CHAPTER 5

Increased N-Methyl-D-Aspartate receptor expression in sweat glands of Complex Regional Pain Syndrome type 1 patients

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Abstract
Sensory abnormalities in Complex Regional Pain Syndrome type 1 (CRPS 1) have been associated with changes in dorsal horn N-Methyl-D-Aspartate (NMDA) receptor activity resulting in central sensitization. However, little is known about changes in NMDA receptor expression in peripheral tissue for this syndrome. The goal of the present study was to evaluate peripheral NMDA receptor expression to address the contribution of peripheral sensitization in CRPS.
A qualitative immunohistochemistry analysis was performed to assess the expression of NMDAR1, phosphorylated NMDAR1 (pNMDAR1), NMDAR2B and NMDAR2D receptor subtypes in skin obtained from the dorsum of the hand and forearm of amputated upper limbs of 6 CRPS 1 patients. As controls, 4 cadavers not diagnosed with CRPS were evaluated. Furthermore, 1 CRPS 2 patient was evaluated. NMDAR1 and pNMDAR1 positive structures were found in the epidermis and external root sheath of hair follicles in both CRPS 1 and non-CRPS 1 hand tissues. In CRPS 1 hand tissue however, NMDAR1 and pNMDAR1 positive structures were also found in the epithelium of secretory units of sweat glands. Furthermore, sweat gland density appeared to be higher in CRPS 1 patients compared to controls. NMDAR2D and NMDAR2B were not detected in the hand and no positive NMDA structures were found in the forearm.
We conclude that increased expression of NMDAR1 and pNMDAR1 receptors found in sweat glands of CRPS 1 hand tissue may contribute to sensory abnormalities experienced by CRPS 1 patients. We hypothesize that these changes may be the result of pathophysiological changes occurring in CRPS 1. A possible relationship to previously received sympathetic blockades is discussed.
Introduction

Complex Regional Pain Syndrome (CRPS) is a poorly understood pain disorder of the extremities which frequently occurs after trauma and is characterized by autonomic and motor dysfunction (1). CRPS can be divided into 2 types, whereby type 2 occurs as a consequence of a lesion of a major peripheral nerve, whereas CRPS 1 occurs without detectable nerve lesions (2). Many CRPS patients experience sensory disorders such as spontaneous pain, hyperalgesia and allodynia (1;3;4). These sensory disturbances have been related to an increase in dorsal horn N-Methyl-D-Aspartate (NMDA) receptors resulting in central sensitization (5-7).

NMDA receptors have also been found in peripheral coetaneous nerves of rats (8-10), mice (11) and in human hairy skin (12;13). These receptors are reported to contribute to peripheral nociceptive transmission and sensitization (14). During inflammation and repetitive pain stimulation, the number of NMDA receptors on peripheral nerves of rats was shown to be up-regulated (15;16). Additionally, an increase in mRNA and protein for NMDA receptors was found in human inflamed tissue compared to non-infamed tissue from the same subject (17). Furthermore, the number of peripheral NMDA receptors was shown to be increased in the skin of fibromyalgia patients (12) and in tendons of patients with painful tendinopathy compared to healthy controls (18). Possibly, similar changes in peripheral NMDA receptors occur in CRPS as a consequence of underlying pathogenic mechanisms (e.g. neurogenic and non-neurogenic inflammation, ischemia (19)) resulting in increased peripheral neuronal hyperexcitability and sensory disturbances.

The NMDA receptor comprises different subtypes (NMDAR1, 2A-D and 3A-C). The NMDAR1 subtype is required for functioning of the receptor, consequently every NMDA receptor will consist of a NMDAR1 subtype in combination with NMDAR2A-D or 3A-C. NMDA subtypes 2B and 2D have most frequently been associated with pain syndromes (12;20). Furthermore, phosphorylation of the NMDA receptor was shown to increase NMDA receptor activity (21-23), and may therefore enhance (peripheral and central) sensitization.

Although sensory abnormalities in CRPS 1 have been associated with changes in central NMDA receptor activity, possible changes of NMDA receptor expression in peripheral tissue have not yet been evaluated for this syndrome. For this purpose, a qualitative immunohistochemical analysis was performed in order to assess the expression of NMDAR1, phosphorylated NMDAR1 (pNMDAR1), NMDAR2B and NMDAR2D subtypes in skin obtained from amputated upper limbs of CRPS 1 and II patients. In addition, we qualitatively compared the type and the extent of the
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NMDA subtypes expressed in skin of CRPS 1 and CRPS 2 patients with cadavers not diagnosed with CRPS.

Methods

Tissue samples
Tissue was obtained from surgically amputated upper limbs of 6 patients diagnosed with CRPS 1 at the University Medical Centre Groningen, and from 1 amputated arm of a patient diagnosed with CRPS 2 at the Pain clinic at the VU University Medical Center Amsterdam. Patients were diagnosed according to the criteria of the International Association for the Study of Pain (2) and all patients gave (written) permission to perform histological analyses of the amputated limb. Arm tissue of three human cadavers without reported history of local pain (3 women, with the ages of 81, 83 and 87, respectively) and arm tissue from 1 subject diagnosed with polyneuropathy as a consequence of diabetes mellitus type 2 (male, age 72) was obtained from corpses, donated to the Department of Anatomy of the VU University Medical Center.

Tissue preparation
From CRPS 2, non-CRPS (both approximately 1.5 by 1.5cm) and CRPS 1 patients (approximately 0.5 by 1.5cm), skin and subcutaneous tissue were obtained from the dorsum of the hand and forearm (figure 1). These tissue samples belonged to areas of the arm and forearm innervated by the superficial branch of the radial nerve and medial nerve respectively, and were post-fixed in 4% buffered formalin. After being rinsed in phosphate buffer pH 7.4 (PB) for 24 hours, the samples were embedded in 10% gelatine with 30% sucrose in PB. Subsequently, gelatine tissue blocks were fixed in 4% paraformaldehyde in PB for 24 hours. Finally, the pieces of skin were cut 40µm thick using a freezing microtome and stored in the freezer at −20°C.

Figure 1: Schematic presentation of locations of obtained skin tissues from the dorsum of the hand and forearm
Immunohistochemistry
Defrosted free-floating sections were rinsed with 50 mM Tris Buffered Saline pH 7.6 (TBS) (Merck). Endogenous tissue peroxidase activity was blocked by treating the sections with 1% hydrogen peroxide (Sigma) solution in TBS at room temperature (RT) for 15 min. After rinsing with TBS, the sections were treated over 20 min at RT with 5% normal goat serum (DAKO Cytomation, Denmark, code no. X0907) blocking solution in TBS-tx (triton X-100). Thereafter, the sections were incubated with primary antibodies for 45 hours at 4°C in a humid environment. Primary antibodies were anti-NMDAR1 (Upstate International Inc., catalogue no. 06-311), anti-pNMDAR1 (Upstate International Inc., catalogue no. 06-640), anti-NMDAR2B (Chemicon International Inc, catalogue no. AB1557P) and anti-NMDAR2D (Chemicon International Inc, catalog no. sc-1471), all diluted 1:1000. The sections were then rinsed and let to react with the secondary antibody (biotinylated goat anti-rabbit IgG, DAKO Cytomation, Denmark, code no. E0432, 1:200), for 1 hour at RT. After washing with TBS, the sites of antibody binding were visualized using the avidin-biotin peroxidase method (ABC Standard kit, Vectastain, Vector Labs, 1:200), for 1 hour at RT. After rinsing with TRIS-HCl buffer pH 7.6 the sections were incubated with 3,3'-Diaminobenzidine (Sigma) for 20 minutes. Finally, sections were mounted on slides, counterstained with Nissl-thionin stain, cover-slipped with Entellan and were observed in the light microscope. As a negative control, tissues were stained using the same immunohistochemistry procedure, with the exclusion of the primary NMDA antibody.

Results
Characteristics of CRPS patients donating tissue for this study are presented in table 1. Age at amputation ranged from 23 to 58 years. Duration of CRPS varied from 1 to 20 years. CRPS occurred after minor trauma or minor surgery in all patients. Patient 1 to 6 were diagnosed with CRPS 1. Patient 7 was diagnosed with CRPS 2, after dissection of the ulnar nerve due to a glass wound. Complaints of sensory, autonomic and motor nature were reported by these patients. Different treatment methods were attempted to reduce complaints in these patients, but without success. Unbearable pain and a dysfunctional limb interfering with ADL were the most prominent reasons for amputation.
<table>
<thead>
<tr>
<th>Pt</th>
<th>Age</th>
<th>Sex</th>
<th>Duration</th>
<th>Trauma</th>
<th>Signs and symptoms before amputation</th>
<th>Used treatments</th>
<th>Reasons for amputation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>F</td>
<td>7</td>
<td>Lifting a music instrument</td>
<td>Spontaneous pain, allodynia, skin discolouration (red), reduced range of motion</td>
<td>Various pain medications, mannitol infusions, oxygen free radical scavengers sympathetic blockade, physical therapy</td>
<td>Pain, infections, reduced range of motion, dysfunctional limb</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>F</td>
<td>6</td>
<td>Short entrapment of the hand without apparent injury</td>
<td>Spontaneous pain, allodynia, reduced skin temperature, reduced range of motion</td>
<td>Various pain medications, mannitol infusions, oxygen free radical scavengers sympathetic blockade, physical therapy</td>
<td>Pain, ulceration, dysfunctional limb</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>M</td>
<td>1</td>
<td>Amputation MCP dig III</td>
<td>Spontaneous pain, oedema, increased skin temperature, skin discoloration (red), hyperhydrosis, reduced range of motion</td>
<td>Various pain medications, mannitol infusions, physical therapy</td>
<td>Ulceration</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>F</td>
<td>19</td>
<td>Surgery</td>
<td>Spontaneous pain, allodynia, reduced skin temperature, skin discolouration (pale), reduced range of motion</td>
<td>Various pain medications, mannitol infusions, sympathetic blockade, regional blockades, physical therapy, psychological treatment</td>
<td>Pain, reduced range of motion, dysfunctional limb</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>M</td>
<td>3</td>
<td>Sprain</td>
<td>Allodynia, oedema, increased skin temperature, skin discoloration (red/cyanotic), hyperhydrosis, reduced range of motion</td>
<td>Various pain medications, oxygen free radical scavengers, regional blockade, mannitol infusions, ketanserin, carnitine, prednison, physical therapy, psychological treatment</td>
<td>Pain, contractures, dysfunctional limb</td>
</tr>
<tr>
<td>6*</td>
<td>44</td>
<td>F</td>
<td>20</td>
<td>Injection accident</td>
<td>Spontaneous pain, alodynia, oedema, both increased and reduced skin temperature, skin discoloration (red/cyanotic/pale)</td>
<td>Various pain medications, sympathectomy, physical therapy</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>M</td>
<td>2</td>
<td>Glass injury</td>
<td>Spontaneous pain, allodynia, edema, increased skin temperature, skin discoloration (red)</td>
<td>Various pain medications, oxygen free radical scavengers, magnesium infusions, physical therapy</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1: Characteristics of CRPS patients**


* In this patient, skin tissue was only obtained from dorsum of arm.
Histology of the skin
Sweat glands were more abundant in CRPS 1 compared to CRPS 2 and non-CRPS tissue. No additional histological differences were found.

NMDAR1 and pNMDAR1 were detected in both CRPS and non-CRPS hand tissue. NMDAR2D and NMDAR2B were not detected in the hand. No positive structures for NMDAR1, pNMDAR1, NMDAR2D and NMDAR2B were found in the forearm. The negative controls (with omission of the primary antibodies) showed no immunoreactivity (figure 2).

In CRPS 1 hand tissues, NMDAR1 and pNMDAR1 receptors were demonstrated in the stratum basale, stratum spinosum and stratum granulosum of the epidermis, in the external root sheath of hair follicles and in dark and clear cells (epithelium) of secretory units of sweat glands (figure 3A, B and 4A, B for NMDAR1 and pNMDAR1, respectively).

In CRPS 2 and non-CRPS tissues of the hand, NMDAR1 and pNMDAR1 positive structures were found in the stratum basale, stratum spinosum and stratum granulosum of the epidermis and in the external root sheath of hair follicles, but not in the epithelium of secretory units of sweat glands (figure 3C-H and 4C-H for NMDAR1 and pNMDAR1, respectively).

Blood erythrocytes (probably due to the presence of endogenic peroxidases), but not blood vessels (endothelium and pericytes) stained positive for all antibodies in all skin tissue samples. Furthermore, an association between NMDA positive staining and nerve structures could not be identified.

Figure 2: CRPS 1 hand tissues stained with (A) and without NMDAR1 antibody (B)
Figure 3: CRPS 1 and non-CRPS 1 hand tissues stained with NMDAR1 antibody. For CRPS 1, NMDA positive structures were found in the epidermis (arrow), hair (arrow) and sweat gland (arrow head) (A, B). In CRPS 2 (C, D), in polyneuropathy (E, F) and in a control without pain (G, H) hand tissues NMDA positive structures were only found in the epidermis (arrow) and hair (arrow), but not in sweat glands (arrow head). Scale bar is 100μm.
**Figure 4: CRPS 1 and non-CRPS 1 hand tissues stained with pNMDAR1 antibody.** For CRPS 1, NMDA positive structures were found in the epidermis (arrow), hair (arrow) and sweat gland (arrow head) (A, B). In CRPS 2 (C, D), in polyneuropathy (E, F) and in a control without pain (G, H) hand tissues NMDA positive structures were only found in the epidermis (arrow) and hair (arrow), but not in sweat glands (arrow head). Scale bar is 100μm.
Discussion

In this study NMDAR1 and pNMDAR1 positive structures were found in the epidermis and external root sheath of hair follicles in both CRPS 1 and non-CRPS 1 hand tissues. In CRPS 1 hand tissue however, NMDAR1 and pNMDAR1 positive structures were also found in the epithelium of secretory units of sweat glands. Furthermore, sweat gland density appeared to be higher in CRPS 1 patients compared to controls. Although a causal relationship cannot be made at this point, different mechanisms proposed to be involved in CRPS 1 may trigger increase of NMDA receptors in sweat glands. A common denominator for these mechanisms is that they can trigger increased release of glutamate. Pathophysiological levels of glutamate are associated with increased expression of NMDA receptors (24). As increased levels of glutamate have been established in CRPS (25), this feed-forward process may be involved in the increased NMDA receptor numbers found in our study. One such mechanism is neurogenic inflammation, a process in which neuropeptide release of nerves leads to inflammatory symptoms (26). Release of the neuropeptides Calcitonine Gene Related Peptide (CGRP) and Substance P (SP) augment NMDA receptor activation (27). Sensitization of peripheral nociceptive nerve endings due to neuropeptide release can subsequently trigger the release of glutamate. Furthermore, NMDA and SP receptors are co-localized on coetaneous axons, and peripheral SP has been shown to potentiate glutamate induced pain behavior, most likely by potentiating NMDA receptor functioning (28). Increased levels of CGRP (26) and SP (29;30) have been found in CRPS 1 patients, possibly providing a base for a cascade leading to increased glutamate release.

An additional explanation may be provided by immune mediated inflammation in CRPS 1 patients. Increased levels of pro-inflammatory cytokines IL-6 and TNF-α have been found in artificially obtained blister fluid in CRPS 1 affected compared to non-affected extremities (31;32), pointing towards a classic inflammatory process in CRPS 1. Spinal TNF-α was shown to induce glutamate release from terminals of primary afferents and spinal NMDA currents (33). As pro-inflammatory cytokines exhibit effects on central and peripheral level, peripheral cytokine release might induce local increase of NMDA receptors. Evidence supporting peripheral interaction between cytokines and NMDA receptors can be deducted from the increase of TNF-α levels after peripheral application of glutamate agonists (24). Hypoxia, as a consequence of a disturbed sympathetic function (34), endothelial dysfunction (35) or ischemia–reperfusion injury (36), has also been proposed to be involved in CRPS 1. Decreased blood flow and lowered oxygen levels may trigger the generation of reactive oxygen species (ROS). Indeed, elevated levels
of malondialdehyde (a biomarker for oxidative stress) were found in serum and saliva of CRPS 1 patients (37) and treatment with free radical scavengers has been proven beneficial in CRPS 1 (38;39). ROS are known to activate the NMDA receptor via protein phosphorylation (40). More importantly, ROS have been reported to modulate glutamate homeostasis by reducing glutamate uptake by glutamate-transporters (41). Locally elevated levels of ROS may therefore be implicated in the observed up-regulation of NMDA receptors in sweat glands. Finally, possible damage to sympathetic nerves may provide an explanation for our observations, which may be a consequence of small fiber neuropathy associated with CRPS 1 (42). Sympathetic nerves innervate sweat glands using acetylcholine and Vasoactive Intestinal Peptide (VIP) as primary mediators (43). VIP and NMDA receptors were found to be co-localized on neurons (44;45), and VIP has been shown to prevent glutamate dependent up-regulation of NMDA receptors (46). Associations between peripheral neuropathy and reduced levels of VIP around sweat glands have been reported elsewhere (47). Possibly, a decrease of VIP around sweat glands as a consequence of sympathetic nerve damage may have occurred in the present sample, resulting in an increase in sweat gland NMDA receptors. Closely related to this hypothesis, is the observation by Ramos et al. (48) who detected an increase in active sweat glands in patients following sympathectomy for the treatment of hyperhidrosis. As all but one CRPS 1 patient in our sample had received either a sympathetic blockade or sympathectomy for the treatment of pain and/or vasoconstriction, we hypothesize that sympathetic blockade may have led to increased sweat gland density as a consequence of local sympathetic suppression and a subsequent decrease in VIP. Sweat glands have been suggested to mediate cutaneous sensation independently of nociceptive fibers (49). Altogether, the increased number of sweat glands and NMDA receptors may therefore contribute to the sensory disturbances in CRPS 1. These observations are in line with findings by Finch et al (50), who evaluated the effect of topical ketamine (a NMDA receptor antagonist) in 20 CRPS 1 and 2 patients. They showed that topical ketamine was effective in reducing sensory complaints in these patients. NMDA positive structures were also found in the epidermis and external root sheath of hair follicles in both CRPS 1 and non-CRPS 1 skin tissue. In the present study no neuronal structures were observed. This is possibly due to their small detection size and because no nerve specific staining procedures were carried out. Nociceptive nerve endings have been located in all layers of the epidermis (51) and NMDA receptors have been identified on peripheral nerves in the stratum basale (13). Therefore, we expect a subgroup of the found NMDA receptors to be
associated with nerves. NMDA receptors have also been located on cell membranes of keratinocytes, where the receptor has been implicated in keratinocyte growth and differentiation (52;53). Quite likely, a part of the positive staining in the epidermis and the external root sheath of hair follicles is located on keratinocytes. Keratinocytes also express other receptors and secrete chemical substances that have been implicated in pain. Furthermore, have they been suggested to be at the forefront of skin sensory perception (54;55). Possibly, NMDA receptor initiated signal transmission in keratinocytes is, besides skin development, also involved in epidermal sensory processes. As mentioned above, glutamate levels are increased in CRPS 1 (25). The enhanced NMDA receptor function in the epidermis and external root sheath of hair follicles could therefore (directly by stimulation free nerve endings or indirectly via keratinocytes) contribute to peripheral and central sensitization processes seen in CRPS 1.

Some critical remarks have to be made regarding our findings and above mentioned hypotheses. Firstly, key constructs to above mentioned hypotheses, such as the presence of elevated levels of glutamate, cytokines and neuropeptides were never evaluated in the present CRPS 1 patient sample at time of amputation, nor for the controls used in this study.

Furthermore, no abnormal VIP levels have been found in a study of 8 CRPS patients (56). Additionally, no increase in NMDA receptors was observed in the CRPS 2 patient and control subject with polyneuropathy, in which as a consequence of dissection of the ulnar nerve and diabetes type II respectively, sympathetic fibers may also have been affected. Therefore, possible mechanisms proposed to account for observations made in the present study will remain speculative, and have to be regarded as study hypotheses for further research.

In this study only a limited number of patients and controls were used. Control skin tissues were obtained from subjects that varied from 72 to 87 years. Both a decrease in the number of NMDA receptor and sweat glands have been reported with increasing age (57;58).

Contrary to our study, Fischer et al. (59) observed NMDA receptors in sweat glands and blood vessels in skin tissue of healthy subjects. Furthermore, positive staining for NMDAR2B and NMDAR2D was found in tissues of fibromyalgia and healthy subjects by Kim et al. (12), suggesting possible sampling bias with regard to our tissues. However, these authors used skin tissues obtained from the shoulder region and since expression of NMDA subtypes may vary throughout the skin, this might in part explain the differences between both studies. Differences between the results of Fischer and coworkers and our own study may be explained by differences in
antibody dilutions used in both studies (1:1000 in our study vs. 1:500 by Fischer et al). Moreover, staining intensities for epidermis and external root sheath were equally intense in CRPS 1 as in non-CRPS 1 tissue. Additionally, for the patient with CRPS 2, being 46 years at the time of the amputation, lower sweat gland density was observed compared to the patients with CRPS 1. Although age related factors and sampling error cannot be ruled out at this point, we are convinced that other factors (e.g., related to disease mechanisms or previous intervention) provide a more likely explanation for the differences found between CRPS 1 and non-CRPS 1 tissues.

We have performed a qualitative study, therefore we cannot be absolutely certain that observed inter-individual differences in staining intensity are actually related to the number of NMDAR1 receptors. Furthermore, NMDAR1 receptors have been detected on various neuronal and non-neuronal tissues and cells (e.g. nociceptive fibers, post-sympathetic fibers, keratinocytes, T-cells (60;61)). Additional staining, more quantitative assessment and specific identification techniques (e.g., confocal laser scanning microscopy, electron microscopy) are necessary to address issues of density and specific localization more accurately.

Since the patients that donated the skin tissue are still alive we did not have the possibility to study brain and spinal cord tissue. Although indirectly an increase in peripheral activation (in this case the increase in peripheral NMDAR1 receptors) may contribute to central sensitization (62), no conclusions on a possible involvement of central sensitization can be drawn from the present investigations.

In conclusion, the increased expression of (p)NMDAR1 receptors found in sweat glands of CRPS 1 hand tissue may contribute to sensory abnormalities experienced by CRPS 1 patients. The increased sweat gland (p)NMDAR1 receptors may be related to pathophysiological changes occurring in CRPS 1, possibly influenced by previously received sympathetic blockade.

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