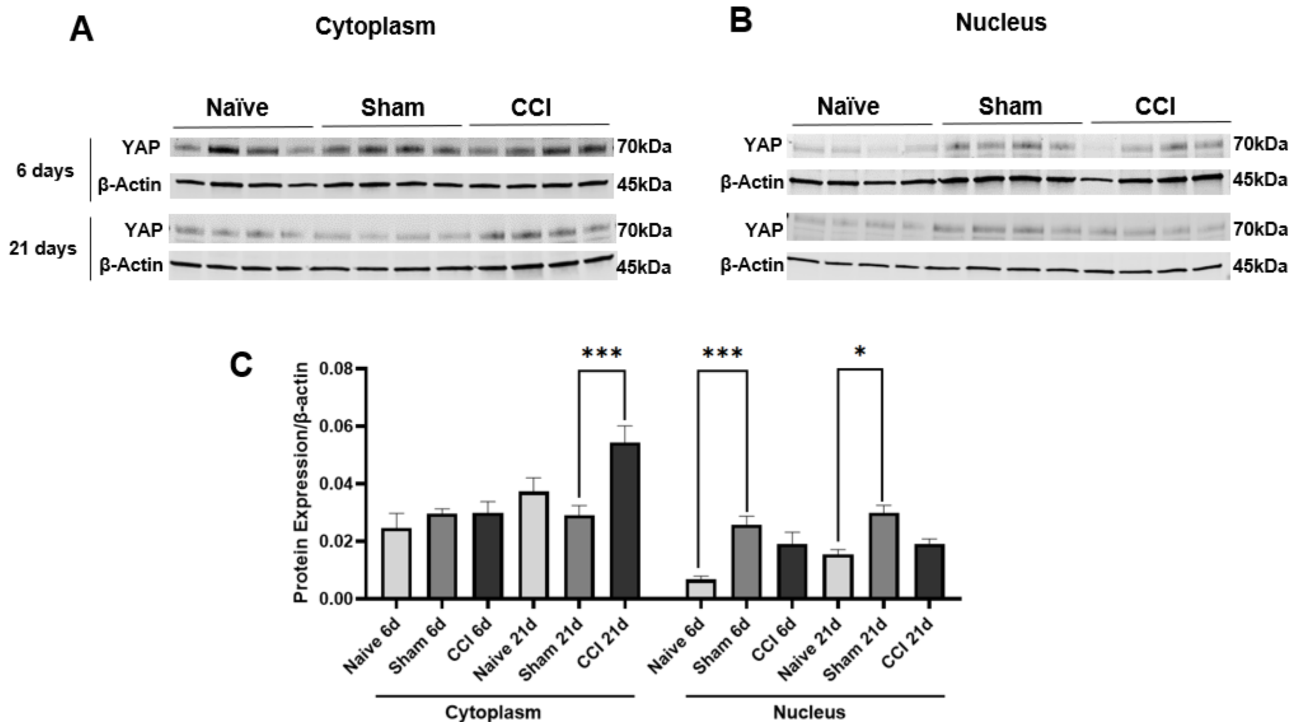

Figure 5. YAP protein levels are overexpressed in the cytoplasm of spinal cord sample after 21 days from CCI. A–B. Cytoplasmic and nuclear YAP were analyzed using fluorescent Western blot. A. Cytoplasmic YAP is overexpressed after 21 days. B. Nuclear YAP seems to overexpress in sham animals. C. Four animals (N=4) in each group. Cytoplasmic and nuclear repetitions 6 days: naïve n=12, n=12; sham n=8, n=8; CCI n=9, n=9. 21 days: naïve n=4, n=4; sham n=4, n=4; CCI n=4, n=4. Two-way ANOVA. 6 days: naïve p=0.5020, p=0.0008; CCI p=0.9980, p=0.3496. 21 days: naïve p=0.2154, p=0.0239; CCI p=0.0002, p=0.0966. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 cytoplasmic/nuclear versus sham, mean with SEM.

The amount of TAZ (50 kDa) was increased too in the cytoplasm after CCI operation. The increase was already shown after 6 days although it was not statistically significant when compared to sham (p=0.5868). The cytoplasmic expression of TAZ after 21 days seemed to be lower than after 6 days but the difference was still statistically significant when compared to sham group (p=0.0191). When comparing nuclear expressions, no significant differences between sham and CCI groups were noticed (p=0.9884, p=0.5982). Additionally, there were no differences between naïve and sham groups. (Figure 6)

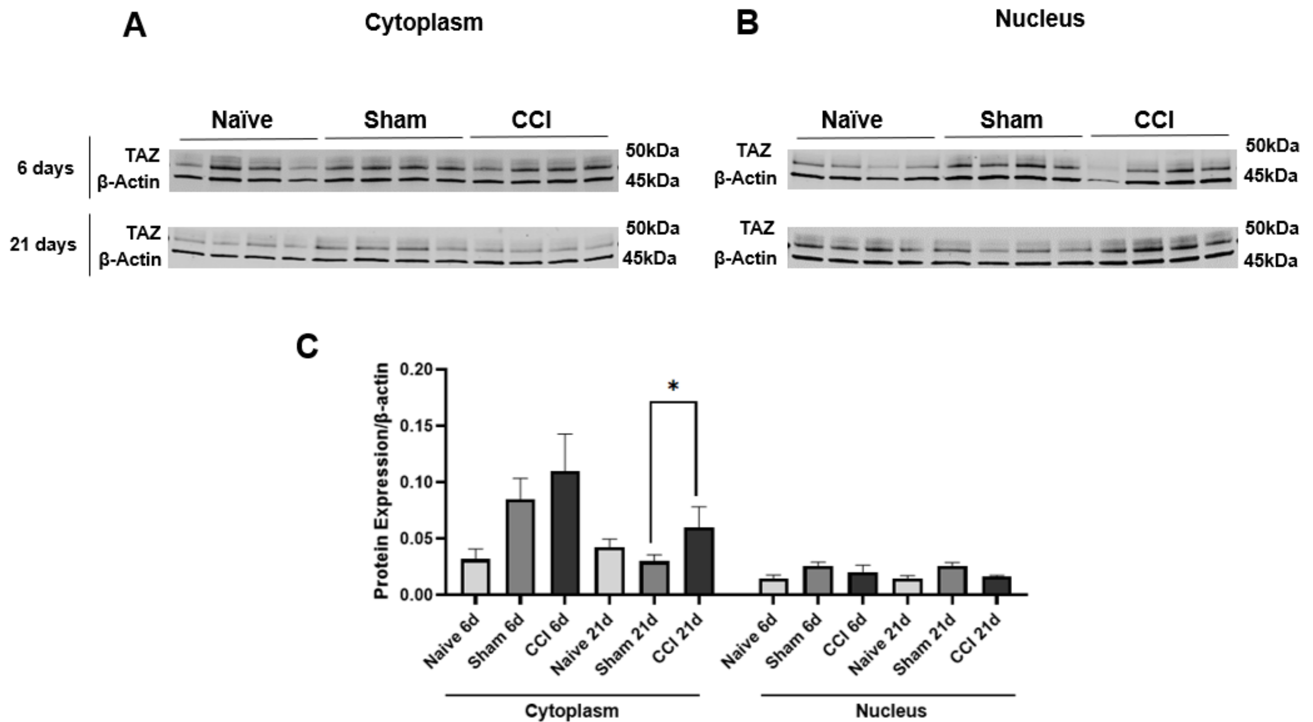

Figure 6. TAZ protein levels increased in the cytoplasm of spinal cord after CCI. A–B. Cytoplasmic and nuclear TAZ were analyzed using fluorescent Western blot. A. TAZ expression is increased in the cytoplasm after CCI. B. In the nucleus, CCI does not change TAZ expression levels. C. Four animals (N=4) in each group. Cytoplasmic and nuclear repetitions 6 days: naïve n=4, n=4; sham n=8, n=4; CCI n=8, n=4; 21 days: naïve n=4, n=4; sham n=4, n=4; CCI n=2, n=2. Two-way ANOVA. 6 days: naïve p=0.2339, p=0.9436; CCI p=0.5868, p=0.9884. 21 days: naïve p=0.2618, p=0.3372; CCI p=0.0191, p=0.5982. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 cytoplasmic/nuclear versus sham, mean with SEM.

There were no changes in the expression of β-catenin (92 kDa) in the spinal cord after a nerve injury. The expression levels were overall a bit higher in 21-day group than in 6-day group but no differences between naïve, sham or CCI were detected. (Figure 7)

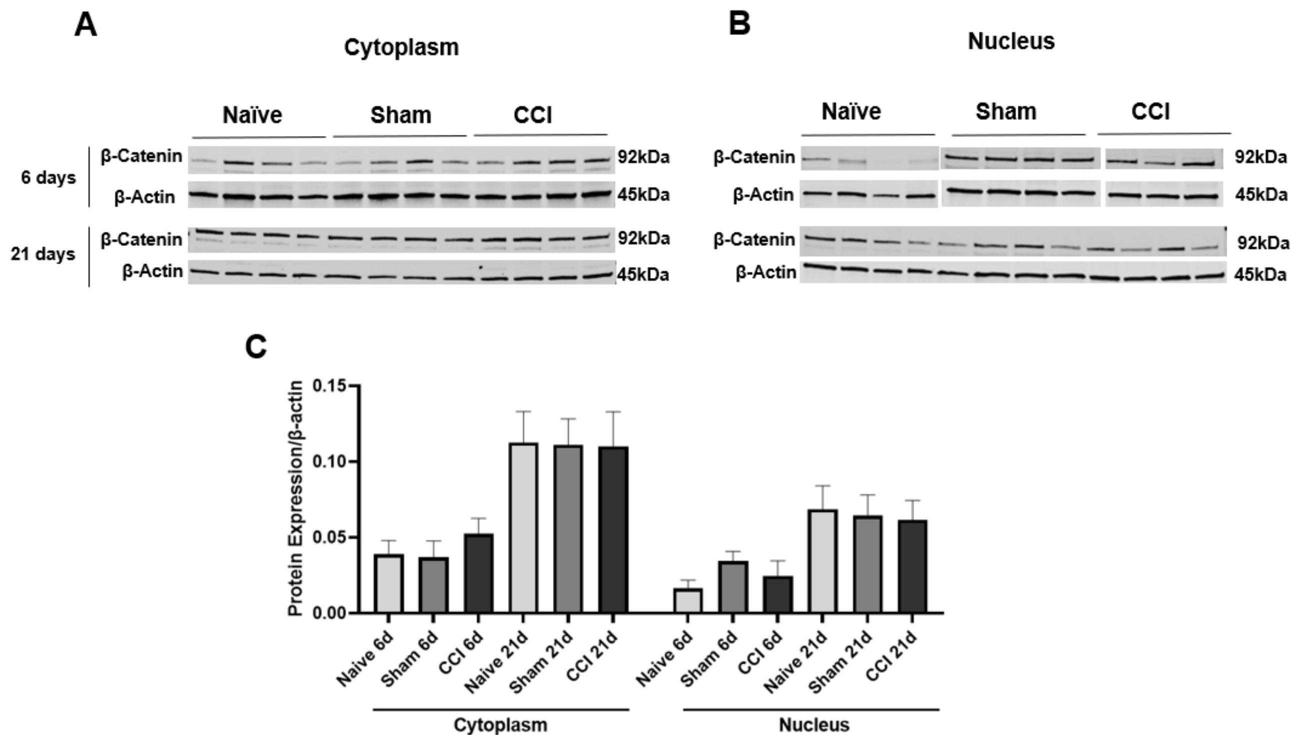

Figure 7. CCI did not affect β-catenin protein levels in the spinal cord. A–B. Cytoplasmic and nuclear YAP were analyzed using fluorescent Western blot. Controls and CCI did not differ in expression levels of β-catenin within the day group. C. Four animals (N=4) in each group. Cytoplasmic and nuclear repetitions 6 days: naïve n=14, n=10; sham n=8, n=8; CCI n=9, n=9. 21 days: naïve n=8, n=4; sham n=8, n=4; CCI n=8, n=4. Two-way ANOVA. 6 days: naïve p=0.9820, p=0.3013; CCI p=0.4181, p=0.6980. 21 days: naïve p=0.9974, p=0.9888; CCI p=0.9990, p=0.9961. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 cytoplasmic/nuclear versus sham, mean with SEM.

2.3.2 Nerve injury increases the expression of YAP, TAZ and β-catenin in dorsal root ganglia

During purification, DRG tissue samples from same animal group were combined to have higher protein concentrations. Similarly, as with spinal cord samples, the animal groups were defined based on the time from CCI or sham operation, either 6 (N=7) or 21 days (N=12). Within these groups the animals were further separated into naïve (N=2 or 4), sham (N=4 or 4) or CCI (N=1 or 4). Naïve and sham animals from different day group were not combined because their protein expressions were not always similar. The expression of YAP, TAZ and β-catenin was studied with these DRG samples. All samples were blotted twice with each antibody. The amount of protein loaded was 20 µg/ml, and β-actin (45 kDa) was used as a loading control. They were imaged with fluorescence.

The expressions of YAP and TAZ were both increased after CCI operation compared to sham. Both YAP (p=0.0365) and TAZ (p=0.0017) increased already after 6 days from CCI operation. However, there was a greater statistical difference after 21 days when comparing YAP (p=0.002) and TAZ (p=0.0001) expressions between CCI and sham. (Figure 8A–B)

Similarly, the amount of β-catenin was increased after a sciatic nerve injury when compared to sham. Especially after 6 days, the β-catenin expression was high (p<0.0001). Instead, the expression decreased slightly after 21 days but was still significantly different compared to sham (p<0.0001). Although there was also a significant difference between naïve and sham groups after 6 days (p=0.0005). (Figure 8C)

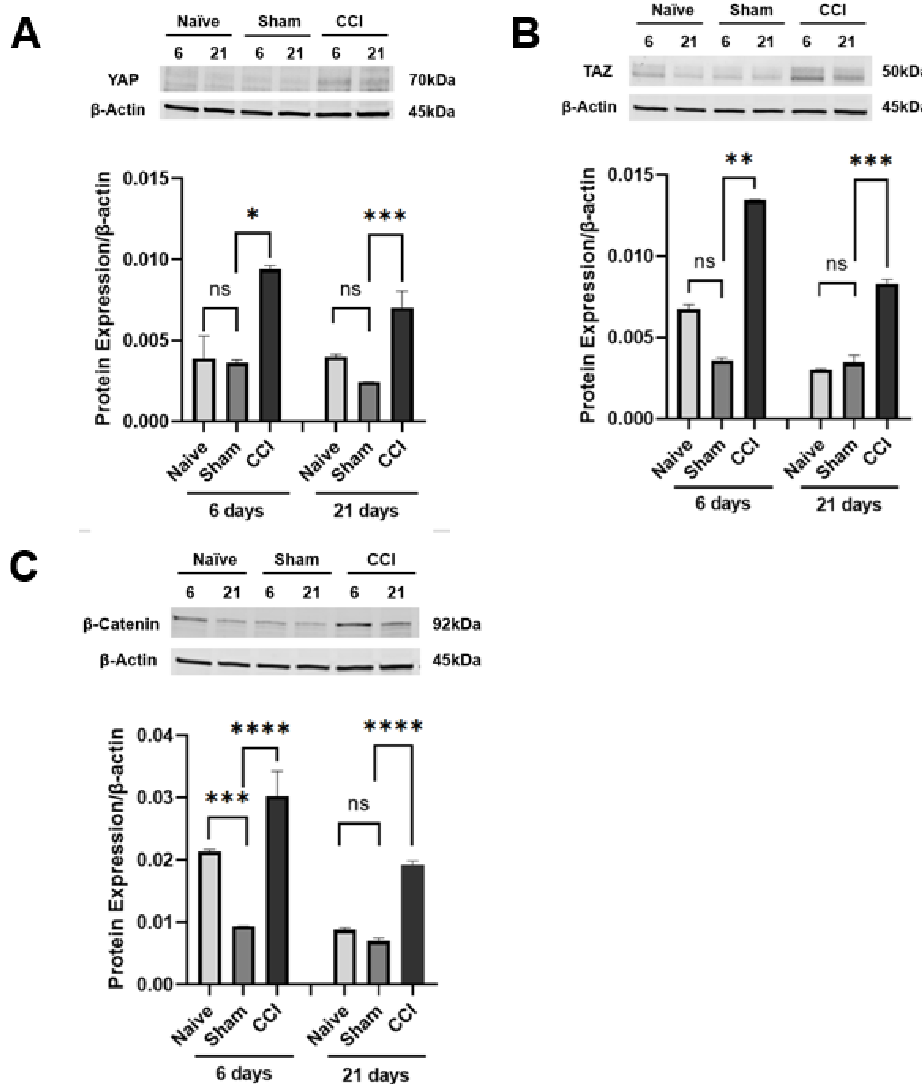

Figure 8. Overexpression of YAP, TAZ and β-catenin in the dorsal root ganglia after CCI when imaged with fluorescence. A. YAP levels were overexpressed after CCI throughout the study (p=0.0365, p=0.002 when compared to sham). B. TAZ levels were increased after 6 and 21 days from CCI (p=0.0017, p=0.0001 when compared to sham). C. β-catenin was overexpressed after CCI (p<0.0001, p<0.0001 when compared to sham). There was difference between naïve and sham groups after 6 days (p=0.0005). A–C. 6 days: naïve N=2, n=2; sham N=4, n=2; CCI N=1, n=2. 21 days: naïve N=4, n=2; sham N=4, n=2; CCI N=4, n=2. Two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus sham, mean with SEM.

2.3.3 Expression of phosphorylated YAP/TAZ increases after a nerve injury in spinal cord whereas nuclear TEAD stays stable

Additionally, phosphorylated YAP/TAZ and TEAD were studied with same naïve, sham and CCI spinal cord protein samples. Antibody against phosphorylated YAP (70 kDa) detected also phosphorylated TAZ (50 kDa). The protein levels of TEAD were studied only in nuclear samples because TEAD was beforehand confirmed to locate mainly in the nucleus. The amount of protein loaded was again 20 μg/ml, and β-actin (45 kDa) was used as a loading control. They were imaged with fluorescence as well.

After 6 days, the expressions of phosphorylated YAP/TAZ were not changed in the cytoplasm (p=0.9226, p=0.7943) when comparing CCI and sham groups. However, p-YAP levels were slightly higher in sham group when compared to naïve (p=0.0389). There were no results of p-TAZ in naïve animals. Instead, the expressions of phosphorylated YAP/TAZ were increased in the cytoplasm after 21 days from CCI operation (p<0.0001, p<0.0001) when compared to sham. After 21 days, a slight difference was shown in cytoplasmic p-YAP and p-TAZ between sham and naïve groups too (p=0.0026, p=0.0252). In the nucleus, the levels of p-YAP and p-TAZ appeared to be low. (Figure 9)

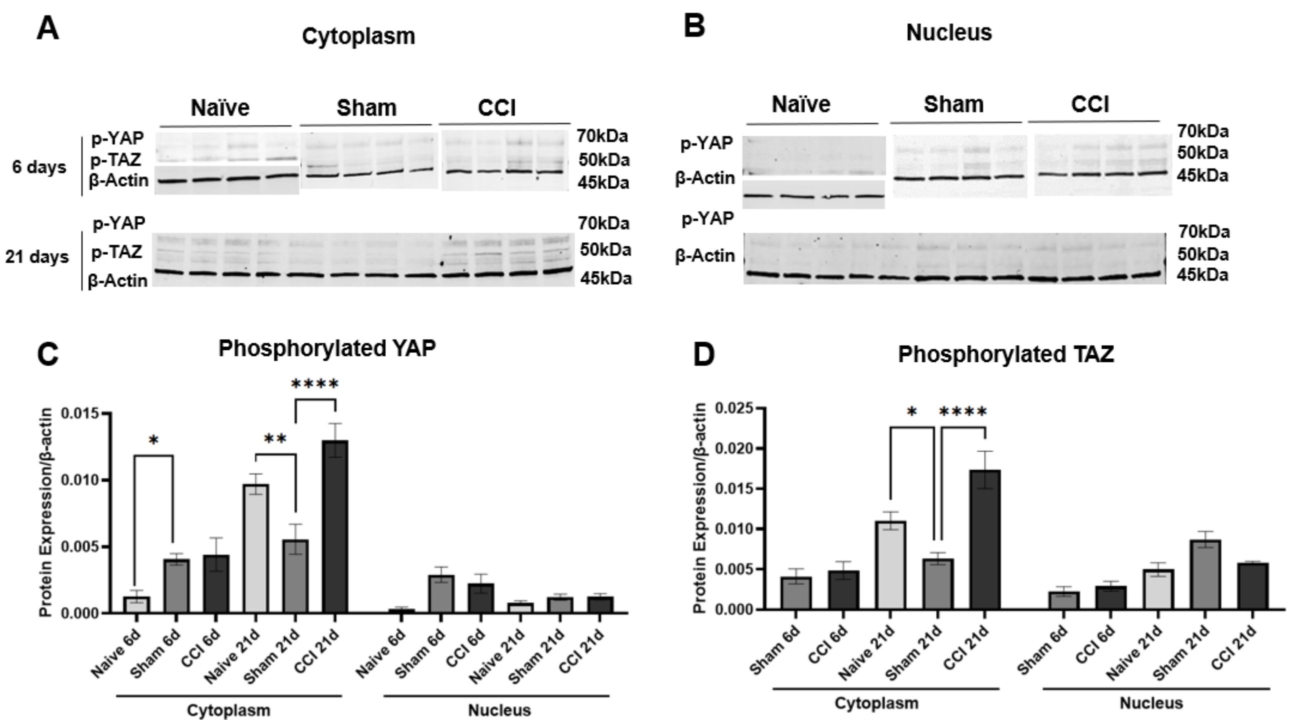

Figure 9. Phosphorylated YAP/TAZ were overexpressed in the cytoplasm after CCI. A. Western blot image of cytoplasmic expression of phosphorylated YAP/TAZ 6 and 21 days after CCI. B. Western blot image showing nuclear expressions of phosphorylated YAP/TAZ. C. The level of phosphorylated YAP increases after 21 days from CCI. Four animals (N=4) in each group. 6 days: naïve n=4, n=4; sham n=4, n=4; CCI n=5, n=5. 21 days: naïve n=4, n=4; sham n=4, n=4; CCI n=4, n=4. Two-way ANOVA. 6 days: naïve p=0.0389, p=0.0579; CCI p=0.9226, p=07547. 21 days: naïve p=0.0026, p=0.8918; CCI p<0.0001, p=0.9993. *p<0.05, **p<0.01,

***p<0.001, ****p<0.0001 cytoplasmic/nuclear versus sham, mean with SEM. D. Phosphorylated TAZ is overexpressed after 21 days from CCI. Four animals (N=4) in each group. 6 days: sham n=4, n=4; CCI n=4, n=4. 21 days: naïve n=4, n=4; sham n=4, n=4; CCI n=4, n=4. Two-way ANOVA. 6 days: CCI p=0.7943, p=0.8239. 21 days: naïve p=0.0252, p=0.0798; CCI p<0.0001, p=0.1944. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 cytoplasmic/nuclear versus sham, mean with SEM.

During characterization of cytoplasmic and nuclear spinal cord samples, TEAD transcription factor (50 kDa) was ensured to be only in the nucleus. To verify that TEAD is independent of chronic pain, its expression levels at naïve, sham and CCI samples were compared. There were no significant differences between these groups. (Figure 10)

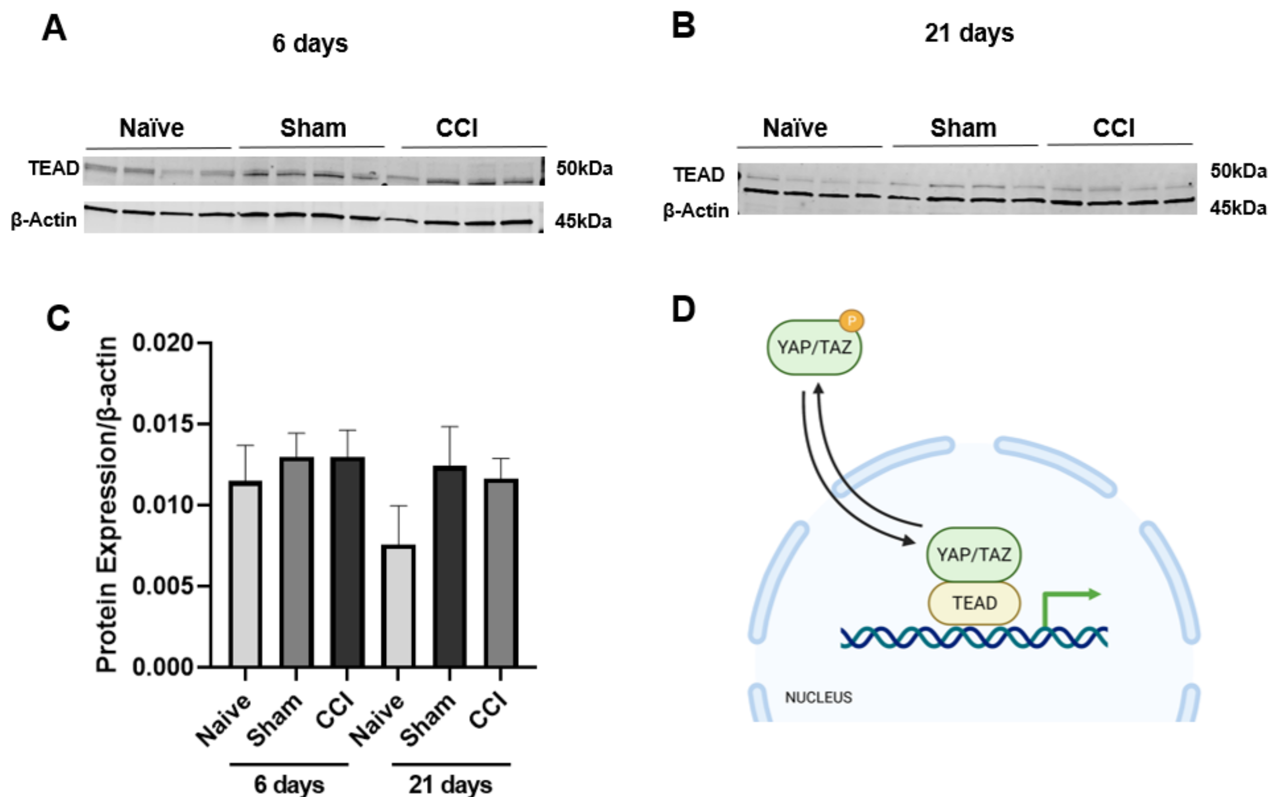

Figure 10. TEAD expression in the nucleus of naïve, sham and CCI spinal cord samples. A–B. TEAD expression do not change due to CCI. C. Four animals (N=4) in each group. One-way ANOVA 6 days: sham/CCI p>0.9999; sham/naïve p=0.7871. 21 days: sham/CCI p=0.9511; sham/naïve p=0.2247. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus sham, mean with SEM. D. Illustration of unphosphorylated YAP/TAZ binding to transcription factor TEAD in the nucleus.

2.4 YAP, TAZ and β-catenin are expressed in human spinal cord

To ensure that YAP, TAZ and β-catenin are expressed in human spinal cord too, fluorescent Western blot and immunohistochemistry were conducted with healthy human spinal cord samples. Additionally, the expression of β-catenin and phosphorylated YAP/TAZ were checked in human SW620 colorectal carcinoma cell line. It was used a positive control because it has been reported to overexpresses YAP/TAZ.

Western blot results showed that YAP/TAZ, phosphorylated YAP/TAZ and transcription factor TEAD were expressed in healthy human spinal cord. (Figure 11A, C and D) The protein expression of β-catenin was studied too but the results were uncertain because there was no clear band at 92 kDa. (Figure 11B) The protein levels were not normalized with any control nor quantitative analyses were not performed because the aim was only to see if these proteins are present in human too. Therefore, the results are only qualitative.

Furthermore, human spinal cord was compared to CCI rat cytoplasmic spinal cord. When comparing the appearance of YAP in healthy human spinal cord and in CCI rat spinal cord, it was noticed that YAP had a higher molecular weight in human sample. The same was seen with TAZ. (Figure 11A) The molecular weight of β-catenin was similar in CCI rat spinal cord and in cancer cells. (Figure 11B) Phosphorylated YAP/TAZ had also a higher molecular weight in human than in CCI rat spinal cord and in colorectal cancer as well. (Figure 11C) Whereas molecular weight of TEAD was similar in human and rat spinal cord. (Figure 11D)

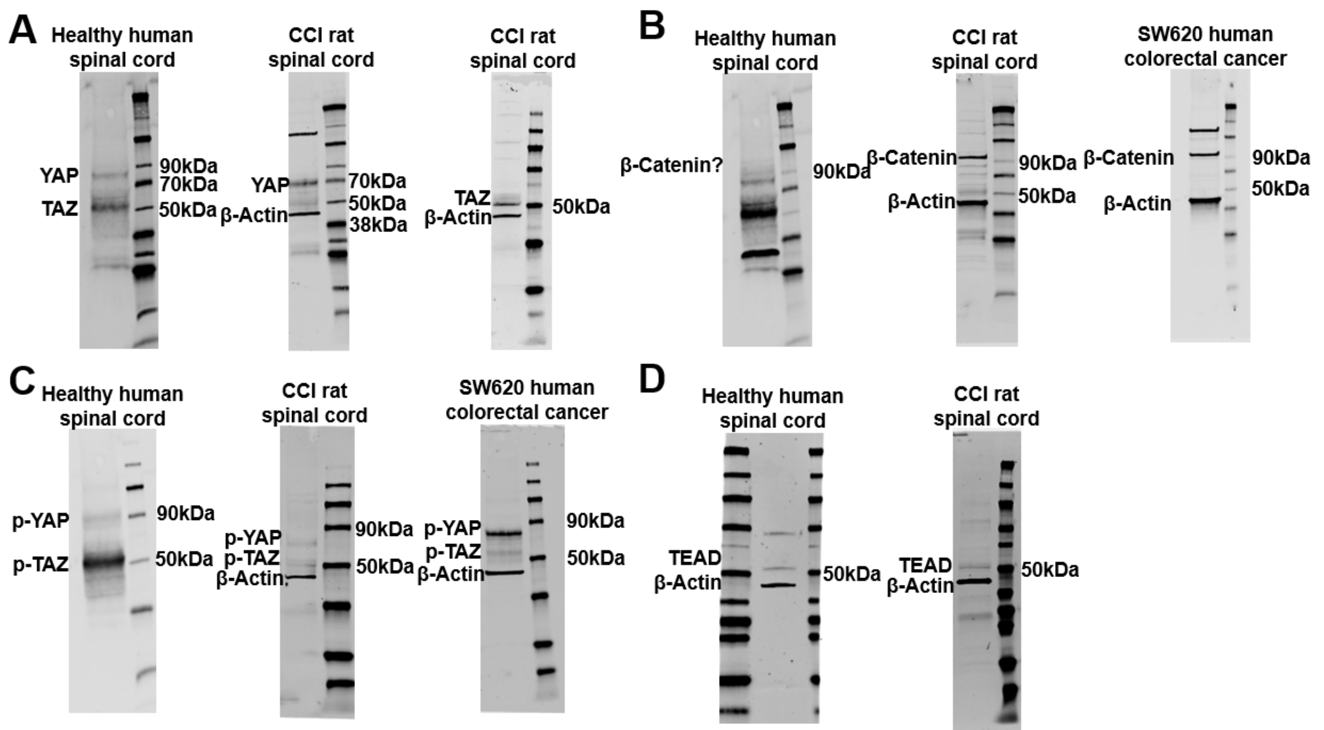

Figure 11. Expression of YAP, β-catenin, phosphorylated YAP/TAZ and TEAD in healthy human spinal cord, CCI rat spinal cord and SW620 human colorectal cancer. A–D. The protein expressions were studied with fluorescent Western blot. A. YAP and TAZ had a higher molecular weight in healthy human spinal cord than in CCI rat spinal cord. B. β-catenin expressed similarly in CCI and colorectal cancer. C. Phosphorylated YAP/TAZ had a higher molecular weight in healthy human spinal cord than in CCI rat spinal cord and colorectal cancer. D. TEAD seemed to have a similar molecular weight in both healthy human spinal cord and CCI rat spinal cord.

Furthermore, YAP was proved to localize in humans' healthy spinal cord lumbar area when imaging paraffin sections with fluorescence. (Figure 12) Black and white picture resembles the typical "butterfly" shape of spinal cord. Within the human spinal cord, YAP was mostly located in the cytoplasm of neurons. In the dorsal area, there were a lot of neurons with larger cell bodies. These motoneurons were prominent stained against YAP, and they are marked with red arrows in Figure 12.

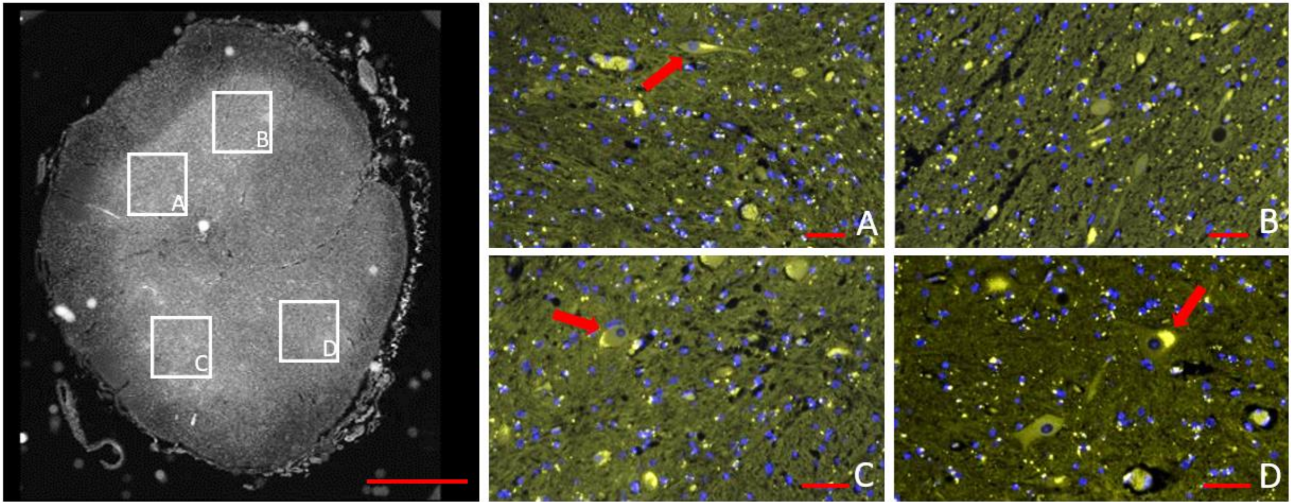

Figure 12. Localization of YAP and DAPI in healthy human spinal cord paraffin sections. A, C. YAP is accumulated in the cytoplasm in the ventral horns B, D. YAP is located in the cytoplasm in the dorsal horns. A–D. Red arrows point motoneurons that express YAP in the cytoplasm. Scale bar, 25 μm.

3 Discussion

3.1 In physiological conditions, YAP, TAZ and β-catenin are accumulated in cytoplasm

Neuropathic pain mechanisms remain still unclear. During the last years, evidence for changes in expression and transcription factor activity have been accumulated. The aim of our research was to study the role of three transcriptional regulators downstream of the Hippo pathway. The study was started with comparing cytoplasmic and nuclear spinal cord samples of naïve rats to understand transcriptional mechanisms in normal control individuals. In addition, it was studied how sham surgery affects to the protein expression of YAP/TAZ and β-catenin.

The Western blot results of spinal cord samples confirmed the assumptions that YAP/TAZ and β-catenin are mostly located in the cytoplasm when animals are not suffering from chronic pain. Xu et al. paper suggested earlier the same. However, we found relatively small amounts of each of these proteins in the nucleus too even in naïve animals. It could be explained by limitations in protein purification. For instance, some of the cytoplasmic proteins could have been mixed into nuclear proteins if cytoplasmic supernatant was not properly removed. However, it can be possible that minor nuclear shuttle of YAP/TAZ and β-catenin happens in control conditions too. Anyway, it was clear that proteins were mostly located in the cytoplasm.

The inactive forms of YAP/TAZ were expected to localize only in the cytoplasm in naïve animals because only unphosphorylated YAP/TAZ tend to enter the nucleus. Although most of the phosphorylated YAP/TAZ were expressed in the cytoplasm, there were some in the nucleus too. According to Wada et al., phosphorylated YAP/TAZ can shuttle into the nucleus, but it is unclear whether they bind to transcription factor TEAD and cause gene activation.

The difference between naïve and sham controls groups was checked to ensure that the surgery itself had no effect on the nuclear shuttle of proteins. In case of the spinal cord, the protein expression of β-catenin in naïve and sham rats were similar. However, in the DRG β-catenin was expressed differently in naïve and sham groups. As regards YAP, its protein levels were increased in nuclear spinal cord sham samples when compared to naïve. Nevertheless, YAP was expressed similarly in naïve and sham groups in DRG samples. Yet, TAZ protein levels were similar in naïve and sham groups in both spinal cord and DRG samples.

The reason for increased protein expression in sham animals can be cleared. The Hippo pathway reacts quite easily to a mechanical stress that the surgery itself could cause to the body (Piccolo et al., 2014). This could lead to an overexpression of some proteins. Therefore, it is vital to compare primarily CCI animals with sham animals and not naïve. Earlier studies have noticed similarly that sham surgery can affect protein expressions. For instance, sham surgeries can cause pain-like changes because of the enormous tissue damage. (Odem et al., 2019) However, some studies have claimed that especially β-catenin is expressed similarly in both naïve and sham groups in the spinal cord (Zhang et al., 2013). Most studies concerning YAP, TAZ and β-catenin, like Li et al., have not made the comparison between naïve and sham animals at all. Finally, we can say that sham surgery causes minor protein changes in individuals but when there is enough animals and repetitions it will not affect greatly on the results.

3.2 Sciatic nerve injury causes overexpression of YAP and TAZ in spinal cord

CCI surgery caused alterations in the protein expression levels in the spinal cord. The level of YAP and TAZ increased after CCI in the spinal cord as assumed beforehand. The increase was more significant in the cytoplasm than in the nucleus. According to earlier studies, these transcriptional regulators should shuttle into the nucleus after a nerve injury to activate proliferative genes. Therefore, it was assumed that the protein level increase would have been shown in the nucleus too. In our experiments, this result was not repeated. However, it was shown that there is overexpression of YAP and TAZ at least in the cytoplasm due to CCI surgery when comparing to sham surgery. Instead, similar changes in β-catenin protein levels were not noticed.

The increase in YAP expression levels in the cytoplasm was noticed after 21 days from CCI. Instead, YAP nuclear expression levels maintained the same throughout the study and did not deviate from sham as mentioned. In Xu et al. paper they compared only the nuclear expression of YAP in sham and CCI. In that study, YAP increased in the nucleus already after 1 day, peaked after 7 days and was already decreased after 14 days. The YAP expression levels were already back to control after 21 days. Instead in Li et al. study, YAP had its concentration peak in the spinal cord 14 days after CCI surgery. Hence, protein levels can alter very differently depending on individuals and studies.

TAZ protein levels changed slightly differently compared to YAP's. TAZ levels peaked already after 6 days and noticeably decreased after 21 days from CCI. Whereas in Xu et al. paper, TAZ shown to maintain its expression level similar throughout the study and was only slightly decreased after 21 days (Xu et al., 2016). Nevertheless, they had different timepoints which could explain some of the variations in results. However, another study had similar results that TAZ peaks in the early phase

after CCI surgery (Li et al., 2013). A reason for an early decrease in TAZ expression levels could be explained by its degradation. As said earlier, TAZ is degraded easily in the cytoplasm because of its two phosphodegrons. The degradation could also happen during the tissue isolation. This could falsify the actual level of TAZ in tissue and protein samples. Maybe for the same reason, we did not see the nuclear shuttle of TAZ. Nonetheless, it was proved that TAZ is overexpressed in the cytoplasm in the spinal cord due to CCI.

As mentioned, the increases in protein levels of YAP and TAZ varied from each other. This was expected because the same has been noticed earlier (Xu et al., 2016). Although YAP and TAZ are structurally quite similar and they are activated through the same pathway, they seem to have different roles during the formation of neuropathic pain. YAP and TAZ have noticed to have distinct roles during cancer progression too. For instance, the expression of YAP/TAZ has depended on the cancer cell type. (Piccolo et al., 2014) Furthermore, YAP is regulated mainly by phosphorylation whereas TAZ is regulated by degradation. As said, degradation is far harder to detect reliably so it could explain the variation between YAP and TAZ too.

Eventually, Western blot was utilized in comparing spinal cords of healthy human and CCI rat. Although YAP and TAZ had a higher molecular weight in human sample than in rat, the results proved that they are expressed in both species. This supports the translation of this study's results to humans. To study further the role and location of YAP in the spinal cord, immunohistochemistry was performed with human healthy spinal cord paraffin sections. The spinal cord was shown to have the "butterfly" shape where the dorsal horn was also pointed. In these physiological conditions, YAP was mostly located in the cytoplasm of neurons which goes together with assumptions. To prove that a peripheral nerve injury causes the shuttle of YAP into the nucleus, disease model should use in further studies. As the immunohistochemistry was done only to visualize the location of YAP, only DAPI was used as a nuclear marker. To verify where exactly YAP is localized within the cells and the whole spinal cord, another marker should be used in the future. YAP localization was studied on CCI and sham rats too but due to practical issues the images were not successful.

Unexpectedly, β-catenin protein levels did not elevate after CCI surgery neither in the cytoplasm nor in the nucleus. Although a change in β-catenin levels between 6 and 21 days after CCI could be seen. However, β-catenin levels changed similarly in sham and naïve animal groups resulting having no statistical differences between CCI and control groups. Earlier studies have stated that β-catenin have an important role in pain formation in the spinal cord through the Wnt signaling pathway (Zhang et al., 2013; Xu et al., 2016). More specific, an excess of Wnt and Fz receptors increased the amount of

both dephosphorylated and phosphorylated β-catenin (Zhang et al., 2013). In both studies, β-catenin had higher expression level the longer it had been from CCI surgery. However, they did not study β-catenin expression in the nucleus after 21 days. β-catenin has also several other roles within the cells which are unrelated to pain formation. This could affect the expression levels of β-catenin and clarify the results of this study. β-catenin can for instance bind to multiple different nuclear factors which promote several target genes (Komiya and Habas, 2008). Furthermore, we showed that β-catenin is expressed in both colorectal cancer and in cytoplasmic CCI spinal cord. Meaning that β-catenin could have similar roles in pain than it does in cancer. To prove that β-catenin really has a role in pain chronification after a peripheral nerve injury, this study should be repeated.

3.3 Phosphorylated YAP/TAZ can shuttle into the nucleus in spinal cord after CCI

The peripheral nerve injury caused an increase in both phosphorylated YAP and phosphorylated TAZ in the cytoplasm after 21 days. Although there were statistically significant differences between naïve and sham animal groups as well. In advance, it was assumed that a peripheral nerve injury decreases the amount of phosphorylated YAP/TAZ in the spinal cord because Hippo pathway is no longer active. In addition, some changes in expression were seen in the nucleus too. Although it was stated earlier that phosphorylated YAP/TAZ should degrade or bind to the destruction complex in the cytoplasm, small amounts can apparently shuttle into the nucleus based on the Western blot results. According to Wada et al. phosphorylated YAP/TAZ can shuttle into the nucleus, but it is unclear whether they can bind to transcription factor TEAD or not. (Wada et al., 2011).

Earlier neuropathic studies have not concentrated on studying the expression on phosphorylated YAP/TAZ after CCI. When studying them, some considerations should be made. Phosphorylated proteins are usually hard to detect because they are degraded easily, especially phosphorylated TAZ. The hydrolysis causes variation to the results, but this was expected beforehand. Even though phosphatase inhibitor was used in the study, some phosphatase enzymes might have cleaved phosphate groups from YAP and TAZ. In addition, the antibody against phosphorylated YAP could have detected also active YAP because their molecular weight does not differ much. This could have distorted the results greatly. Nonetheless, the analyses of phosphorylated YAP/TAZ should be continued to clear their mechanisms.

Finally, the expression of TEAD transcription factor was assessed in the spinal cord too. Western blot results showed that it is expressed only in the nucleus and not in the cytoplasm. According to earlier studies TEAD factors should localize only in the nucleus but in rare cases they can shuttle into the cytoplasm due to stress. (Lin et al., 2017) In advance, its expression was assumed to stay stable in the

nucleus regardless of chronic pain. The results proved this assumption to be right because no difference between naïve, sham and CCI was detected. So, a nerve injury causes only variation in YAP and TAZ expressions which then translocate into the nucleus to bind with TEAD. Lastly, no differences in molecular weight were noticed when spinal cords of healthy human and CCI rat were compared. This finding supports the assumption that TEAD expression does not change due to chronic pain.

3.4 Peripheral nerve injury increases the expression of β-catenin, YAP and TAZ in dorsal root ganglia

Along with spinal cord, a peripheral nerve injury changed the protein expression levels of YAP and TAZ in the DRG too. Depart from the spinal cord, β-catenin was overexpressed in the DRG after CCI. The DRG tissue samples were not purified into separate cytoplasmic and nuclear samples. Instead, all proteins were purified together. Therefore, cytoplasmic and nuclear expression levels cannot be separately compared like spinal cord samples. However, the Western blot results showed that all three transcription regulators were overexpressed due to CCI in the DRG.

YAP expression levels increased already in the beginning and continued increasing throughout the study. The expression of TAZ changed similarly. Instead in the spinal cord, the expression levels of YAP and TAZ changed differently when compared to each other. Earlier studies have noticed that YAP and TAZ stay mainly in the cytoplasm in the DRG and not accumulate in the nucleus due to CCI (Xu et al., 2016). Our study concentrated only on the total expression changes of YAP and TAZ in the DRG. Xu et al. did not study that so comparing the results is challenging. However, they noticed that YAP and TAZ do not shuttle into the nucleus in the DRG and concluded that peripheral injury causes changes only in the spinal cord. Again, the results vary.

We showed that β-catenin is overexpressed in the DRG after CCI surgery. Other studies have come up with different outcomes. Zhang et al. study stated that pain hypersensitivity in the DRG neurons is not dependent on β-catenin-dependent Wnt signaling. Simonetti et al. study had the same conclusion. However as mentioned earlier, there are only a few studies available which have concentrated on the role of YAP, TAZ and β-catenin during neuropathic pain. Especially DRG is not well studied on this field and hence results can vary. According to these results, we believe that β-catenin has a role in the formation of neuropathic pain in the periphery too.

The study of DRG concentrated only on YAP, TAZ and β-catenin. Therefore, phosphorylated YAP/TAZ were not studied. YAP and TAZ expression values were already quite low, and probably

these inactive forms would be even less expressed. Hence, the results of phosphorylated YAP/TAZ might have been unreliable.

3.5 Conclusions and study limitations

As mentioned earlier, there is only few studies that have been clarifying the role of YAP, TAZ and β-catenin in the chronic pain formation. Therefore, the results between different studies can vary considerably. This could also hamper the reading of results. Some differences between studies can be pointed out that could further explain the variation in results. In Xu et al. study most animals were hypersensitive already after a couple of hours whereas in our study the animals were hypersensitive only after 6 days from CCI. In general, the development of hypersensitivity differs greatly between individuals (Austin et al., 2012). Also, protein levels can vary among control animals too. For instance, in spinal cord β-catenin results sham and naïve groups from different day groups had completely dissimilar protein expression levels. Therefore, sham animals were separated based on the postoperative days. This was done only in our study. Additionally, Xu et al. study did not include naïve animals in their Western blots. We wanted to compare naïve and sham animals to clarify the effect of surgery itself on protein expression levels. In addition, Xu et al. used different housekeeping protein.

Another source to the variation of results can arise from the isolated tissue samples. First, the number of animal tissues was limited because of their high costs which straightly affected the reliability of results. As known, the size of spinal cord and DRG is small, but the operation was conducted by professionals, so the isolation hardly impacted on the results. Beforehand it has been shown that transcriptional changes in neuropathic pain happen in the dorsal horn of spinal cord (Xu et al., 2016). However, study's tissue samples included the whole spinal cord area because it would have been impossible to isolate only the dorsal horn part. This could have distorted the results because dorsal horn represents around 25% of the whole spinal cord area. Hence the minor changes in the dorsal horn were harder to detect.

The purification and separation of samples into cytoplasmic and nuclear proteins requires a lot of accuracy. Besides, the nuclear proteins are harder to purify than cytoplasmic proteins because there is less protein in the nucleus when compared to the amount in the cytoplasm (Goldstein and Ko, 1981). This could explain why the protein levels changes were hard to detect in the nucleus. Moreover, frozen tissue samples were used instead of fresh ones which was against the recommendations told by the NE-PER protocol. Nevertheless, we still succeeded to get reasonable results which allowed us to make conclusions.

Furthermore, the main study method Western blot contains multiple steps and manual work where errors can happen. Anyway, Western blot have maximum of 30% variation of samples, and therefore the results were expected to vary from earlier studies' results. Because the concentrations of protein samples were quite low, not many repetitions could be done with same sample. Hence, membranes were reblotted with different primary antibodies to increase the number of repetitions and reliability. Also, when fluorescence signals were analyzed with Imaging System, different sizes of boxes were used in marking the bands. Finally, the study was conducted as a thesis project which caused some limitations. Most significantly, there were a specific time limit, and therefore not all repetitions were done.

Nonetheless, with the help of these results, further studies can be easily conducted. First, the Hippo pathway's kinases that phosphorylate YAP and TAZ could be studied. Hence, these results could be compared with the results of Xu et al. study. Furthermore, by adding more time points to the study, the protein level changes of YAP, TAZ and β-catenin could be analyzed in more detail. It would be reasonable to study also the inhibition of YAP, TAZ and β-catenin. Moreover, immunohistochemistry could be performed with rats' spinal cord and DRG tissue samples. Then, immunohistochemistry results could be compared more easily with Western blot results. They were tested in this study as well but due to time limit and technical issues they were not properly conducted. Finally, different techniques could be used as well like qPCR and immunocytochemistry with DRG cells. With them, the current results could be confirmed.

As a conclusion, this research indicates that a peripheral nerve injury causes an overexpression of YAP, TAZ and β-catenin in the peripheral and central nervous system strongly suggesting the involvement of the Hippo signaling pathway in chronic pain. Secondly, the comparative analysis between spinal cord and DRG suggests that pain formation starts in the periphery and proceeds into the central nervous system because the protein expression changes are noticed in the DRG earlier than in the spinal cord. Meaning that both peripheral and central drugs could have an impact on treating neuropathic pain. It was also proved that the Hippo pathway components are expressed in humans' spinal cord too. Therefore, the results could be translated to humans. In the future, these transcriptional mechanisms should be further studied to develop more specific analgesics.

4 Materials and methods

4.1 Animals

Wistar Han juvenile male rats (160–180 g) from Charles River Laboratories (Germany) were used in the study. All protocols were approved by the Regional State Administrative Agency for Southern Finland.

4.2 Chronic constriction injury of the sciatic nerve

Briefly to introduce chronic constriction injury (CCI) animal model, rats' sciatic nerve, proximal to the sciatic nerve trifurcation, was loosely tied. Sham animals received similar surgery than CCI rats without the actual nerve ligation. CCI animals are usually most hypersensitive 2–3 weeks after the procedure but it varies among individuals, and they recover from the operation eventually. To ensure that the rats had developed a hypersensitivity after CCI surgery, mechanical allodynia was tested at days -5, 3, 6, 14 and 21 *in vivo* before tissue isolation. To this end, the up-down von Frey test was employed where plastic filaments were applied to ipsilateral hind paw and reaction was detected. These operations were conducted prior to this thesis project by Orion's *in vivo* site. Therefore, they are not properly included in the thesis itself.

The spinal cord and DRG tissue samples (10–60 mg) were isolated at L4–L6 spinal segments from naïve, sham and CCI rats 6 and 21 days after operation.

4.3 Protein purification

While setting up the protocol for spinal cord and DRG tissue purification, different methods were tried. To isolate cytoplasmic and nuclear samples separately, NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific #78833) was used for spinal cord tissue samples. They were purified according to the manufacturer's instructions.

In the case of DRG tissue samples, NE-PER purification produced too low protein concentrations. Hence N-PER Neuronal Protein Extraction Reagent (Thermo Scientific #87792) was used for their purification which ended up having cytoplasmic and nuclear proteins in same sample. DRG samples were first weighted without any liquid inside 2 ml tubes. N-PER lysis buffer (10 ml to 1 g of tissue) was added prior to homogenization with IKA T25 Basic Ultra-Turrax. Afterwards, homogenized DRG samples were incubated 10 minutes on ice and centrifuged at 16 000 x g for 10 minutes at +4 °C. Finally, the supernatants were moved into micronic tubes and stored in -80 °C.

To inhibit protease and phosphatase activity in final samples, protease and phosphatase inhibitor cocktails were used along with NE-PER kit's reagents or N-PER buffer. One Protease inhibitor tablet (Roche #11873580001) was dissolved in 2 ml milli-Q water (4 µl for 100 µl final buffer). One Phosphatase inhibitor tablet (PhosSTOP, Roche #04906837001) was diluted in 1 ml ice-cold RIPA lysis buffer to get 10x stock solution (10 µl for 100 µl final buffer).

The protein concentrations of samples were analyzed using BCA Protein Assay Kit (Pierce™, Thermo Scientific #23235). The concentrations were measured with Enspire Multimode Plate Reader (PerkinElmer) using Bradford96 562 nm program.

4.4 Western blot

Western blot analyses were performed using enhanced chemiluminescence (ECL) and fluorescence techniques. The amount of protein in samples was equalized. The samples were prepared on ice adding loading buffer (4x Laemmli Sample Buffer, Bio-Rad #161-0747) or (4x Protein sample Loading Buffer, LI-COR #928-40004) and an appropriate amount of N-PER lysis buffer (Thermo Scientific #87792). The samples were boiled at +95 °C and centrifuged 10 000 x g for 10 seconds.

The protein samples were separated with 4–15% Criterion™ TGX Stain-Free™ Protein Gel (12+2 well, 45 µl, Bio-Rad #5678083). The chambers were filled with 1x Tris/Glysine/SDS buffer (Bio-Rad #161-0772). Wells were rinsed with the same buffer before adding the samples. Depending on the imaging technique, chemiluminescence (Precision Plus Protein™ WesternC™ Blotting standards, Bio-Rad #1610376) or fluorescence marker (Chameleon Duo Prestained Protein Ladder, LI-COR #928-60000) was added too. The gel was run 80 V for 15 minutes and 120 V for 70–80 minutes until the marker was at the bottom of the gel cassette.

Afterwards, the gel was transferred to Trans-Blot Turbo Midi Nitrocellulose blotting membrane (Bio-Rad #1704159). The blotting cassette was run with Trans-Blot Turbo Mixed MW>150 program for 7 minutes. After the transfer, the membrane was blocked using either 5% milk (Blotting Grade Blocker, Bio-Rad #1706404) 0.1% Tween-20 in 1x TBS (Pierce 20x TBS Tween 20 Buffer, Thermo Scientific #28360) or Odyssey Blocking Buffer (LI-COR #927-60001) 0.1% Tween-20 for 1 hour at RT on a continuous agitation.

After removing the blocking buffer, appropriate primary antibodies were added to membranes which were then incubated overnight at +4 °C on a continuous agitation. The primary antibodies that were used are listed in Table 1. On the next day, membranes were rinsed and washed with 1x TBST (Tris Buffered Saline, Thermo Scientific #28360) for 3x 5 minutes on a continuous agitation. Either Goat

anti-mouse IgG-HRP (1:3000, Bio-Rad #1706516) or Goat anti-rabbit IgG-HRP (1:3000, Bio-Rad #1706515) secondary antibodies were added to chemiluminescence membranes together with Precision Protein StrepTactin-HRP Conjugate (1:10 000, Bio-Rad #161-0381). Instead for fluorescent membranes, Goat anti-mouse 800CW (1:10 000, LI-COR #925-32210) or Goat anti-rabbit 680RD (1:10 000, LI-COR #925-68071) secondary antibodies were added. Secondary antibodies were incubated 1–2 hours at RT on a continuous agitation. Afterwards, membranes were rinsed and washed with 1x TBST for 3x 5 minutes on a continuous agitation before imaging. Chemiluminescence membranes were imaged with Azure c400 (Azure Biosystems) and fluorescence membranes with Odyssey DLx Imaging System (LI-COR).

For chemiluminescence membranes, antibodies were diluted in 5% milk (Blotting Grade Blocker, Bio-Rad #1706404) 0.1% Tween-20 in 1x TBS (Thermo Scientific #28360) and for fluorescence in Odyssey Blocking Buffer (LI-COR #927-60001) 0.1% Tween-20.

Table 1. Primary antibodies and their dilutions used in Western blot.

Antibody	**Final dilution**	**Supplier**
Monoclonal Anti-YAP	1:1000	Cell Signaling Technology #12395
Polyclonal Anti-TAZ	1:1000	Cell Signaling Technology #4883
Monoclonal Anti-p-YAP	1:1000	Cell Signaling Technology #13008
Monoclonal Anti-β-catenin	1:2000	Sigma-Aldrich #05-665
Monoclonal Anti-pan-TEAD	1:1000	Cell Signaling Technology #13295
Monoclonal Anti-DCC	1:1000	Enzo Life Sciences #ALX-804-853
Monoclonal Anti-β-Actin	1:1000	Cell Signaling Technology #3700
Polyclonal Anti-β-Actin	1:500	Sigma-Aldrich #A2066

Finding a relevant housekeeping antibody for protein of interest was challenging because proteins are rarely evenly distributed into cytoplasm and nucleus. Eventually, anti-β-actin antibody was decided to be used as a housekeeping and loading control for other antibodies because it manifests as a clear band in both cytoplasmic and nuclear protein samples. Other considered controls were GAPDH, TOM20 and Lamin B. They were tested with naïve samples but ruled out because they did not express similarly in cytoplasmic and nuclear samples.

4.5 Immunohistochemistry

Healthy human adult spinal cord tissue slides were purchased from Novus Biologicals (#NBP2-77800). The sections were deparaffinized and rehydrated by washing them with Xylene (2x 7.5 min),

100% Ethanol (2x 10 min), 94% Ethanol (2x 5 min), 70% Ethanol (2x 5 min) and Deionized water (1x 30 s). Simultaneously, citrate buffer (pH=6, 10x, Antigen Retriever, Sigma-Aldrich #9999) was pre-warmed for 20 minutes in a hot air cooker. After the washings, the sections were heated in the hot air cooker for 20 minutes. Then the tissues were cooled in citrate buffer for 20 minutes.

The tissue sections were washed with 1x PBST (Pierce™ 20x PBS Tween™ 20 Buffer, Thermo Scientific #28352) for 3x 10 minutes. Then the liquid was removed, and the sections were circled with PAP-pen. The sections were incubated with 10% BSA goat serum (Bovine Serum Albumin, Sigma Life Sciences #A9647) in 1x PBST for 30 minutes at RT. After the incubation, solution was removed from the sections and primary antibody diluted in 1% BSA goat serum in 1x PBST was added. The tissue sections were incubated overnight at +4 °C. The primary antibody used was anti-YAP (1:100, Cell Signaling Technology #12395).

On the next day, primary antibody solution was removed from the sections. They were afterwards washed in 1x PBST for 3x 5 minutes. The AlexaFluor conjugated secondary antibody (1:500, Donkey anti-Mouse IgG Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen, Thermo Scientific #A21202) diluted in 1x PBS was added and incubated for 1 hour at RT in a humified chamber.

The sections were rinsed in 1x PBS once. The DAPI solution (1:10 000, Roche #10236276001) in 1x PBS was added and incubated for 10 minutes at RT. Finally, the tissue sections were washed with 1x PBS for 3x 5 minutes. For mounting the slides, Prolong™ Glass Antifade Mountant (Thermo Scientific #P36980) was used. The slides were let dry for 24 hours protected from light prior to imaging. Then they were imaged with Olympus VS200 digital slide scanner.

4.6 Statistical analyses

The Western blot fluorescence signals were analyzed with help of Image Studio™ software. The signals were normalized with loading control and within the same blot to ensure a reliable comparison of protein samples. The calculations were performed using Excel (Version 2102), and from there the equalized protein values were transferred to GraphPad Prism 9 which was used to create graphs and perform statistical analyses. The statistical tests were conducted by comparing naïve or CCI values to sham values from same day group (6 or 21 days after surgery). The differences were analyzed using two-way or one-way ANOVA. When probability (p) values were less than 0.05, differences were considered statistically significant. All data is presented mean with SEM.

In immunohistochemistry, negative controls were incubated only with 1x PBS and DAPI solution. The data analysis was only quantitative, and hence no statistical tests were conducted. Hence, the images of tissue sections were only visually compared to each other.

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6 List of abbreviations

CCI	chronic constriction injury
DRG	dorsal root ganglia
Fz	Frizzled
hDRG	human dorsal root ganglia
LATS1/2	large tumor suppressor kinases 1/2
LEF	lymphoid enhancer-binding factor
MST1/2	mammalian Ste20-like kinases 1/2
p-TAZ	phosphorylated WW-domain-containing transcription regulator 1
p-YAP	phosphorylated Yes-associated protein 1
TAZ	WW domain-containing transcription regulator 1
TCF	T cell-specific factor
TEAD	Transcriptional enhancer associate domain
YAP	Yes-associated protein 1

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