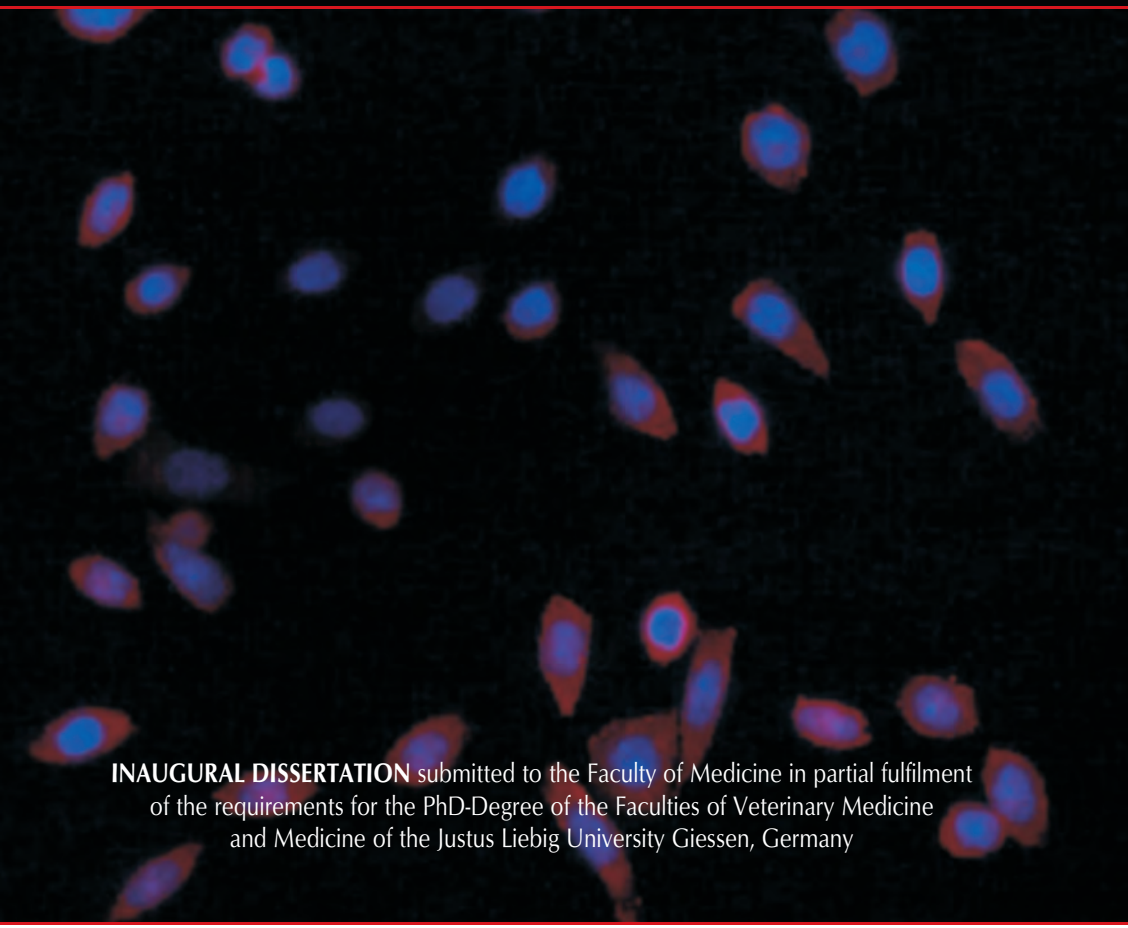
AUTOIMMUNITY IN COMPLEX REGIONAL PAIN SYNDROME

DANIELLE KOHR



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by
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Giessen 2011

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Dedicated to my loving family
Markus, Carlos and Bruno.

Declaration

I declare that the present thesis is my original work and that it has not been previously presented in this or any other university for any degree. I have also abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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LIST OF ABBREVIATIONS

nAchR	Nicotinic acetylcholine receptor
AP	Alkaline phosphatase
BCIP/NBT	5-bromo 4-chloro 3-indolyl phosphate / nitro-blue tetrazolium chloride
β1AR	Beta 1 adrenergic receptor
β2AR	Beta 2 adrenergic receptor
BMP-2	Bone morphogenic protein-2
CGRP	Calcitonin gene-related peptide
CRPS	Complex regional pain syndrome
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulphoxide
dNTP	Deoxyribonucleotidtriphosphat
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescinisothiocyanate
Fura-2AM	Fura-2 acetoxymethylester
HC	Healthy control
HLA	Human leukocyte antigen
IASP	International Association for the Study of Pain
IgG	Immunoglobulin G
IL	Interleukin
IvIg	Intravenous immunoglobulin
LDH	Lactat dehydrogenase
LEMS	Lambert-Eaton myasthenic syndrome
NF-kB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

M2R	Muscarinic 2 receptor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPN	Myenteric plexus neurons
NL	Nerve lesion
NP	Polyneuropathy
PBGD	Porphobilinogen deaminase
RA	Retinoic acid
SN	Sympathetic neurons
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor alfa

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I – INTRODUCTION

1.1. Complex Regional Pain Syndrome

Complex regional pain syndrome (CRPS) is a painful condition affecting one or more extremities of the body, marked by a wide variety of symptoms and signs. The syndrome was first described as causalgia by Silas Weir Mitchell in 1865 during the American Civil War in soldiers who developed burning pain and trophic disturbances after gunshot wounds associated with nerve injuries. In 1900, Paul Sudeck, a german surgeon, described a similar syndrome with pain, edema and trophic changes after a limb trauma without nerve lesion, and termed as post traumatic bone dystrophy. He was the first to hypothesize on the pivoting role of an inflammatory process. As the sympathetic nervous system seems to be overactive at the first glance, the term reflex sympathetic dystrophy was used for many years. The descriptive term CRPS was then established after some studies questioned the role of sympathetic nervous system in the pathophysiology of this condition (Stanton-Hicks, Janig et al. 1995). Two types of CRPS are distinguished: CRPS 1 without and CRPS 2 with clinically evident peripheral nerve lesion. There is neither clinical nor pathological evidence to suggest that the mechanisms are any different in these two types of CRPS, and the response (or lack thereof) to treatments are similar (Oaklander; Oaklander and Fields 2009; Oaklander 2010). The disease course varies from relatively mild and self-limiting to a chronic devastating disease with a high impact on the daily functioning and quality of life of the patients, associated with a substantial enhancement of societal and economic burdens (Albrecht, Hines et al. 2006).

1.1.1. Incidence

Few prospective, epidemiological studies have been published on CRPS; nevertheless, population-based cohort studies estimate its incidence between 5.46 in the U.S.A. and 26.2 in the Netherlands per 100,000 person per year (Sandroni,

Benrud-Larson et al. 2003; de Mos, de Bruijn et al. 2007). This discrepancy might be due to differences in population characteristics or diagnostic criteria applied. Both European and North American studies agree that the upper extremities are more affected than the lower extremity. Women are clearly more affected than men with a ratio of 3-4:1, and the peak of incidence lies between 50 and 70 years of age. Menopausal woman between 61–70 years appeared to be at the highest risk for the development of CRPS. It appears though frequently in almost every age group except children. CRPS type 1 has been described in children, but the incidence is much lower than in adults. The risk is considered to be much higher in certain subgroups of patients exposed to a trauma or surgery, exceeding 30% following some surgical procedures or fracture (Sandroni, Benrud-Larson et al. 2003; de Mos, de Bruijn et al. 2007).

1.1.2. Etiology

One exact initial event in CRPS is not yet known. There have been reported a number of events that can lead to CRPS. Most authors agree that the common event is a physical injury affecting the distal part of an extremity. The symptoms exceed in both magnitude and duration the expected clinical course of the inciting event, and often result in a significant impairment of motor function. Symptoms appear in one or more extremities after even a relative mild trauma, or many times spontaneously with no association with previous inciting injury. In many cases patients are not able to relate any initial event to the onset. Fracture is the most common precipitating event (44%) beside post-surgical conditions, contusions, and strain or sprain. Less common causes are central nervous system lesions, including spinal cord injuries and cerebrovascular accidents (Braus, Krauss et al. 1994; Kocabas, Levendoglu et al. 2007; Chae 2010), and cardiac ischemia (Ahmed 2003). A variety of other events have been lately related to the onset of CRPS such as infections (van de Vusse, Goossens et al. 2001; Goebel, Vogel et al. 2005; Gross, Tschernatsch et al. 2007) or autoimmune diseases (Ostrov, Eichenfield et al. 1993; Tsutsumi, Horita et al. 1999; Ahmed 2003; Schwartzman,

Gurusinghe et al. 2008). Recently, some studies demonstrated familial occurrence of CRPS as well (Shirani, Jawaid et al.; de Rooij, de Mos et al. 2009).

1.1.3. Pathogenesis and mechanisms

How CRPS sometimes progresses into being such a destructive condition is not clearly known. It is difficult to explain how a possible initial minor injury can result in a condition with far greater pain and discomfort well after this initial injury has healed. There are a number of hypotheses on the pathophysiology of CRPS based on recent experimental and clinical findings. However, all studies on the pathophysiology revealed that the mechanisms are multifactorial and complex, and therefore not completely understood (Bruehl 2010). The following mechanisms have been identified: (1) Exaggerated inflammation after trauma, in particular a neurogenic inflammation at the site of the lesion disturbing the somatic sensation, (2) hypoxic changes, (3) dysfunctions of the peripheral or central sympathetic nervous system, and (4) profound cortical reorganization processes (see Figure 1) (Wasner, Schattschneider et al. 2001; Huygen, De Bruijn et al. 2002; Leis, Weber et al. 2003; Maihofner, Handwerker et al. 2003; de Mos, Sturkenboom et al. 2009). Certain mechanisms seem to predominate depending on clinical presentation or CRPS stage (Eberle, Doganci et al. 2009). Additionally, there is increasing evidence of an involvement of the immune system as pivotal mechanism in CRPS. Since the first description of the disease 150 years ago, the development of these dysfunctions in only a part of trauma patients remains obscure.

1.1.3.1. Inflammation, neurogenic inflammation and hypoxic changes

Paul Sudeck already hypothesized in his first description on the pivotal role of an inflammatory process. He described in the clinical picture of CRPS the major signs of inflammation - *calor, rubor, dolor* and *functio leasa* - as an indication that at least in the initial phase of the disorder an inflammatory process is playing a role in the pathophysiology of this condition (Sudeck 1900). Classic inflammation is marked by the presence of proinflammatory cytokines. In CRPS, analysis of blister fluid

from the affected limb revealed increase of the cytokines TNFα, IL-6 and tryptase (a product of mast cell degranulation) (Huygen, De Bruijn et al. 2002). IL-1 and IL-6 were also found to be increased in spinal fluid (Alexander, van Rijn et al. 2005).

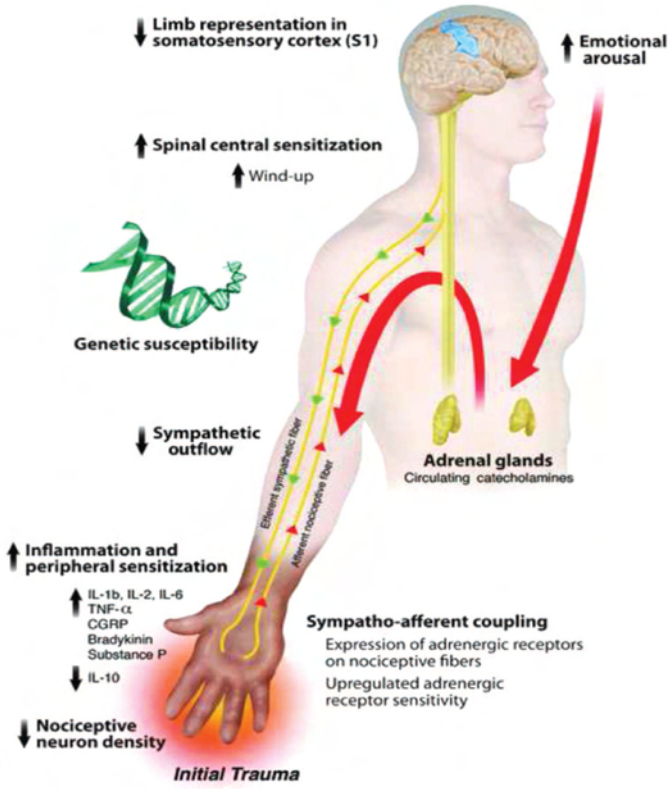


Figure 1. Possible interaction between pathogenic mechanisms that might lead to the florid clinical picture of CRPS. (Extracted from (Bruehl 2010)). CGRP, calcitonin gene-related protein; IL, interleukin; TNFα, tumor necrosis factor alpha.

Additionally to classical inflammation, a neurogenic inflammation is an important local abnormality observed in CRPS (Vacariu 2002). It is characterized by release of neuropeptides from afferent (and nociceptive) nerve endings causing inflammatory effects such as edema, increased permeability of blood vessels, and histamine release from mast cells (Weber, Birklein et al. 2001). Neuropeptides are thought to be released mainly by “sleeping” nociceptors. Trauma and the following release of inflammatory mediators “wake up” these afferent fibers leading to long-term sensitization of nociceptors mediated by neuropeptides including substance

P, calcitonin gene related peptide (CGRP), neuropeptides Y, bradykinin and vasoactive intestinal protein. While a limited inflammatory response is expected after trauma, studies have demonstrated robust and persistent amplification of cytokine (Wesseldijk, Huygen et al. 2008), endothelin-1 (Groeneweg, Huygen et al. 2006) and neuropeptides (CGRP, substance P) (Birklein, Schmelz et al. 2001; Weber, Birklein et al. 2001) signaling in patients who developed CRPS after injury. Recently, a loss of inhibition of these neuropeptides has been suggested to explain at least a part of CRPS symptoms (Birklein and Kingery 2009). Deficiency or blockage of peptidases such as neutral endopeptidase and angiotensin converting enzyme, which participate in the post-release and degradation of neuropeptides, has been implicated to predispose to CRPS (Kramer, Schmidt et al. 2005; de Mos, Huygen et al. 2009; Kramer, He et al. 2009). Moreover, endothelial dysfunction, hypoxic changes, and free radical damage have also been suggested as important processes in the pathogenesis of CRPS. An impaired microcirculation as a result of free radicals, lactate acidosis and altered nitric oxide synthesis after inflammation and ischemia seems to underlie a cold extremity that is observed in some CRPS patients (de Mos, Sturkenboom et al. 2009).

1.1.3.2. Autonomic nervous system dysregulation

Most authors suggest CRPS to be a syndrome where the autonomic nervous system is “out of control”. Disturbances of the sympathetic nervous system were classically supposed as main feature of CRPS. Changes in skin blood flow, temperature, and sweating as well as the symptom of sympathetically maintained pain have been ascribed to sympathetic dysregulation in CRPS.

Physiologically, sympathetic preganglionic neurons, which are involved in regulation of effector cells in somatic tissues, project to the paravertebral ganglia of the sympathetic trunk and synapse with postganglionic neurons that innervate the effector cells. These preganglionic sympathetic neurons are under central control originating in the hypothalamus, and the pattern of ongoing and reflex discharges is characteristic for each type of sympathetic pathway, e.g., skin vasoconstrictor,

muscle vasoconstrictor and sudomotor neurons, and varies according to the innervated target cells. Sympathetic vasoconstrictor pathways that regulate cutaneous blood flow are mainly under thermoregulatory as well as respiratory control. Under normal condition sympathetic activity does not interact with the nociceptive neurons in the periphery (Wasner 2010).

Sympathetically maintained painful sensations described in CRPS are provoked by sympathetic outflow through sympathetic-afferent coupling in which adrenergic receptors are expressed on primary afferent nerve endings. The sympathetic hyperactivity was the traditional rationale for clinical use of selective sympatholytic blockers in CRPS. Indeed, pain, in a subset of CRPS patients, can be relieved by blockage of the efferent sympathetic nerve supply to the affected limb (Schattschneider, Binder et al. 2006). Moreover, it is known that after injury new communication pathways are formed between sympathetic terminals and sensory neurons (Watkins and Maier 2002). This underlying sympathetic sprouting in the dorsal horn and/or in the skin has been referred to as one explanation for the pathogenic sympathetic activity in CRPS.

The common autonomic features of CRPS, such as cool, bluish limb as a result of vasoconstriction also reflect excessive sympathetic outflow. However, most CRPS patients in early acute stages often show increased skin temperature associated with increased sweating. This pattern of excessive functional inhibition of cutaneous sympathetic activity associated with enhanced sympathetic sudomotor activity in the affected limb suggest a profound inhibition of central sympathetic structures leading to thermoregulatory dysfunction (Birklein, Riedl et al. 1998).

Further studies have given strong evidence that sympathetic vasoconstrictor activity is inhibited rather than enhanced in acute CRPS (Wasner, Heckmann et al. 1999). Profound vasodilatation due to neurogenic inflammation interferes with sympathetic outflow and mimic loss of vasoconstrictor response. Sympathetic vasoconstrictor reflexes and venous levels of norepinephrine and its metabolites are diminished as a result of decreased release of transmitter from postganglionic

sympathetic fibers on the affected side (Wasner, Schattschneider et al. 2001).
Some authors suggest that decrease in blood flow and skin temperature often observed in late stages might reflect an secondary adrenergic supersensitivity, consequence of receptor upregulation that occur in response to the initial functional inhibition of vasoconstrictor activity and diminished levels of neurotransmitters observed in the acute stage (Baron and Maier 1996). This secondary hyperactivity could lead to cold bluish affected limb, even though sympathetic neuronal activity is reduced (Wasner 2010).

1.1.3.3. Sensitization and cortical reorganization

The peripheral alterations give rise to a series of changes at the level of the central nervous system. The neural plasticity that happens after an injury, described as the capacity of neurons to change their structure and functional profile, is mediated by the neuropeptides described above, and is implied as one mechanism behind the sensorial disturbances in CRPS. Sensitization leads to painful response to a normally not painful stimulus, resulting in allodynia and hyperalgesia. CRPS patient display an increased excitability of spinal cord neurons to repeated stimuli applied to the affected limb in comparison to the contralateral side (Bruehl 2010).
Following the central sensitization, alterations at the supraspinal level might evolve, resulting in the altered sensory mapping in the cerebral cortex observed in CRPS patients. Neuroimmaging studies showed a reduction in size of the representation of the CRPS-affected limb in the somatosensory cortex compared with the non-affected limb (Juottonen, Gockel et al. 2002; Maihofner, Handwerker et al. 2003; Pleger, Ragert et al. 2006). This might implicate an additional contribution to the sensational disturbance in CRPS (Juottonen, Gockel et al. 2002; Maihofner, Handwerker et al. 2003; Pleger, Ragert et al. 2006). Recently, an altered central opioidergic neurotransmission has been described (Klega, Eberle et al. 2010). However, the importance of the cortical reorganization and central alterations in CRPS remains controversial, and is currently under investigation. The origin and at

which point in the development of CRPS somatotopic reorganization occurs is not known (Bruehl 2010).

1.1.3.4. Possible involvement of the immune system

Some studies showed an increasing evidence for the involvement of the immune system in the physiopathology of CRPS. The HLA alleles DQ1, DR13, DR15 and the centromeric regions of the HLA class 1 antigens were linked to CRPS (Kemler, van de Vusse et al. 1999; van de Beek, Roep et al. 2003). Local and systemic disturbances of the cytokine network have been described, and raised up the idea of an immune-mediated process (Kemler, van de Vusse et al. 1999; Huygen, De Bruijn et al. 2002; Alexander, van Rijn et al. 2005; Maihofner, Handwerker et al. 2005). Analysis of blister fluid of affected extremities, venous blood and liquor revealed a pro-inflammatory cytokine expression profile indicating an ongoing immune process, and reinforce that the immune system might play an underestimated role in CRPS (Maihofner, Handwerker et al. 2005; Alexander, Perreault et al. 2007). Additionally, one study showed evidence of an enhanced immune cell activation with migration of leukocytes and nonspecific immunoglobulins (IgG) towards the CRPS affected limb (Tan, Oyen et al. 2005). Notwithstanding controversial reports about the efficiency of corticosteroids, some patients with acute CRPS could be sufficiently treated by using systemic corticosteroids (Christensen, Jensen et al. 1982; Braus, Krauss et al. 1994; Zyluk and Puchalski 2008). Additionally, an improvement of CRPS after treatment with the TNF-α antagonist infliximab or intravenous immunoglobulins (IvIg) has been observed in some cases (Huygen, Niehof et al. 2004; Goebel, Vogel et al. 2005; Bernateck, Rolke et al. 2007; Goebel, Baranowski et al. 2010).

Autoantibodies specifically targeting autonomic (e.g. sympathetic) nervous system structures have been demonstrated in our group for the first time (Blaes, Schmitz et al. 2004). These autoantibodies, detected by immunofluorescence, have been found to be directed against antigens of sympathetic ganglia neurons. Additionally, Goebel et al. (2005) could also demonstrate increased binding of CRPS sera to

various peripheral and central nervous system structures (Goebel, Vogel et al. 2005). These autoantibodies might be transferable since IgG derived from CRPS serum samples, which was injected in mice, led to abnormal behaviour (Goebel, Stock et al. 2005). The underlying antigens and the pathophysiological role of autoantibodies are entirely unknown.

1.1.4. Clinical features

Many symptoms observed in CRPS patients are normal during the recovery period of a trauma or a surgical procedure. The characteristic feature of CRPS is that this normal reaction does not stop; the normal inflammation seems to continue and gets out of control.

The most prominent feature is pain, including spontaneous pain, allodynia and hyperalgesia. Additionally, the affected extremity can display autonomic abnormalities e.g. changes in color and/or temperature (vasomotor disturbances), edema, alterations in transpiration (sudomotor disturbances), trophic changes such as abnormal hair and nail growth; and, muscular atrophy and/or dysfunction (motortrophic disturbances).

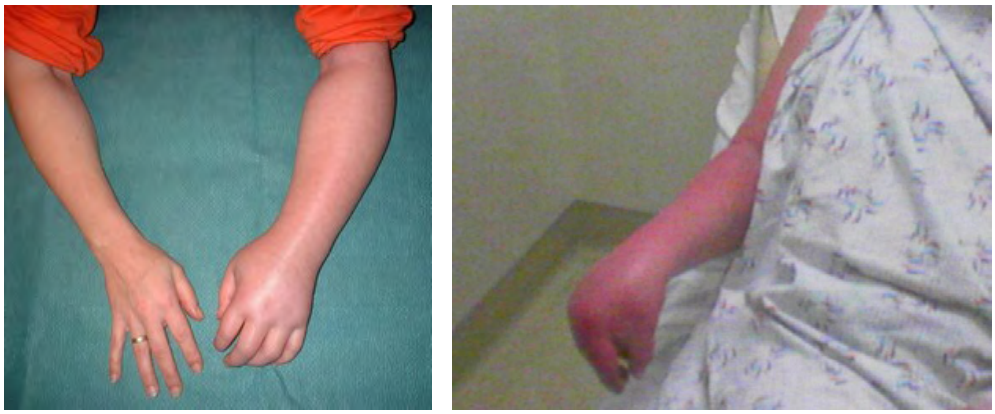


Figure 2. Picture of moderate (left) and severe (right) CRPS on the upper limb. Debilitating pain, edema and trophic changes impairs the function of the affected limb and consequently the quality of life in both cases (extracted from www.foot-pain-explained.com/CRPS.html and http://en.wikipedia.org/wiki/File:Severe_CRPS.jpg).

The initial stage of the disease is characterized by the appearance of the major signs of inflammation: *calor, rubor, dolor* and *functio leasa*. The functionality of the affected limb is often impaired. The most important sensory disturbances are pain and hyperalgesia. The patients report often burning spontaneous pain in the distal part of the affected limb, sometimes the pain has an aching or pricking character. Nearly all patients experience hyperalgesia, basically to thermal stimuli or mechanical impact. Hyperalgesia to cold is significantly more frequent, and is regarded as a symptom of sympathetically maintained pain. Sensory disturbances appear usually in early stages, are predominantly distally located, and display no consistent spatial relationship to individual nerve territories or to the site of the inciting lesion.

In addition, motor symptoms are usually present from the beginning (Veldman, Reynen et al. 1993; Birklein and Handwerker 2001), and may progress with ongoing duration (van Hilten, van de Beek et al. 2000). Weakness of all muscles of the affected distal extremity is present in 77% of CRPS patients. Small accurate movements and range of motion are often impaired. In acute cases movement limitations are determinated by edema and in chronic stages by atrophic changes, especially on palmar and plantar sides of hands and feet. About 50% of the patients have a postural or action tremor; and, 5-10% of patients develop dystonia of the affected hand or foot (van Hilten 2010) (Figure 3).

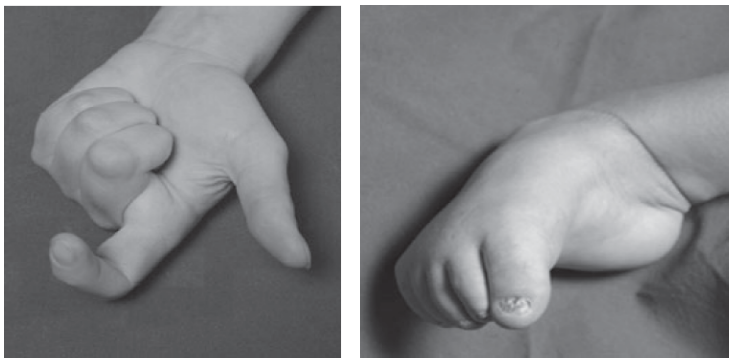


Figure 3. Extremity dystonia in CRPS patients (Extracted from (van Hilten 2010).

During the acute stage the affected limb is usually red and hot, warmer than the contralateral limb. Additionally, edema and sweating abnormalities like hyper- or hypohidrosis are pronounced, and affect almost all CRPS patients. Later on in chronic stages, the affected skin turns to bluish and cold; however, 30% percent of all patients have a decreased skin temperature from the beginning and usually stay cold in CRPS course. The typical temperature difference between affected and unaffected side is more than 1 ºC.

Three stages of CRPS have been classified. Although a consensus panel recommended that staging be eliminated, it is important to have an awareness of potential disease progress. Disease progress is very variable.

Stage I or early CRPS: This stage is chracterized by severe pain, more than what would be expected from the injury, with a burning or aching quality. It may be increased by dependency of the limb, physical contact, or emotional upset. The affected area becomes edematous, may be hyperthermic or hypothermic, and shows increased nail and hair growth. Radiographs may show early bony changes. Duration is usually 3 months from onset of symptoms. Some patients remain in one stage or another for many months or even years. They may never progress or they may progress quickly to late stage. Physical findings may be minimal, especially in those who remain in stage I or progress slowly.

Stage II or established CRPS: As diseases progresses, affected area becomes indurated, cool and hyperhidrotic with livedo reticularis or cyanosis. Hair may be lost, and nails become ridged, cracked, and brittle. Hand dryness becomes prominent, and atrophy of skin and subcutaneous tissues becomes noticeable. Intense debilitating pain remains the dominant feature. It usually is constant and is increased by any stimulus to the affected area. Stiffness of affected limb develops at this stage. Radiographs may show diffuse osteoporosis. Duration is usually 3-12 months from onset.

Stage III or late CRPS: In the late stage pain starts spreading proximally. Although it may diminish in intensity, pain remains a prominent feature. Flare-ups

may occur spontaneously. Irreversible tissue damage occurs. Skin is thin and shiny. Edema is absent. Contractures may occur. Radiographs indicate marked demineralization.

1.1.5. Diagnosis

Until the present date, there is no objective diagnostic method to confirm the diagnosis of CRPS. Diagnosis is possible based on the clinical presentation of the syndrome. The clinic of CRPS is based on the findings during the history and physical examination, for which several diagnostic criteria sets have been developed. According to the International Association for the Study of Pain (IASP), established during a consensus of experts in 1994 (Merskey and Bogduk 1994), the diagnosis of CRPS can be made if the following criteria are fulfilled:

1. The presence of an initiating noxious event, or a cause for immobilization, without (CRPS 1) or with nerve lesion (CRPS 2);
2. Spontaneous pain or hyperalgesia/hyperesthesia not limited to a single nerve territory and disproportionate to the inciting event in severity;
3. Evidence at some time of edema, changes in skin blood flow (temperature) or sudomotor abnormalities, motor symptoms or trophic changes are present on the affected limb, in particular at distal sites;
4. Exclusion of conditions that would otherwise account for the degree of pain and dysfunction.

The IASP criteria are the most widely accepted. However, these criteria were never fully validated. Many studies have reported high sensitivity (0.98) which makes them valuable for clinical use but the low specificity (0.36) limits its use for research purpose (Bruehl, Harden et al. 1999). In the practice, the application of IASP criteria may result in the overdiagnosis of CRPS due to a poor specificity. The combination of multiple elements like vasomotor, sudomotor and edema-related changes into one diagnostic criterion, and the failure to include motor/trophic changes has been reported to be responsible for the limited specificity of the IASP criteria. For research purpose a more restricted definition made by Bruehl and

co-workers (1999) (Bruehl, Harden et al. 1999) has been recommended. These authors proposed extra criteria in which the patient tells his/her own story about the complaints, and the observer confirms a number of these complains by physical examination. The proposed modified diagnostic criteria for CRPS extracted from Bruehl et al. (1999) include the following extra criteria:

1. Continuing pain which is disproportionate to any event
2. Must report at least one symptom in each of the following four categories:
    - **Sensory**: Reports of hyperesthesia and/or allodynia
    - **Vasomotor**: Reports of temperature asymmetry and/or skin colour changes and/or skin colour asymmetry
    - **Sudomotor/edema**: Reports of edema and/or sweating changes and/or sweating asymmetry
    - **Motor/trophic**: reports of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)
3. Must display at least one sign in two or more of the following categories:
    - **Sensory**: evidence of hyperalgesia (to pinprick) and/or allodynia (to light touch)
    - **Vasomotor**: evidence of temperature asymmetry and/or skin colour changes and/or asymmetry
    - **Sudomotor/edema**: evidence of edema and/or sweating changes and /or sweating asymmetry
    - **Motor/trophic**: evidence of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)

These criteria lead to a gain in specificity (0.94) of the IASP criteria, and thus improve the ability to discriminate accurately between CRPS and other types of neuropathic pain. For that, it should be observed at least one symptom in all four symptom categories and at least one sign (at evaluation) in two or more sign

categories, as revised by Harden and co-workers (Harden, Bruehl et al. 2007).

1.1.6. Treatment

The treatment of CRPS still is an interdisciplinary challenge. Because there is no cure, treatment is aimed at relieving painful symptoms. Most of the treatment regimens including analgesics, antiepileptics, or antidepressants are only symptomatic treatment. These treatment strategies have failed to produce consistent long-lasting improvement in symptoms. Other treatments include physical therapy, sympathetic nerve block, spinal cord stimulation, intrathecal drug pumps to deliver opioids and local anesthetic agents via the spinal cord; and, as a last resort for pain relief, amputation of the affected extremity (Albrecht, Hines et al. 2006). Corticosteroids have been used successfully in some small studies (Christensen, Jensen et al. 1982; Braus, Krauss et al. 1994; Zyluk and Puchalski 2008). Huygen and co-workers reported successful treatment of CRPS with a TNF-α antagonist infliximab in a small study (Huygen, Niehof et al. 2004); these authors failed to confirm this effect in a controlled study using TNF-α antagonist (personal communication). Improvement of CRPS after treatment with IvIg has been observed (Huygen, Niehof et al. 2004; Goebel, Vogel et al. 2005; Goebel, Baranowski et al. 2010). Recently, we reported that both pain and autonomic disturbances ameliorated after plasmapheresis (Blaes, Tschernatsch et al. Manuscript under peer-review).

1.2. Autoimmunity

The immune system is the body's natural guardian against disease. It basically defends our body from invading pathogenic organisms like bacteria, parasites and viruses. It displays several remarkable characteristics which include distinction between "self" and "non-self", and has the ability to remember previous experiences (Goldsby, Kindt et al. 2000; Janeway, Travers et al. 2001). The concept of autoimmunity was first predicted by Nobel Laureate Paul Ehrlich. He described it as 'horror autotoxicus'. His experiments led him to conclude that the

immune system is normally focused on responding to foreign materials and has an inbuilt tendency to avoid attacking self-tissues. But when this process goes wrong, the result is an inappropriate response of the immune system against self-components resulting in autoimmune disease. An autoimmune disease can be either organ-specific or systemic.

In an organ-specific autoimmune disease, the immune response is directed to a target antigen unique to a single organ or gland, so that the manifestations are largely limited to that organ. The cells of the target organs may be damaged directly by humoral or cell-mediated effector mechanisms. Alternatively, the antibodies may overstimulate or block the normal function of the target organ. Some examples of this type of autoimmune disease are Hashimoto's thyroiditis, Goodpasture's syndrome, Insulin-dependent diabetes mellitus, which are mediated by direct cellular damage; and, others are Graves' disease, Myasthenia gravis, which are mediated by stimulating or blocking autoantibodies (Besinger, Toyka et al. 1983; Bach 1994; Drachman 1994).

In systemic autoimmune diseases, the response is directed towards a broad range of target antigens, and involves a number of organs and tissues. These diseases reflect a general defect in immune regulation that results in hyperactive T and B cells. Tissue damage is widespread, both from cell-mediated immune responses and from direct cellular damage caused by autoantibodies or by accumulation of immune complexes (Klinman 1989; Lin, Mamula et al. 1991). Myasthenia gravis usually presented in young adult or later adult life as muscle weakness and excessive fatigue during repetitive movements is one example. It often involves the extraocular muscles of the eye with double vision and ptosis at onset, but usually progresses to a generalized weakness. It is the prototype autoimmune disease mediated by pathogenic antibodies. These autoantibodies are directed against the nicotinic acetylcholine receptor (nAChR) on the neuromuscular junction or helper proteins of the receptor such as the muscle-specific kinase MUSK (Vincent and Newsom-Davis 1985; McConville, Farrugia et al. 2004). Interestingly, the anti-

nAChR antibodies do not block the receptor directly, but lead to a cross-linking of receptor molecules and an internalization of the receptor-antibody complexes (Elias, Butler et al. 1978; Appel, Elias et al. 1979).

1.2.1. Mechanism of autoimmunity

Autoimmunity is not set off by a single cause. The molecular and cellular mechanisms of autoimmune responses and their origins are numerous and varied; and, even if a given autoimmune disease was initiated primarily by a single trigger, many other events and regulating mechanisms come into play thereby adding complexity to the process. Autoimmune diseases result from interaction of genetic, immune, hormonal and environmental components (Ermann and Fathman 2001). For a specific autoimmune condition to develop, a particular combination of these factors is required.

1.2.1.1. MHC control and genetic background

Genetic markers were delineated, involving specific chromosomal aberration and specific genes. Predisposition to a given autoimmune response requires the requisite allele(s) that controls antigen presentation by antigen-presenting cells for T cell recognition. A striking characteristic of human autoimmune diseases is the increased frequency of certain HLA class II alleles in affected individuals. Since alleles positively associated with autoimmune diseases share amino acid residues in the hypervariable HLA regions involved in peptide binding, it is likely that disease associated class II molecules have the capacity to bind the autoantigen and present it to T cells, thereby inducing and maintaining the autoimmune disease (Adorini 1992; Shoenfeld, Gilburd et al. 2008). For instance, in celiac disease the antigluten T cell response is strongly associated with histocompatibility antigen HLA-DQ2.5 homozygosity (Fallang, Bergseng et al. 2009; Pietzak, Schofield et al. 2009).

1.2.1.2. Disruption in the level or activity of regulatory proteins and cells

Immune disturbances can occur because of disruption in the levels or activity of regulatory proteins, and consequently regulatory cells. An abnormal T cell or B cell activation and cell death signaling underline the pathology of systemic lupus erythematosus (Kyttaris, Juang et al. 2005). Moreover, in lupus, B cells exhibit abnormal signaling through the B-cell receptor (Jenks and Sanz 2009). The crucial role of regulatory cells in self-tolerance and autoimmunity has been clearly established in numerous types of regulatory cells, the majority of which are $CD4^+$ T cells. Much focus has been placed on thymus derived $CD4^+CD25^+$ regulatory T cells, given that the depletion of this subset in murine models results in the spontaneous development of autoimmune diseases (Asano, Toda et al. 1996).

1.2.1.3. Altered proteins

Proteins to which the immune system is self-tolerant might, if altered, elicit autoimmune responses. Mutation and altered expression of proteins trigger autoimmune responses that cross-react with unaltered forms of the protein and compromise important feedback inhibitory pathways that downregulate antigen receptor signaling (Yu, Mamchak et al. 2003). Posttranslational modifications of self-proteins can also affect intracellular signaling and protein recognition by the immune system. It generates neo-epitopes from self-proteins that are not subjected to immune tolerance; as consequence, leads to overt increase in affinity of MHC or T-cell receptor binding, and also affect activity of antigen processing enzymes (Anderton 2004). These processes as well as covalent modifications of proteins are frequently reported after inflammation. Oxidant species that play a role in acute and chronic inflammation are able to modify proteins and generate neo-autoantigens (Nissim, Winyard et al. 2005). Denatured, misfolded or disordered proteins have been shown to trigger autoimmune response in similar manner (Bouvet and Zouali 2005).

1.2.1.4. Apoptosis and autoimmunity to sequestered proteins

Dysregulated apoptosis, both interrupted as well as accelerated apoptosis, have been suggested as central defects in diverse autoimmune disease. Apoptosis results in release of cell components that are ordinarily sheltered from the immune system, and can be a significant trigger of autoimmunity (Wucherpfennig 1994; Zandman-Goddard and Blank 2002). Autoantigens targeted in systemic autoimmune diseases share little in terms of structure, subcellular distribution or function in normal cells. These molecules are unified by becoming clustered and concentrated in the surface blebs of apoptotic cells. Their structure is altered during some types of cell death to generate structures not previously generated during development and homeostasis (Rosen and Casciola-Rosen 2004). On the other hand, apoptosis is essential for the prevention of immunity against self-antigens, since it enables the disposal of reactive T cells, homeostasis and downregulation of autoimmune responses. Abnormal clearance mechanisms can allow the persistence of antigenic stimulation; for instance, immune responses that specifically target apoptotically modified form of lupus autoantigen have been identified in lupus patients (Greidinger 2001). An abnormal B or T cell apoptosis seems nevertheless to play an important role since impaired clearance of apoptotic cardiomyocytes, in infants born to mothers with Systemic Lupus erythematosus or Sjögren's syndrome, has been linked to anti-SSA/Ro and –SSB/La antibodies in the pathogenesis of congenital heart block (Clancy, Neufing et al. 2006).

Defect or deficiency in subunits of the proteasome pathway leads to reduced poly-ubiquitination of altered proteins. Proteolytic processing required for production and activation of NF-kB is also impaired in the presence of defective proteosomes. Such defect has been suggested to play an important role in immune and inflammatory responses, as it increases susceptibility of the affected cells to apoptosis induced by TNF-α (Hayashi and Faustman 2000).

1.2.1.5. Molecular mimicry

A major trigger of autoimmune responses and diseases has been shown to originate from immune responses to antigens on human-infecting microbes (Sfriso, Ghirardello et al. 2010). An autoimmune disease can be induced or triggered by infectious agents, whose protein(s) possess homology or structural similarities in some of its determinants to regions on proteins of the host; however, they differ sufficiently to induce an immune response when presented to T cells. Thus, the tolerance to autoantigens can breakdown, and antibodies evoked against a pathogen might cross-react with self-proteins and act as autoantibodies. The involved autoantigen then provides a source for persistent stimulation (Blank, Barzilai et al. 2007).

In Guillain-Barré syndrome antigenic epitopes are shared between *Campylobacter jejuni* glycoproteins and structures of the myelin sheath (Albert and Inman 1999; Goodyear, O'Hanlon et al. 1999). Although the triggering event in most autoimmune diseases is unknown, an infectious cause has long been postulated to explain the development of many autoimmunities. It is possible that more than one pathogen act as a trigger for an autoimmune disease; for instance, scanning of protein sequences databases yielded approximately 100 candidate microbial determinants regions on different microbial proteins that display similarities to autodeterminant epitopes of human acetylcholine receptor, and many displayed the ability to bind autoantibodies in sera of myasthenia gravis patients (Deitiker, Ashizawa et al. 2000).

1.2.1.6. Natural antibodies and autoantibodies

Immunoglobulins that arise independently of known and/or deliberate immunization have been termed as natural antibodies. Wide-ranging natural antibodies were found to react with self-molecules, and therefore are defined as autoantibodies (Atassi and Casali 2008). Natural autoantibodies possess in general a low intrinsic affinity for antigens, but can function as templates for the generation of pathogenic autoantibodies, that emerge through a process of clonal selection. When self-

tolerance is disturbed as a result of inflammatory processes, exposure to chemicals, molecular mimicry with pathogens, vaccination, receptor editing, radiation or genetic background, it can be followed by exposition of autoantigens and/or dysregulation of the immune system resulting in the emergence of an autoimmune disease. Elevated autoantibody titers will be detected, followed by autoantibody spread. Nevertheless, many antibodies can be detected already in the pre-clinical phase of autoimmune diseases. The detection of serum autoantibodies serve as biomarker for different autoimmune disease diagnostic and activity, for the classification and prognosis; and more recently, emerged as a predictive tool for development of autoimmunity (Shoenfeld, Blank et al. 2008).

II - AIM

CRPS remains one of the most enigmatic and difficult to treat of all pain conditions, probably in large part due to the fact that the pathophysiology remains poorly characterized. Recent evidence has begun to focus research on the involvement of the immune system in CRPS pathogenesis. Our group showed in a previous report that CRPS patients have autoantibodies against nervous system structures. Putative autoantibodies have not yet been characterized. Therefore, the aim of this study was:

1. to screen for autoantibodies against surface epitopes of differentiated autonomic neurons in CRPS patients,
2. to identify and characterize autoantibodies using different autoantibody detection methods and pharmacological approaches,
3. to identify target autoantigens and
4. to assess the functional activity of autoantibodies.

III – MATERIALS AND METHODS

3.1. Patients

Serum was obtained from patients with clinically defined CRPS, according to the revised IASP diagnostic criteria (Bruehl, Harden et al. 1999; Harden, Bruehl et al. 2007) after informed consent and approval of the local ethical committee (JLU Giessen, No 105/02; Landesärztekammer Rheinland Pfalz, No 4208). Sera of healthy controls (HC), patients with non-inflammatory neuropathy (NP) and patients with peripheral nerve lesions (NL) served as controls. The control groups were age- and sex-matched with patients group. Detailed description of patient samples are given in result section. For some experiments, we randomly selected some serum samples and isolated IgG using affinity chromatography, as described below.

3.2. Materials

3.2.1. Chemicals and solutions

Table 1. General laboratory chemicals

Acetic Acid 100%	Merck
Agarose multipurpose	Bioline GmbH
Ammoniumpersulfat APS	Roth
all-trans retinoic acid	Sigma Aldrich
Atropine	Sigma Aldrich
5-bromo 4-chloro 3-indolyl phosphate / nitro-blue tetrazolium chloride (BCIP/NBT)-Blue Liquid Substrate	Sigma Aldrich
BDH crystalline trypsin (bovine pancreas)	Sigma Aldrich
Bisoprolol hemifumarate salt	Sigma Aldrich
Bovine serum albumine, fraction V (BSA)	Sigma Aldrich
BQ-610	EMD4 biosciences
Bromphenolblue	Neolab
Carbamoylcholine chloride	Sigma Aldrich
Carbonate-bicarbonate buffer	Sigma Aldrich
CelLytic^{TM} M cell lysis reagent	Sigma Aldrich
Clenbuterol hydrochloride	Sigma Aldrich

Collagenase Worthington type CLS II	Biochrom
Deoxiribunuclease 1, Type 4 bovine pancreas	Sigma Aldrich
Destilled water „Aqua ad injectabilia“	Braun
D(+)Glucose	Gibco
Dimethylsulfoxide (DMSO)	Roth
Ethylendiamintetracetic acid (EDTA) 0,5M	Roth
Ethanol 100 %	Sigma Aldrich
Ethanol for molecular biology 100%	Merck
Ethidiumbromid	Merck
Ficoll-PaqueTM Plus	Amersham
Fura-2-acetoxymethylester (fura-2/AM)	Invitrogen
5-Fluoro-2´-deoxyuridine	Merck
Glycerol	Roth
Glycin	Merck
H-EBSS	Gibco
Hepes	Sigma
ICI 118,551 hydrochloride	Sigma Aldrich
IgG-Standard	Sigma
Isopropanol	Merck
Potassiumchlorid (KCl)	Merck
Potassiumdihydrogenphosphat (KH_2PO_4)	Merck
Methanol	Merck
Magnesiumsulphate ($MgSO_4$)	Sigma
Sodiumchloride (NaCl)	Roth
Sodiumhydrogencarbonate ($NaHCO_3$)	Merck
Disodiumhydrogenphosphate (Na_2HPO_4)	Merck
Sodiumdihydrogenphoaphate (NaH_2PO_4)	Merck
Sodiumazide (NaN_3)	Merck
Paraformaldehyde (PFA)	Sigma Aldrich
Pluronic acid	Invitrogen
Poly-L-lysin-hydrobromide	Sigma
Propranolol hydrochloride	Sigma Aldrich
Protein G-sepharose	GE Healthcare
Quick load 1kb DNA ladder	Biolabs
Recombinant human BMP-2	Biovision
RNAse free water	Fermentas GmBH
Rotiphorese gel 30 = 30 % Acrylamide-mix	Applichem
Saponin	Invitrogen
TMB peroxidase substrate	KPL
Transforming growth factor-ß1	Sigma
Tris-acetat-EDTA buffer (TAE) 10x	Neolab
Trichlor-acetic acid (20%)	Roth
Trishydroxymethylaminomethan (Tris)	Roth

Tris-HCl	Sigma
Trypanblue	USB
Trypsin (2,5g/l)	Roth
5% Trypsin-EDTA (10x)	Gibco
Trypsin inhibitor Type I-S: from Soybean	Gibco
Trypsin Type III-S and XII-S	Sigma Aldrich
Tween20	Sigma Aldrich

3.2.2. Consumables

Table 2. General laboratory consumables

Cellstar® 6 Well Cell Culture Plate	Greiner bio-one
Cellstar® Plastikpipettes (5 ml, 10 ml)	Greiner bio-one
Cellstar® U-shape with Lid, TC-Plate, 96 well, sterile	Greiner Bio-one
Cellstar® 75 cm^2 Cell cultur flasks	Greiner Bio-one
Cell scrapper	Greiner bio-one
Cryobox 136x136x130 mm	Ratiolab GmbH
Cryo Tube™ vials (1,8 mL; 4,5 mL)	Nunc
Disposable scalpel, sterile	Feather safity razorco
Disposable cuvettes	Ratiolab
FACS-Tubes 0,5 mL 38x6,5 mm PS	Sarstedt
Falcon 5 mL Polystyrene Round-Bottom Tube	Becton Dickinson
Falcon® Plastic pipettes 25 mL	Becton Dickinson Labware
Falcon tubes (15 und 50 mL)	Becton Dickinson
Gel documentation Thermal Image System FTI-500	Fuji Film
Glas Pasteur pipettes 150 mm	Brand
Glaswares (different sorts)	Fisherbrand; IDL; Schott&Gen; Simax
Kodan® Tinktur Forte (alcoholic skin disinfectant)	Schülke & Mayr
LightCycler® Capillaries [20 µL] (for Real time-PCR)	Roche
Minisart single use filter (0,2 µm, 0,45 µm)	Biotech
Neubauer improved	Brand
Nitra-Tex® powder free	Ansell
Nitrocellulose membrane	Biometra
NobaGlove® – Latex powder free	NOBA GmbH
Nunclon™ surface 96-Well plates with flat bottom	Nunc™
Parafilm	American National Can
Pipette tipps (10 µL, 100 µL, 1000 µL)	Sarstedt
PP-PCR-Tubes 0,2 mL thin walled	Greiner bio-one
Grid inserts for Cryobox	Ratiolab GmbH
Reaction tubes 1,5 mL	Sarstedt

Safety-Multifly®–Set, sterile, pyrogenfree (Cannulae)	Sarstedt
Servapor® dialysis tubing (6mm, 25mm)	Serva Electrophoresis GmbH
S-Monovette® 7,5 mL Z (Serum-Tubes)	Sarstedt
Sterile Pipette tips with filter	Nerbe Plus
Tissue culture dishes steril 35,0 / 10 mm	Greiner bio-one
UV-spectroscopic cuvettes	Bio-Rad
Whatmann-Filterpaper 3 mm	A. Hartenstein

3.2.3. Instruments

Table 3. Laboratory instruments

BEP 2000 Advance (ELISA-Reader)	Dade Behring
Centrifuge typ 2-6	Sigma
Centrifuge Universal 32 R (cell culture)	Hettich
ClasII type A/B3 (Sterilbank)	Nuaire Biological Safety Cabinets
Easia shaker	Medgenix diagnostics
FACSCalibur	Becoton Dickinson
Fluorescence microscope DM RB	Leitz
Gel documentation image Masters VDS	Pharmacia Biotech
Gel electrophoresis chamber	Peqlab
Gel trays und gel combs	Peqlab
Heating block / Thermoshaker	Peqlab
HiTrapTM protein G HP (1 mL und 5 mL Protein G columns)	Amersham Biosciences
Inverse light microscope MBL 3100	A.Krüss Optronic
Inverse fluorescence/Light microscope	Olympus IX-50
Image analysis system	Till Photonics
Light Cycler 1.5	Roche Diagnostics
LightCycler centrifuge adapter	Roche Diagnostics
Liquid nitrogen tank	Arpege 75
Magnetic mixer	IKA® Werke
Microwell	SHARP Electronics
Multiscan Ex (ELISA-Reader)	Thermo Electron Corporation
Nalgene™ Cryo 1°C Freezing container	Nalgene®
PC-System, Printer	Hewlett Packard
pH-Meter	Schott Geräte
Pipettes (different volumes)	Gilson, Eppendorf
Pipette boy	Integra Biosciences
Power pack	Peqlab
ProSpec (Nephelometer)	Dade Behring
Pump P-1 (Pump for IgG purification)	Pharmacia Biotech

Refrigerators and freezers	Different companies
Rotamax 120 (Shaker)	Heidolph
Swivel platform	Peqlab
SmartSpec™ Plus spectrophotometer	Bio-Rad
Steri-Cult 200 incubator for cell culture	Labotec GmbH
Sterile bench	Köttermann
Thermocycler	Biometra
Table top centrifuge EBA 20	Hettich
Table top centrifuge micro 120	Hettich
Universal 32 R (centrifuge)	Hettich
Vortex minishaker	IKA® Werke
Vortexer vortex-Genie2	Scientific Industries
Weighing balance	Sartorius AG
Waterbath	Memmert

3.2.4. Molecular biology and biochemistry kits

Table 4. Detection kits

Cell proliferantion reagent WST-1	Roche Applied Science
Cytotoxicity detection kit (LDH)	Roche Applied Science
QIAamp® RNA Blood Mini kit	Qiagen
Quanti Fast™ SYBR Green PCR kit	Qiagen
RevertAid™ First Strand cDNA Synthesis kit	Fermentas
RNeasy Mini kit	Qiagen

3.2.5. Buffers and solutions

Table 5. General buffer and solutions

DNA-loading buffer (10x)	250 mg 33 mL 60 mL 7 mL	Bromphenolblue Tris (150 mM, pH 7.6) Glycerol H_2O
Ethidium bromide staining solution	2.5 mg 1 L	Ethidiumbromide 1x TAE
FACS buffer	500 mL 5 mL 5 mL	1x PBS 10 % NaN_3 Fetal calf serum (FCS)
Glycine buffer	3.75 g In 500 mL	Glycine dH_2O pH 9.0
IgG-Elution buffer	0.75 g 100 mL	Glycine dH2O pH 2.7 (= 0.1 M)
Blocking buffer	0.5 g 10 mL	BSA PBS Tween

1 % Paraformaldehyde (PFA)	1 g 100 mL	PFA 1x PBS
PBS (10x)	80 g 2 g 14.4 g 2.4 g Dissolve in 1 L	NaCl KCl Na_2HPO_4 KH_2PO_4 dH_2O
1x PBS	900 ml 100 mL	dH_2O PBS (10x)
PBS Tween	1 L 100 µL	1x PBS Tween 20
Permeabilisation buffer	500 mL 0.5 g	FACS buffer Saponin
Poly-L-lysin	60 mL 3 mg	dH_2O Poly-L-Lysin
Ponceau S-solution	0.25 g 15 mL Make up 100 mL	Ponceau S Trichloracetate (TCA) with H_2O (protect from light)
TBS-buffer	4.5 g 0.71 g 0.15 g 5.5 g 2.5 mL 500 mL	NaCl Na_2HPO_4 NaH_2PO_4 Non fat dry milk Tween 20 dH_2O
Trypsin-solution	10 mL 2.5 mg	Solution 1H Trypsin
Trypsin inhibitor solution	10 mL 1.6 mL	Solution 1H Konz. D/T-I Lsg

3.2.6. Media and solutions for cell culture

Table 6. Cell culture media

HEK 293	RPMI1640	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
SH-SY5Y	RPMI1640	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
SN	MEM	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco

MPN	DMEM	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
Cardiomyocytes	Halle Sm 20-I	10 % 2 µmol/L	Fetal calf serum (FCS) Fluorodeoxyuridine	Gibco Merck
CHO WT	DMEM/HAMs F-12	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
CHO- hβ1AR - hβ2AR - hM2R	DMEM/HAMs F-12	10 % 2mM 1 % 63 mg/L	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin) Geneticin	Gibco Hyclone Gibco Gibco Gibco

3.2.7. Cell lines

Table 7. Cell lines

Name	**Origin**	**Source**
HEK 293	Fibroblast cells	AG Blaes, Neurology - Giessen
SH-SY5Y	Neuroblastoma	AG Blaes, Neurology - Giessen
CHO-WT	Chinese hamster ovary	kindly provided by Dr. Mohr - Bonn, Germany
CHO-hβ1AR	Chinese hamster ovary, transfected with hβ1AR	kindly provided by Dr. Gerd Wallukat - Berlin, Germany
CHO-hβ2AR	Chinese hamster ovary, transfected with hβ2AR	kindly provided by Dr. Gerd Wallukat - Berlin, Germany
CHO- M2R	Chinese hamster ovary, transfected with hM2R	kindly provided by Dr. Mohr - Bonn, Germany

3.2.8. Antibodies

Table 8. Primary and secondary antibodies

Antibodies-Name	**against**	**raised in**	**Conjugation**	**Company**
Neurofilament	human	mouse	-	Dako
HuD	human	mouse	-	Mol. probes
IgG	human	rabbit	peroxidase	Dako
IgG	human	rabbit	FITC	Dako
IgG	mouse	rabbit	FITC	Dako
IgG	rabbit	swine	FITC	Dako

IgG 1	human	mouse	-	Sigma
IgG 2	human	mouse	-	Sigma
IgG 3	human	mouse	-	Sigma
IgG 4	human	mouse	-	Sigma
Isotype-control		mouse	FITC	R&D Systems
Isotyp-control		mouse	-	Dako
Isotyp-control		rabbit	FITC	Dako
M2R	human	mouse	-	Santa Cruz
β2AR	human	rabbit	-	Abcam

3.2.9. Primers

Table 9. Primer sequences

Description	**Nucleotide sequence**	**NCBI GenBank**
PBGD 8F fwd	5´-TGCAACGGCGGAAGAAAAC-3´	NM_00190
PBGD 3.1 rev	5´-GGCTCCGATGGTGAAGCC-3´	
DßH fwd	5’ - ACTGTCCACTTGGTCTACGGG - 3’	NM_000787
DßH rev	5’ - CGGTCGGGTTTCATCTTG - 3’	
VAChT fwd	5’ - GGGGCATCCTCTATGAGTTCG - 3’	HSU09210
VAChT rev	5’ - GTGGCAATGGTGGGTTCG - 3’	
M2R fwd	5’ - CCAATGCTGCTGTCACCT - 3’	NM_000739
M2R rev	5’ - TTGGCTTCACTATCCTTCCT - 3’	
β2AR fwd	5’ - CTTCTGGTGCGGAGTTTTGGAC - 3’	NM_000024
β2AR rev	5’ - AATCTTCTGGAGCTGCCTTTT - 3’	

3.2.10. Software

BEP® 2000 SW V.1.23.4 (ELISA-Reader)

CellQuest® BD (FACSCalibur)

Excel 2002 Microsoft

Graph Pad Prism Software Version 4.02 (Statistical analysis + Grafics)

Microsoft Office PowerPoint 2003

Microsoft Office Word 2003

QC-Net (Unity Real Time) Bio Rad (Nephalometer analysis)

Roche Molecular Biochemicals Light Cycler Software Version 3.5 (LightCycler)

Windows 2002 Microsoft

WinMDI 2.9 (analysis of FACS data)

3.3. Methods

3.3.1. Experiment set 1: Autoantibody detection by flow cytometry on the surface of differentiated autonomic cells

For this group of experiment, serum samples of controls, CRPS patients or PNP/NL were tested in non-neuronal cells, autonomic primary neurons, undifferentiated and autonomic differentiated neuroblastoma cells for surface binding using basically flow cytometry (FACS). Isolation of neurons from rats were approved by the local ethical committee and followed International Guidelines for the Care of Research Animals.

3.3.1.1. Cell culture

The neuroblastoma cell line SH-SY5Y, which originates from a tumour of the sympathetic nervous system, were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1% penicillin, 1% streptomycin. The cultures were maintained in a 37°C humidified atmosphere of 95% air and 5% CO_2. To test for surface autoantibody binding, we used the SH-SY5Y undifferentiated or differentiated according to the protocol given below. The epithelial cell line HEK 293 cultured in the same conditions served as a non-neuronal control cell line. We additionally tested primary cultures of sympathetic neurons and gut myenteric plexus neurons from rats isolated and cultured as described below. The experiment was approved by the local ethical committee and followed International Guidelines for the Care of Research Animals.

3.3.1.2. Differentiation of neuroblastoma cells

For differentiation experiments, SH-SY5Y cells were plated at 20,000 cells/cm^2 and cultured for 2 days prior to differentiation. Cells were differentiated by incubation with the following agents for 4 days: all-trans retinoic acid at 10 µM; BMP-2 at 10 ng/mL; transforming growth factor (TGF)-β1 at 10 ng/mL. We also used combinations of RA and BMP-2 or RA and TGF-β1 under the same conditions.

3.3.1.3. Isolation of sympathetic neurons

Adult Wistar rats were anaesthetised with halothane and sacrificed by cervical dislocation. The whole vertebral column was then removed and the sympathetic trunk on either side was dissected over its full length including cervical and lumbar ganglia. All ganglia were cut out of the trunk, cleaned from connective tissue and placed into calcium- and magnesium-free phosphate-buffered saline (PBS). The ganglia were incubated in 6 mg/mL collagenase Worthington type CLS II and 2 mg/mL trypsin type III-S for 40 min in a shaking water bath at 37°C. Afterwards, the ganglia were washed three times with plating medium (minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% penicillin / streptomycin and 1% glutamine). Cells were then dissociated by repeated passages through three Pasteur pipettes with decreasing diameter, and then stored in plating medium under a humidified 5% CO_2 atmosphere at 37°C.

3.3.1.4. Isolation of gut myenteric plexus

As described previously in (Schafer, Klotz et al. 2000), myenteric plexus from 12- or 13-day-old Wistar rats was obtained. After decapitation the whole gut was removed and stored on ice-cold MEM eagle. In the small intestine the smooth muscle layer was separated from the submucosal layer under microscopic control at 15x magnification. The colon was flushed, opened and incubated as a whole in 1 mL of a Collagenase solution (Worthington CLS II, 1 mg per mL in : Hank's balanced salt solution) at 37°C for 1.5 h. After the digestion step the vials were mechanically agitated using a vortex. The already visible plexus pieces were collected and transferred into another petri dish with ice-cold MEM. The remaining undigested tissue was re-digested with a fresh collagenase solution at 37°C for another 15 min. The procedure was repeated again until muscle pieces were digested. Finally, the plexus pieces were collected and centrifuged at 1000 rpm for 2 min. The supernatant was decanted and the pellet was incubated with 1 mL trypsin for 5 min. After centrifugation at 1000 rpm for 10 min, the trypsin solution was replaced by Dulbecco's Modified Essential Medium (DMEM) containing 10%

fetal calf serum (FCS), 1% penicillin / streptomycin and 1% glutamine. Then the plexus was dissociated by aspirating through a 27-gauge needle.

3.3.1.5. Autoantibody detection by flow cytometry

We introduced flow cytometry (FACS) to detect autoantibodies in neuroimmunological diseases (Blaes, Beeson et al. 2000). To avoid unspecific binding to ubiquitous antigens, we pre-absorbed the sera by incubating in PBS containing 1% FCS and 0.1% NaN_3 (FACS buffer) with HEK 293 cells for 24 h at 4^oC (serum dilution 1/50) prior to incubation of the primary cell cultures. The different cell lines and primary cultures described above were then incubated with the pre-absorbed HC, CRPS or PNP/NL patients' sera for 30 min at 4^oC, washed, and incubated again with FITC-conjugated anti-human IgG (30 min, 4^oC in dark). After washing again, binding was analyzed in a FACScalibur using CellQuest® software. After measurement of the mean fluorescence intensity (MFI), the mean MFI of the controls of each experiment has been set as 100%. For each serum, the percentage of difference from the controls was calculated. This standardization is necessary since the binding, given as MFI, of patients sera to a mixed primary cell culture can vary between the experiments. A percentage above mean + 2.5 standard deviations of the controls was considered positive. To avoid irrelevant binding to intracellular antigens, we tested the primary cell cultures with trypan blue for viability and also measured binding of anti-neurofilament antibody to non-permeabilized cells.

3.3.1.6. Identification of neuronal cells

To show neuronal specific-binding, cells were incubated with the patients sera and FITC-conjugated anti-human IgG as described above, fixed with 2% paraformaldehyde (20 min, 4^oC), permeabilized with FACS-buffer containing 0.1% saponine and then incubated with the neuronal-specific anti-HuD antibody. Cells were than gated according to their reactivity to the neuronal anti-HuD protein and only positive (neuronal) cells were analyzed in flow cytometry.

3.3.1.7. PCR experiments

Total RNA was isolated using RNeasy Mini Kit as per manufacturer's instructions. RNA quantity was determined with a BioRad spectrophotometer. Total RNA (1 µg) was subjected to reverse transcription using Oligo(dT)18 Primer. The RNA/primer mixture was denatured at 70°C for 5 min before the addition of a MasterMix containing 1 µL RiboLock™ RNase Inhibitor (20 u/µL), 2 µL of 10 mM dNTP mix, 4 µL 5× Reaction buffer and 1 µL RevertAid™ M-MuLV Reverse Transcriptase (200 u/µL) in diethylpyrocarbonate treated H2O. The reactions were incubated for 60 min at 42°C before quenching for 10 min at 70°C. cDNA was stored at 4°C until further use. Primer sequences used for PCR were designed using Primer Premier 5® software. Expression of dopamine-β-hydroxylase (DßH) and vesicular acetylcholine transporter (VAChT) was assessed by polymerase chain reaction (RT-PCR). Primer sequences used are DßH: F: 5' - ACTGTCCACTTGGTCTACGGG - 3' and R: 5' - CGGTCGGGTTTCATCTTG - 3'; VAChT: F: 5' - GGGGCATCCTCTATGAGTTCG - 3' and R: 5' - GTGGCAATGGTGGGTTCG - 3'; and porphobilinogen deaminase (PBGD): F: 5' - TGCAACGGCGGAAGAAAAC - 3' and R: 5' - GGCTCCGATGGTGAAGCC - 3'. PBGD was used as a housekeeping gene because its level is not affected by the differentiation protocol used. Each PCR amplification contained 1 µL cDNA, 2.5 µL 10x reaction buffer, 1 µL each of forward and reverse primers for gene of interest and 0.5 µL Taq polymerase. The reaction was supplemented with nuclease free H2O to bring the volume to 25 µL. Thermal cycling conditions used are as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30s, 72°C for 30 sec and a final extension at 72°C for 5 min. A negative template control containing 1 µL nuclease free H_2O was included for each set of primers. To confirm presence of product and amplicon size, PCR products were separated on 1% agarose gel and stained with ethidiumbromide.

3.3.2. Experiment set 2: Identification of putative autoantigens targeted by CRPS serum antibodies

In this section, we randomly selected 10 HC, 20 CRPS, 10 PNP/NL patients, no matter whether positive or negative in previous experiments, and isolated IgGs. Affinity-purified IgGs were tested in a well-established method to detect autoantibodies against autonomic receptors, the spontaneously beating neonatal rat cardiomyocytes assay (Wallukat and Wollenberger 1987; Wallukat and Wollenberger 1991). Further, target protein sequences were identified and re-tested in an Enzyme immunoassay.

3.3.2.1. Cell culture

Chinese hamster ovary (CHO) cells wild-type or overexpressing human muscarinic 2 receptor (kindly provided by Dr. Mohr - Bonn, Germany), β1- or β2-adrenergic receptor (kindly provided by Dr. Gerd Wallukat - Berlin, Germany) were cultured in DMEM/HAMs F-12 containing 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin and 1% streptomycin. The medium for stable transfected cells was enriched additionally with geneticin (63 mg/L) as selection antibiotic. SY5Y neuroblastoma cells were cultured and differentiated as described before.

3.3.2.2. Affinity chromatography

Serum was diluted 1:3 with glycine buffer (0.1 Mol, pH 9), applied to a protein G-sepharose column (HiTrap - GE) that binds exclusively IgG, and the rest of the serum was washed out using glycin buffer (pH 9). IgG-fractions were eluted by changing the pH from 9 to 2.7. The IgG-concentration was determined by nephelometry (Boehring). Each IgG-fraction was dialysed against PBS to eliminate the glycin and increase the pH up to 7.4

3.3.2.3. Preparation of cardiomyocyte cultures

Neonatal rat cardiomyocytes were isolated and cultured as described by Wallukat and Wollenberger (Wallukat and Wollenberger 1987). The experiment was approved by the local ethical committee and followed International Guidelines for

the Care of Research Animals. Hearts were removed aseptically from 1- to 2-day-old Wistar rats. Single cells were dissociated from the minced heart ventricle with a 0.25% solution of trypsin. The cardiomyocytes were cultured as monolayers with a density of 800 cells/mm^2 on the bottom of 45-mL Muller bottles (1.2x 10^6 seeded cells in 3 mL medium) in Halle SM20-I medium containing 10% heat-inactivated calf serum and 2 µmol/L fluorodeoxyuridine, the latter to prevent proliferation of nonmuscle cells. The flasks were attached to a plate that was continuously rocked back and forth at two cycles of 30 each per min to ensure an adequate gas and metabolite exchange. After first 24 h of incubation, additional 400 µL of fresh medium was added to each flask, and cells were further cultivated for additional 4 days before the experiments.

3.3.2.4. Peptides

Peptides corresponding to the sequence of the second extracellular loop of human M2R (residues 169-193): VRTVEDGECYIQFFSNAAVTFGTAI; and, β2AR (residues 172-197): HWYRATHQEAINCYANETCCDDFFTNQ were synthesized by Thermo Fisher Scientific (Ulm, Germany) in short overlapping peptides containing 6 to 7 amino acids each, and used as described below in each experimental section.

3.3.2.5. Pharmacological assays

The flasks containing spontaneously beating cardiomyocytes were transferred to the heatable stage of an inverted microscope. The contraction rate of seven to ten clusters of synchronously beating cells - in selected ten fields per flask - were counted for 15 s, which served as the basal beating rate. This procedure was replicated in different culture to yield the results, representing a total of up to 30 cells or clusters for each sample of given immunoglobulin. The basal beating rate was 140 ± 20 beats per min. The compounds to be tested were dissolved in the same medium used for the determination of the basal beating rate. Cardiomyocytes were incubated with IgG of HC, CRPS, or PNP/NL at a final concentration of 60 mg/L. Changes in beating frequency of each selected field

were measured 1 h after the addition of IgGs. At this point (without removing the previously added IgG), adrenergic/cholinergic agonist/antagonist drugs (carbachol 10 µM, atropine 1 µM, ICI 118.551 0.3 µM, clenbuterol 3 µM or propranolol 1 µM) were applied to the beating cultures and, after five minutes, beating frequencies were measured again. Additionally, we tested the effect of a selective β1AR-antagonist (bisoprolol 1 µM) and endothelin 1 receptor antagonist BQ-610 (1 µM). All drugs were obtained from Sigma.

In different experiments, we preincubated the cardiomyocytes with antagonists - propranolol or atropine – to block the β2AR or M2R before adding immunoglobulins. This procedure was important to analyze the isolated effect of each pathogenic antibodies since they showed opposite effect on cardiomyocytes.

To map the epitopes in the first, second or third extracellular loops of both receptors, immunoglobulins were preincubated with each short sequence of overlapping peptides of both receptors for 1 h at 4°C, at a final concentration of 1.5 g/L of antibody and 10 mg/L of the peptides. IgG/peptide solution was added to cardiomyocytes (preincubated either with atropine or propranolol) and beating frequencies were then measured after 60 minutes as described above.

To test whether there is cross reactivity between antibodies that bind β2AR or M2R, we preincubated IgG of CRPS patients with sequences of β2AR or M2R peptides, and in parallel incubated cardiomyocytes with propranolol (1 µM) or atropine (1 µM). The intention was to determine if those antibodies responsible for the activation of M2R can be blocked by peptides of the β2AR or vice versa.

3.3.2.6. Enzyme immunoassay

NUNC microtiter plates were coated with 50 µg/mL of synthetic peptides corresponding to the sequences of the second extracellular loop of both the human β2AR and M2R in 100 mM carbonate–bicarbonate buffer (pH 9.6) overnight at 4°C. In order to avoid unspecific binding, blocking solution (phosphate buffered saline + 0.1% Tween 20 – PBST - supplemented with 5% BSA) was added to the wells and the plates maintained at room temperature for 1 h. After three washings, affinity-purified 60 mg/L of immunoglobulins from CRPS patients and HC were allowed

to adsorb on the peptides for 2 h at room temperature. After washing the wells three times with PBST, bound antibodies were revealed by successive 2 h incubation at room temperature with a peroxidase conjugated anti-human IgG (diluted 1:6000 in PBST). This was again followed by three washing procedures, and then a TMB peroxidase substrate was added. After 7 minutes, 100 µL of 1 N HCl were added to stop the reaction. The optical density was measured at 450 nm in an ELISA plate reader.

3.3.3. Experiment set 3: Determination of immunoglobulin subclasses of CRPS pathogenic antibodies

Autoantibodies against the β2AR and the M2R belong to the IgG class. IgG includes the subclasses IgG1-4. These subclasses differ immunologically and functionally, and the subtype identification of pathogenic antibodies might indicate which T helper (Th) cell subset is mostly involved in an autoimmune response, and whether autoantibodies may be able to bind complement. Thus, to determine the immunoglobulin subclasses of pathogenic antibodies we incubated affinity-purified immunoglobulin samples with mouse anti-human IgG1, -2, -3 and -4 antibodies for 1 h at 4°C, centrifuged and used the supernatant to assess changes in the beating rate of heart muscle cells in the same conditions as described above.

3.3.4. Experiment set 4: CRPS pathogenic antibodies bind to the surface of stable transfected cells

In order to determinate whether CRPS autoantibodies identify autoantigens transfected in cells that do not constitutively express them, we performed flow cytometry assay using wild type and stable transfected CHO cells.

3.3.4.1. Antibody binding measured by flow cytometry

We introduced flow cytometry (FACS) assay to detect autoantibodies in Experiment set 1. Binding experiments in this assay have been done using isolated IgG fractions instead sera. To avoid unspecific binding to ubiquitous antigens, we pre-absorbed the IgGs by incubating them in PBS containing 1% FCS and 0.1%

NaN3 (FACS buffer) with wild type CHO cells for 24 h at 4°C (serum dilution 1/50) prior to incubation with receptor-transfected cells. Wild type and receptor-transfected CHO cells were then incubated with the pre-absorbed IgGs for 30 minutes at 4°C, washed, and incubated again with FITC-conjugated anti-human IgG (Dako) (30 minutes, 4°C in dark). After washing again, binding was analyzed in a FACScalibur using CellQuest® software. Mean fluorescence intensity above mean + 2.5 standard deviations of the controls for each cell line was considered positive. To avoid irrelevant binding to intracellular antigens, we tested cell cultures with trypan blue for viability and membrane integrity.

3.3.4.2. PCR experiments

We checked additionally the expression of β2AR and M2R in SH-SY5Y cell differentiated with BMP-2 and/or RA using the method describe above. Primer sequences used are β2AR: F: 5' - CTTCTGGTGCGGAGTTTTGGAC - 3' and R: 5' - AATCTTCTGGAGCTGCCTTTT - 3'; M2R: F: 5' - CCAATGCTGCTGTCACCT - 3' and R: 5' - TTGGCTTCACTATCCTTCCT - 3'; and porphobilinogen deaminase (PBGD): F: 5' - TGCAACGGCGGAAGAAAAC - 3' and R: 5' - GGCTCCGATGGTGAAGCC - 3'. RNA isolation and PCR conditions were same as described in Experiment set 1. The annealing temperature for these primers was set at 55°C.

3.3.5. <u>Experiment set 5:</u> Functional assays

3.3.5.1. Functional cardiomyocyte assay

Affinity purified antibodies from CRPS patients were added to the medium of spontaneously beating cardiomyocytes - pre-treated either with atropine or propranolol - and their effect was investigated as a function of time. IgG-induced changes in beating rate were evaluated after 5, 60, 120, 180 and 400 minutes. Afterwards, antagonists (0.3 µM ICI 118,551 or 1 µM atropine) were add to the solution, after 5 minutes washed out for more 5 minutes, and then the agonists (3 µM clenbuterol or 10 µM carbachol) were added.

3.3.5.2. Receptor expression

hβ2AR- and hM2R-transfected CHO cells were incubated with HC or CRPS IgGs for 24 h and 48 h at 37°C, harvested and incubated with anti- β2AR antibodies (Abcam) or anti-M2R antibodies (Santa Cruz Biotechnology). The surface expression of receptors was analyzed using flow cytometry as described above. The experiment was performed in triplicate.

3.3.5.3. Calcium imaging

Relative changes in the intracellular Ca^{2+} concentration were measured using the Ca^{2+}-sensitive fluorescent dye fura-2, as described previously in (Haschke, Schafer et al. 2002). The maximum excitation wavelength of fura-2 shifts in dependence of the cytoplasmatic Ca^{2+} concentration. CHO cells (wild type or expressing hβ2AR or hM2R) grown on glass slides were loaded for 60 minutes with 5 µmol/L fura-2-acetoxymethylester (fura-2/AM) in the presence of 0.1 g/L pluronic acid. Fura-2 was then washed away. Further, glass slides were transferred into the experimental chamber with a volume about 1 mL, and then cells were superfused hydrostatically with tyrode solution (1 mL/min). The baseline in the fluorescence ratio of fura-2 was measured for some minutes before IgG or drugs were administered.

At this point, HC or CRPS IgG were added to the cells at a final concentration of 60 mg/L, and the effect was recorded for approximately ten minutes. The agonist clenbuterol (30 µM) for hβ2AR-CHO and bethanechol (100 µM) for hM2R-CHO were added to the cells - without washing the previously added IgG - and changes in intracellular Ca^{2+} concentration were measured for further five minutes. Additionally, in another set of experiments, cells were pre-treated with antagonist propranolol (10 µM) for hβ2AR-CHO or atropine (10 µM) for hM2R-CHO five minutes before addition of IgGs and agonists. Experiments were carried out at room temperature on an inverted microscope (Olympus IX-50) equipped with an epifluorescence and an image analysis system (Till Photonics). The emission above 470 nm was measured from several regions of interest, each with the size of

about one cell. The cells were excited alternatively at 340 and 380 nm and the ratio of the emission signal at both excitation wavelengths was calculated. Data were sampled at 0.33 Hz.

3.3.6. Experiment set 6: Cytotoxicity of pathogenic autoantibodies

Here we tested whether surface binding autoantibodies are able to induce cytotoxicity in receptor-transfected cells.

3.3.6.1. LDH

Cytotoxicity was assessed with a lactate dehydrogenase (LDH)-cytotoxicity detection kit, which measures LDH activity released from the cytosol of damaged cells. Cells were grown in 96-well culture plates, at 37°C, for 24 h to near confluence. Cells grown in 96-well plates were exposed to IgGs (120 mg/L) for 24 h. After that, 100 µL of supernatant per well was harvested and transferred into a new 96-well, flat-bottom plate. LDH substrate (100 µL) was added to each well and incubated for 30 minutes at room temperature protected from light. The absorbance of the samples was measure at 490 nm with an ELISA reader. Cytotoxicity was calculated with the formula: % cytotoxicity = (experimental value - low control) x 100/(high control - low control), where low control is assay medium plus cells and high control is assay medium (plus 2% Triton X-100) plus cells.

3.3.6.2. WST-1 assay

The cell proliferation reagent WST-1 is a water-soluble tetrazolium salt that can be used for cell proliferation or cell viability assays. The rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells in the culture. This assay is appropriate not only for the measurement of cell proliferation and growth inhibition, but also for determination of the cytotoxic effect in response to growth factors, cytokines, mitogens or antibodies. CHO cells were plated in 96 well plates containing 100 µL of DMEM medium and cultured for 2 days prior to the stimulation with 120 mg/L IgGs. After 24 h stimulation, WST-1 reagent is added directly to the cells (1/10 of the culture volume) and absorbance measured at 450

nm using an ELISA plate reader following 1 h, 2 h and 3 h incubation at 37°C in a humidified atmosphere.

3.3.7. Statistical analysis

Frequency of autoantibodies and comparison with clinical data was analyzed by Fisher's exact test. Surface binding, ELISA optical densities and changes in the beating rate or in the fura-2 ratio were in part analyzed by analysis of variance (ANOVA) for multiple comparisons followed by Turkey's test for a posteriori comparison of means, or Student's t-test as appropriated. All parametric tests, even those including groups with small sample size (e.g. $n < 10$), were performed after validating the normal distribution of the datasets using the Kolmogorov-Smirnov test; and, the equal variances using Bartlett's test. For the remaining data, we applied the Kruskal-Wallis H test or Friedman test with post Dunn's multiple comparison testing, or Mann Whitney test as appropriated. Unless indicated in the figure legend or elsewhere, data were expressed as mean + S.E.M. The degree of linear relationship between ELISA optical density values for different peptides was tested by Pearson's correlation since all data showed normal distribution; and, between ELISA optical density values for different peptides, changes in the beating rate or the presence of surface binding antibodies and clinical data of CRPS patients were tested by Spearman's correlation. All statistical analyzes were performed using Prism® 4.02 Software (Graph Pad Inc.). The probability level accepted for significance was $p < 0.05$.

IV – RESULTS

4.1. Experiment set 1: Serum autoantibodies in complex regional pain syndrome bind to a differentiation-dependent neuronal surface-autoantigen

4.1.1. Patients

Serum was obtained from 30 patients with clinically defined CRPS (20 CRPS 1, 10 CRPS 2, according to the revised IASP diagnostic criteria (Harden, Bruehl et al. 2007); mean age 48.5 ± 11.8 years; 19 female, 11 male). Sera of 30 HC, 20 NP (10 diabetes type II, 5 hereditary sensorimotor neuropathy, 5 alcoholic neuropathy) and 20 NL patients (6 carpal tunnel syndrome, 6 radial nerve lesion after upper arm fracture, 4 patients with idiopathic plexus neuritis, 4 pressure palsy of the peronaeal nerve) served as controls. The control groups were age- and sex-matched (HC 52.6 ± 12.4 years, NP 58.4 ± 14.0, NL 54.5 ± 17.4). None of the healthy controls or NL and 11/20 NP patients had a history of chronic neuropathic pain. The mean duration of CRPS symptoms in our patients' group was 25.1 ± 23.8 weeks and 287.4 ± 155.5 weeks for the NP group. The clinical and epidemiological data of the CRPS patients are given in Table 10. Twelve of the CRPS patients have been described elsewhere (Blaes, Schmitz et al. 2004).

Table 10. Clinical and epidemiological data of the CRPS patients.

Age	52.8 ± 13.8
Sex	25 f / 15m
CRPS 1 / CRPS 2	20 / 10
Affected limb (upper / lower)	24 / 6
Surgery	17/30
Motor impairment	21/30
Sensory impairment	20/30
Trophic changes	24/30
Time to serum analysis[1] (weeks)	25.1 ± 23.8

[1] *time between onset of symptoms and obtaining of serum*

4.1.2. Surface binding to primary cultured autonomic neurons

Both primary cultures (SN and MPN) showed a high purity for neuronal cells (70.1 – 88.5 % neurons in sympathetic neuronal culture and 68.7 – 83.3 % neurons in myenteric plexus culture). Sera have been tested for surface binding to sympathetic neurons (SN) and myenteric plexus neurons (MPN). Only binding to neuronal cells identified by anti-HuD antibody was considered positive. Flow cytometry analysis (Figure 4A) revealed autoantibody binding to SN or MPN in 13/30 (43.3%) of the CRPS patients, but only in 1/20 NP patients, 1/20 NL patients and none of the healthy controls (one-way ANOVA, $p < 0.0001$). Nine patients showed binding to both SN and MPN, sera of two patients bound exclusively to SM, but not to MPN, and two CRPS sera contained autoantibodies against MPN, but not SN (shown for binding to SN, Figure 4B).

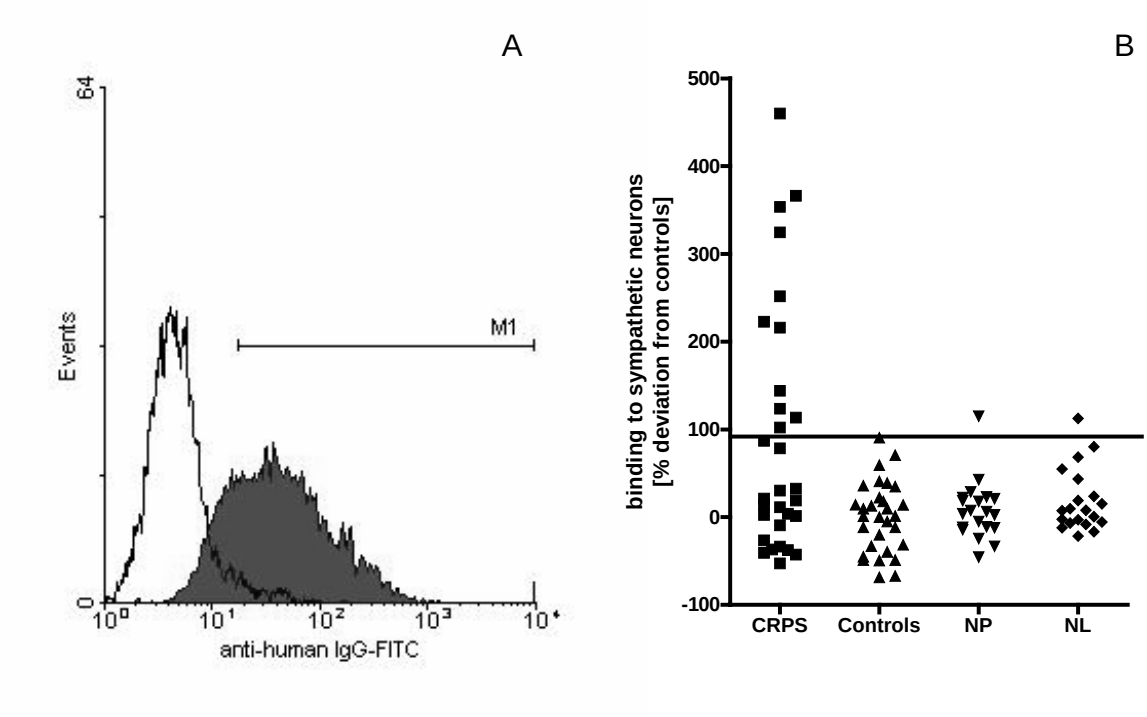


Figure 4. Surface-binding antibodies detected by flow cytometry using sympathetic neurons. (A) Binding of a CRPS serum (filled graph) and a control serum (line graph) to sympathetic neurons. (B) Binding to sympathetic neurons (expressed as percent difference to the mean binding of the controls) after pre-incubation of the sera with HEK 293 cells to remove unspecific binding. Cut-off, marked as horizontal line, was determined as mean of the controls + 2.5x std dev. CRPS patients have more and higher binding to SN than controls, neuropathy patients (NP) or peripheral nerve lesions patients (NL).

4.1.3. Binding to undifferentiated SH-SY5Y neuroblastoma cells

Only 20% (6/30) of the CRPS patients, one of the NP patients and none of the other control groups showed binding to undifferentiated SH-SY5Y neuroblastoma cells (one-way ANOVA, $p < 0.01$, Figure 5).

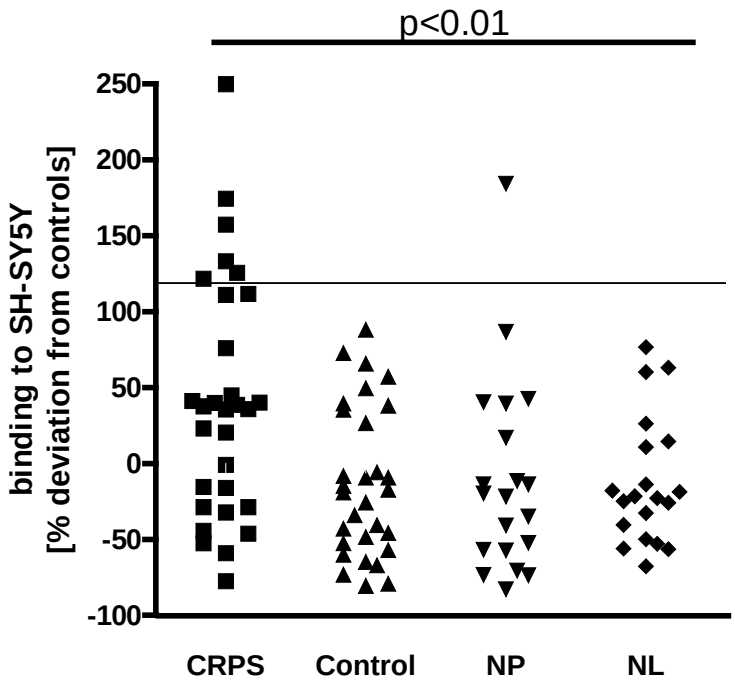


Figure 5. Binding of CRPS and control sera to undifferentiated SH-SY5Y cells measured by flow cytometry. ANOVA, $p < 0.01$. Cut-off, marked as horizontal line, was determined as mean of the controls + 2.5x std dev.

4.1.4. Binding to differentiated SH-SY5Y neuroblastoma cells

Binding to primary cultured (and well-differentiated) autonomic neurons was detectable in more than twice the patients compared to undifferentiated SH-SY5Y. Therefore we differentiated SH-SY5Y neuroblastoma cells into different neuronal phenotypes to test whether the recognized antigen is differentiation-dependent or not. We used a variety of differentiation agents (RA, TGF-β1, TGF-β1/RA, BMP-2 or BMP-2/RA) and checked the differentiated neurons for cholinergic and catecholaminergic markers. Dopamine-β-hydroxylase was induced by differentiation with TGF-β1, but strongly downregulated by BMP-2/RA differentiation (Figure 6).

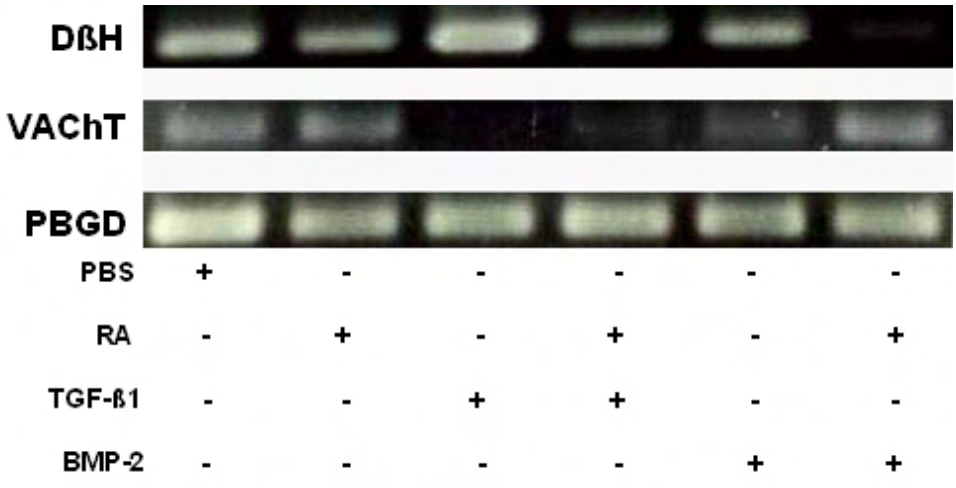


Figure 6. PCR characterization of differentiated SH-SY5Y cells. Different agents induce differentiation of SH-SY5Y cells into two different neuronal phenotype. SH-SY5Y cells were differentiated using TGF-β1, RA, BMP-2 alone or in combinations. After differentiation total RNA was prepared and subjected to cDNA synthesis. This cDNA was used for PCR amplification of VAChT, DβH and PBGD.

In contrast, the cholinergic marker vesicular acetylcholine transporter (VAChT) was downregulated using TGF-β1 and slightly upregulated by RA and BMP-2/RA differentiation. This indicates that TGF-β1 induces a more catecholaminergic neuronal phenotype, whereas BMP-2/RA induces a cholinergic neuronal phenotype. Then CRPS and control groups were incubated with the differentiated neurons to detect specific surface-binding. CRPS and controls did not show significant binding to either TGF-β1-differentiated, RA-differentiated or BMP-2-differentiated SH-SY5Y cells (Figure 7A-C). There was even a tendency to a lower binding of the CRPS sera to the TGF-β1- and the TGF-β1/RA-differentiated cells (Figure 7B,C). However, if SH-SY5Y cells are differentiated with a combination of RA and BMP-2 - inducing a cholinergic neuronal phenotype, 60.0% (18/30) of CRPS sera, but only one of the NL patients and none of the other control patients showed any significant surface-binding (one-way ANOVA, $p < 0.001$, Figure 7D).

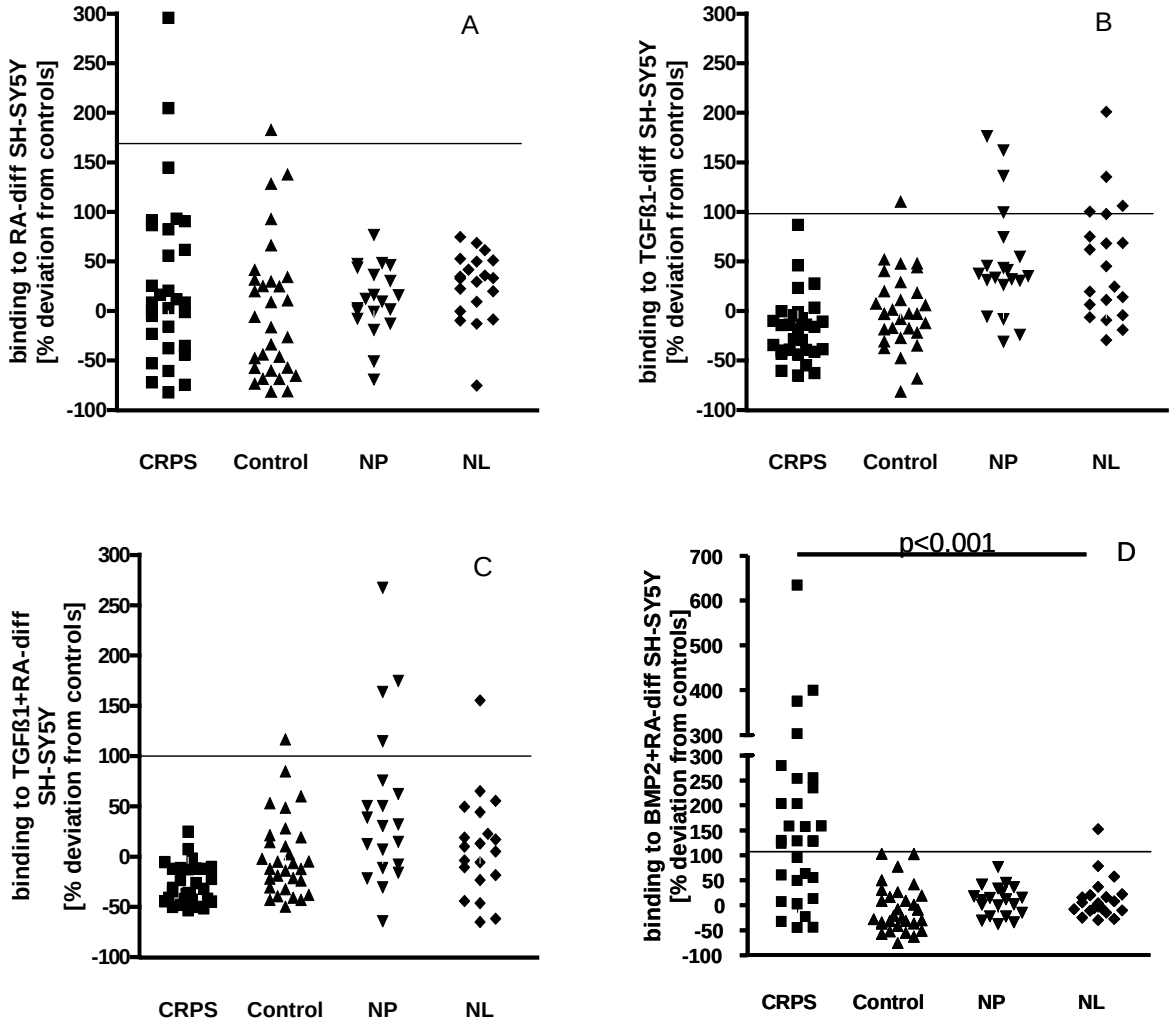


Figure 7. Surface binding of autoantibodies to differentiated SH-SY5Y neuroblastoma cells. SH-SY5Y cells differentiated with (A) RA, (B) TGF-β1, (C) TGF-β1/RA, or (D) BMP-2/RA were incubated with CRPS and control sera and their binding was analyzed by flow cytometry. Only BMB-2/RA-differentiated cells express a surface autoantigen recognized by CRPS sera. (A-C) ANOVA, $p > 0.05$. (D) ANOVA, $p < 0.001$. RA = Retinoic acid; TGF-β1 = Transforming growth factor beta 1; BMP-2 = Bone morphogenic protein-2.

4.1.5. Association with clinical data

We compared surface-binding in CRPS with clinical and epidemiological data. Patients with and without surface-binding autoantibodies to primary cultured autonomic neurons or differentiated cells did not differ in any epidemiological or clinical features tested (Table 11).

Table 11. Clinical data of surface-binding positive and negative patients.

Feature	**Surface-binding (+)** (n = 13)	**Surface-binding (-)** (n = 17)	***p***
Age	52.5 ± 15.6	50.6 ± 10.8	n.s.
Sex	8f / 5m	11f / 6m	n.s.
CRPS type	6 CRPS 1 7 CRPS 2	14 CRPS 1 3 CRPS 2	n.s.
Affected limb	12 upper / 1 lower	12 upper / 5 lower	n.s.
Operation	6/13	11/17	n.s.
Motor impairment[1]	8/13	13/17	n.s.
Sensory impairment[2]	8/13	12/17	n.s.
Skin Temperature[3]	7/10*	10/16*	n.s.
Hair growth[3]	5/10*	6/16*	n.s.
Sweating abnormalities[3]	4/10*	8/16*	n.s.
Time to serum analysis[4] (weeks)	37.6 ± 35.3	20.9 ± 19.2	n.s.

*Fisher's exact test, $p > 0.05$. [1]any paresis in the affected limb, not related to nerve lesion; [2]any sensory disturbances, not related to nerve lesion; [3]increased or decreased; [4]time between onset of symptoms and obtaining of serum; *information not available from all patients*

4.2. Experiment set 2: CRPS immunoglobulins bind to the surface of cardiomyocytes on the M2R and β2AR

4.2.1. Patients

Serum was obtained from 20 clinically diagnosed CRPS patients, according to the IASP diagnostic criteria (Harden, Bruehl et al. 2007) (10 CRPS 1, 10 CRPS 2; mean age 48.5 ± 13.8 years; 12 female, 8 male), who all had autonomic symptoms such as sweating abnormalities or vasomotor changes on the affected limb. Sera of 10 HC, 9 patients with NP (n = 4) or NL (n = 5) served as age- and sex-matched controls. None of the HC, but 4/9 PNP/NL patients had a history of chronic neuropathic pain. The clinical and epidemiological data of the CRPS patients are given in Table 12.

Table 12. Clinical and epidemiological data of the CRPS patients.

Age	48.5 ± 13.8
Sex	12 f / 8 m
CRPS 1 / CRPS 2	10 / 10
Warm / Cold CRPS	15 / 5
Pain Score (Median)	4 (25% Percentile = 0, 75% Percentile = 7)
Affected limb (upper / lower)	18 / 2
Surgery	12/20
Trauma	11/20
Motor impairment[1]	14/20
Sensory impairment[2]	10/20
Trophic changes	11/20
Time to serum analysis[3]	
Minimum	2 weeks
25% Percentile	6 weeks
Median	14.5 weeks
75% Percentile	39 weeks
Maximum	80 weeks

[1]any paresis in the affected limb, not related to nerve lesion, [2]any sensory disturbances, not related to nerve lesion, [3]time between onset of symptoms and obtaining of serum.

4.2.2 - CRPS immunoglobulins exhibit agonistic effects on the M2R and the β2AR in spontaneously beating cardiomyocytes

The addition of affinity-purified IgG fractions of CRPS patients (60 mg/L) changed the beating frequency of spontaneously beating neonatal cardiomyocytes (Figure 8), whereas healthy and disease control (PNP/NL) IgG fractions did not induce substantial change (deviation from basal beating rate: controls 1.02 ± 0.70, n = 10; CRPS 9.78 ± 10.57, n = 20; PNP/NL 0.88 ± 0.66, n = 9, ANOVA, *p* = 0.0041).

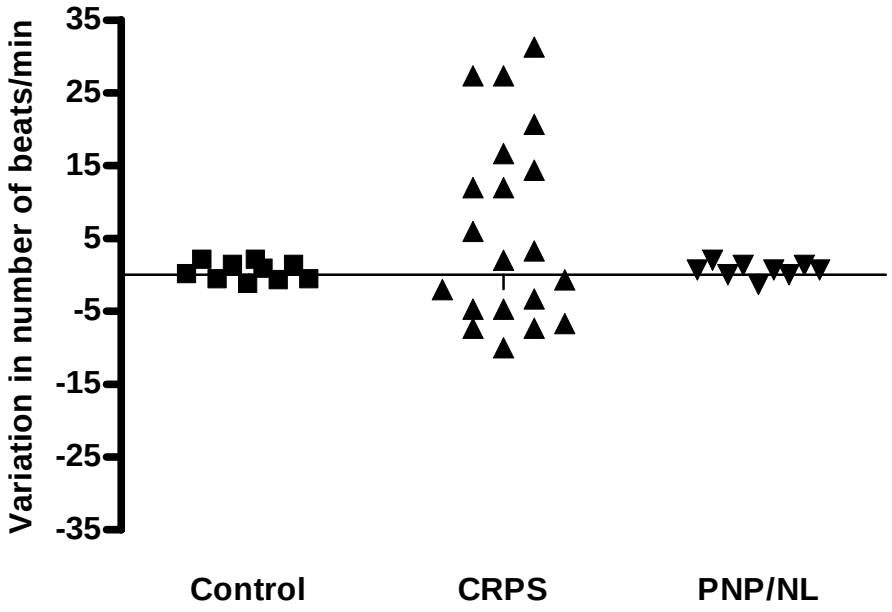


Figure 8. Functional effect of immunoglobulins (60 mg/L) of controls (n = 10), CRPS patients (n = 20) or PNP/NL patients (n = 9) on spontaneously beating cardiomyocytes. CRPS antibodies induced changes (between -10.00 to 31.32 beats/min) in the beating frequency whereas controls and PNP/NL induced only very small changes (not higher than the ± 2.0 beats per min). ANOVA, *p* = 0.0041.

The neonatal cardiomyocyte model was established to measure and identify effects on different receptors, and a positive chronotropic action of CRPS IgG suggests an activation of beta-adrenergic receptors (Wallukat and Wollenberger 1987). Therefore, we tested the IgG effect with the nonselective beta-antagonist propranolol at 1 µM, which does not exhibit an intrinsic effect on the receptor at this dose (Wallukat and Wollenberger 1987).

We confirmed indeed the lack of intrinsic activity of 1 µM propranolol in cells treated with control IgGs (Figure 9). Instead a simply inhibition of the positive chronotropic effect of CRPS IgG by propranolol, we found a significant decrease in the beating rate below the control levels, suggesting that a second effect, previously masked, was present in the CRPS IgG fraction (controls 0.12 ± 0.43, n = 10; CRPS -15.55 ± 1.28, n = 20; PNP/NL 0.36 ± 0.36, n = 9, Kruskal-Wallis test, p = 0.00001) (Figure 9). The addition of atropine (1 µM – which also has no intrinsic effect at this dose) led to a complete normalization of the beating rate (controls 0 ± 0, n = 10; CRPS 0.2 ± 0.55, n = 20; PNP/NL 0 ± 0, n = 9; Kruskal-Wallis test, p = 0.9975) (Figure 9), whereas the addition of 1 µM atropine to the IgG solutions without propranolol shifted the beating rate in 18/20 (90%) samples to a positive chronotropic effect, but had no influence on the control IgG fractions (Figure 10). Thus, it appeared that there were two independent effects in the IgG fraction of CRPS patients.

To analyse the effect of each pathogenic activity, since they showed opposite properties, in further experiments we preincubated the cardiomyocytes with antagonists - propranolol or atropine - to block β2AR or M2R, respectively, before adding the IgG fractions.

The positive chronotropic effect of IgG, in cells pre-treated with atropine, could be blocked by the beta2-specific antagonist ICI 118.551 (0.3 µM), but not with the beta1-specific bisoprolol (1 µM) (Figure 11A), strongly implying that the β2AR is one target antigen in CRPS. Blocking by atropine of the negative chronotropic effect of CRPS IgG, in cells pre-treated with propranolol, suggested that muscarinic acetylcholine receptors must be another antigenic target (Figure 11B). The main

muscarinic receptor expressed on cardiomyocytes is the M2 subtype (Hosey 1992). Since endothelin receptors are also expressed on cardiomyocytes (Allen, Phuong et al. 2003), and their stimulation can induce marked decrease in cardiovascular function (Miller, Redfield et al. 1989), the endothelin receptor antagonist BQ-610 (1 µM) was used in propranolol-pretreated cells to validate that the negative chronotropic effect is not mediated by endothelin receptors (Figure 11B).

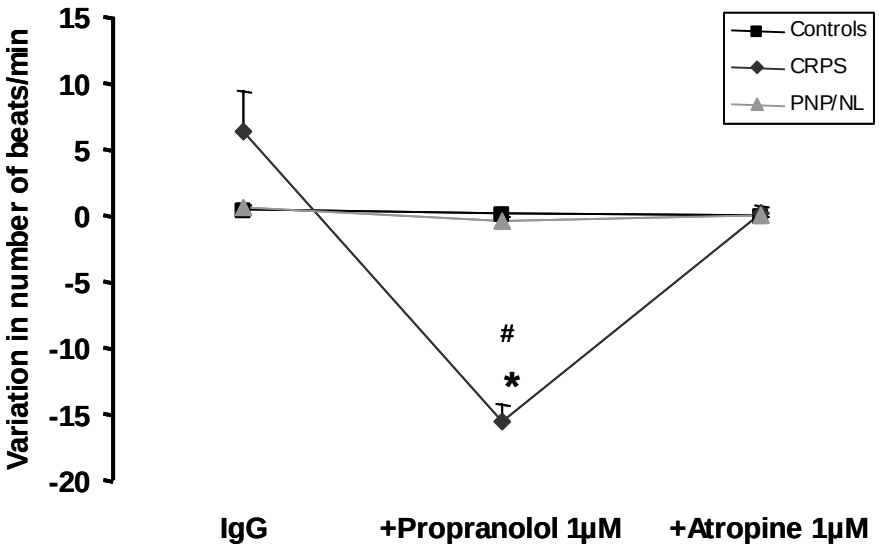


Figure 9. Pharmacological modulation of immunoglobulins' effect on spontaneously beating cardiomyocytes. The treatment with propranolol induced prominent decrease in the beating rate of cells treated with CRPS IgG in comparison with cells treated with control or PNP/NL IgG, showing that these antibodies have a beta-agonist effect along with an additional negative chronotropic effect (Kruskal-Wallis test, followed by Dunn's Test, $p = 0.00001$; *different from CRPS groups in IgG treatment, $p = 0.00001$; $^{\#}$ different from control and PNP/NL groups in propranolol treatment, $p = 0.00001$). Further treatment with atropine reversed the changes in beating rate to the level of controls (Kruskal-Wallis test, $p = 0.9975$) Values are expressed as mean of samples + S.E.M.

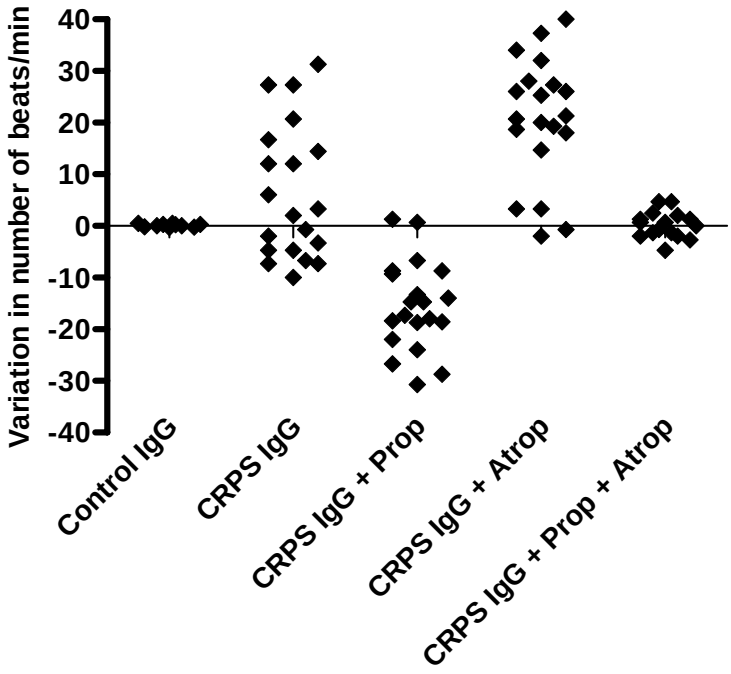


Figure 10. Dot blot representing the effect of each controls or CRPS IgG sample. Addition of propranolol or atropine to CRPS IgG shifted 90% of the samples to negative and positive range, respectively. Both drugs reversed the effect of 75% of all CRPS samples. For statistics see text.

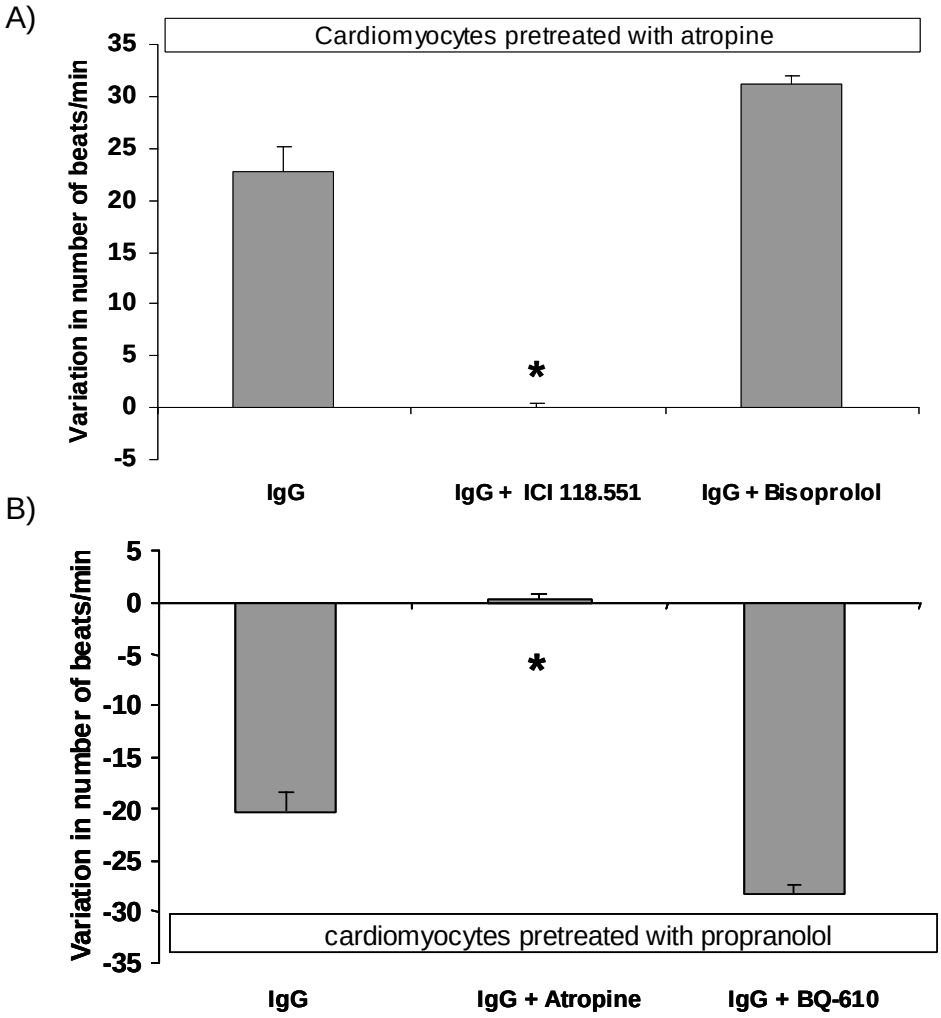


Figure 11. Effect of affinity-purified autoantibodies from CRPS patients in cultured cardiomyocytes and identification of putative target receptors. Cardiomyocytes were pretreated with atropine (A) or propranolol (B) as indicated in the panel. The chronotropic activity was blocked specifically by 0.3 µM of ICI 118.551 (selective β2AR antagonist) and by 1 µM of atropine. The following treatment with antagonist of β1 (bisoprolol, 1 µM) and endothelin receptor-1 (BQ-610, 1 µM) showed that these receptors were not involved in the CRPS IgG activity. *different from IgG treatment (Kruskal-Wallis test followed by Dunn's Test, p = 0.00001). Values are expressed as mean of samples + S.E.M.

4.2.3. The IgG effect of CRPS can be blocked by specific peptides derived from the second extracellular loop of the M2R and the β2AR

Both G protein-coupled receptors (M2R and β2AR) consist of a common structural signature of seven membrane spanning domains, three intra- and three extracellular loops, an extracellular N-terminal, and an intracellular C-terminal tail. The second extracellular loop has been reported to be one of the main immunogenic regions of both receptors as well as one of the main targets for allosteric modulators (Elies, Fu et al. 1998; Peter, Eftekhari et al. 2003; May, Avlani et al. 2007; May, Leach et al. 2007). Therefore, 7 randomly selected CRPS IgGs were preincubated with short overlapping synthetic peptides corresponding to the second extracellular loop of the human β2AR and M2R, and then applied to the neonatal cardiomyocytes.

Peptides with the sequences HWYRAT and ATHQEAI, but no other sequences derived from the second extracellular loop of the β2AR, blocked the positive chronotropic effect of CRPS IgG (Figure 12A). Moreover, synthetic peptides possessing the sequence of the first and third extracellular loop did not neutralize the IgG-mediated chronotropic effects (Figure 13). The peptides VRTVED and EDGECY were the only peptides from the second extracellular loop of the M2R which could block the negative chronotropic effect of CRPS IgG in propranolol-pretreated cardiomyocytes (Figure 12B).

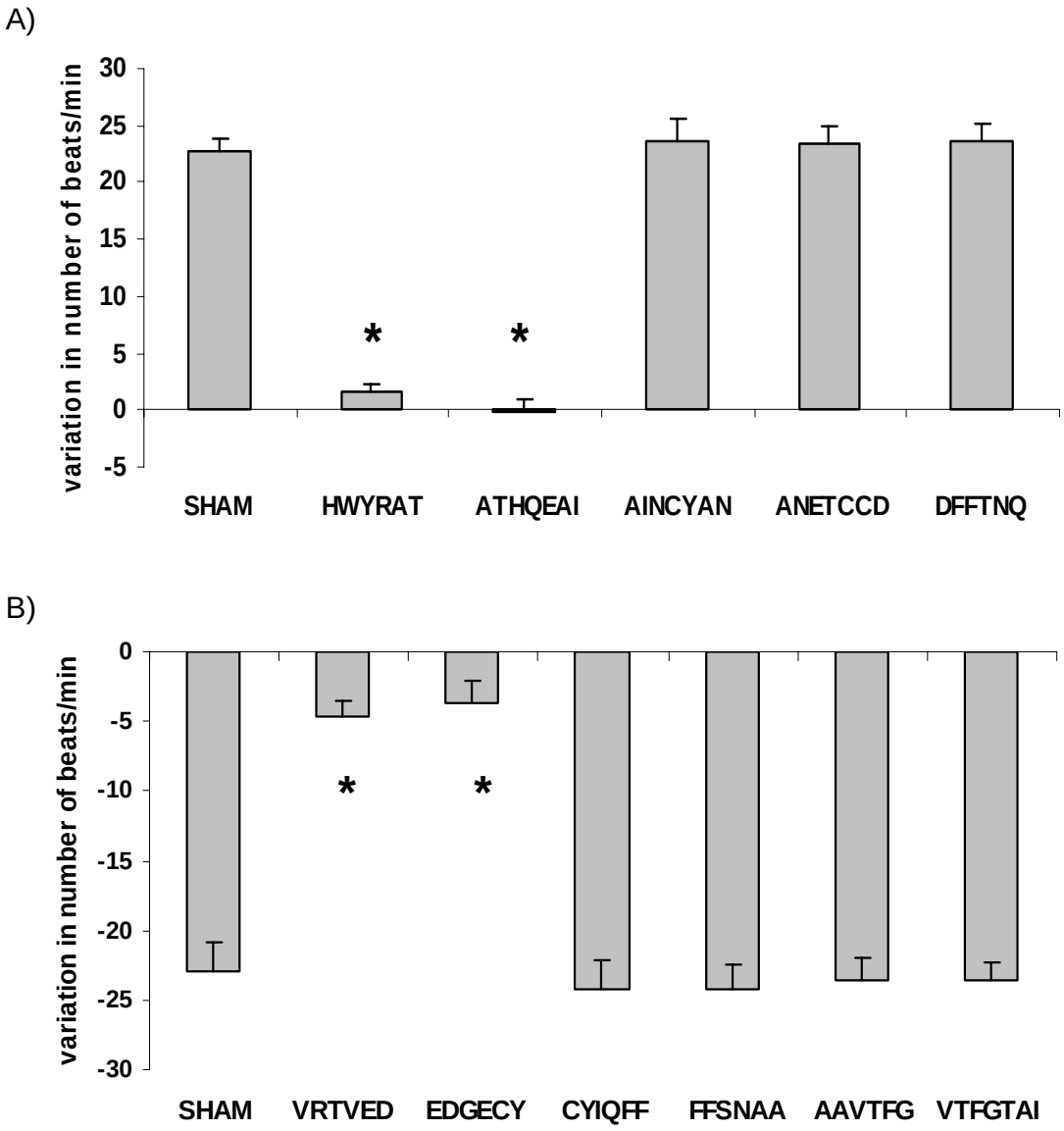


Figure 12. (A,B) Neutralization of CRPS IgG (n = 7) effect by peptides derived from extracellular sequences of the β2AR or the M2R on spontaneously beating cardiomyocyte. Cardiomyocytes were pre-treated with 1 µM propranolol (A) and 1 µM atropine (B). IgG fractions were pre-incubated with the peptides covering residues from the sequence of the extracellular loop of M2R (A) and β2AR (B). Two peptides from both receptors significantly blocked the chronotropic effect of immunoglobulins from CRPS patients. *different from SHAM and other peptides (ANOVA, Tukey's test, $p < 0.001$).

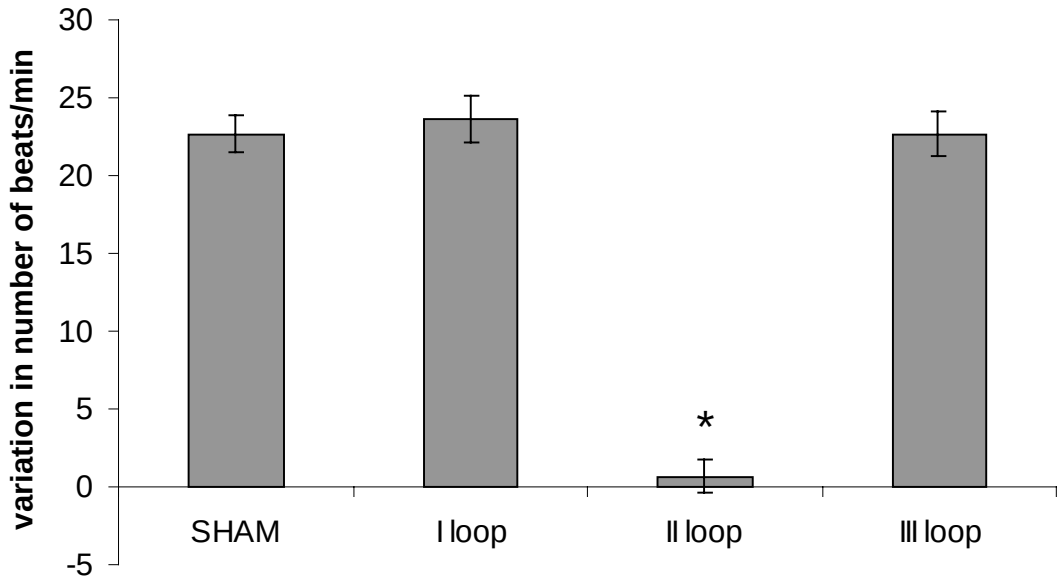


Figure 13. Pre-incubation of CRPS IgGs with peptides derived from the first, second and third extracellular loop sequence of β2AR before addition to cardiomyocytes. Cells were pre-treated with atropine to block the agonistic of CRPS antibodies on M2R. Synthetic peptides possessing the sequence of the first and third extracellular loop did not neutralize the IgG-mediated chronotropic effects. *different from SHAM (n = 7, Kruskal-Wallis test followed by Dunn's Test, p = 0.00001). Values are expressed as mean of samples ± S.D.

4.2.4. Peptide characterization by ELISA

Affinity-purified IgG from CRPS patients and HC were tested by enzyme immunoassay using plates coated with peptides of the second extracellular loop of β2AR (HWYRAT and ATHQEAI) and M2R (VRTVED and EDGECY). Additionally, we tested the peptide sequences AINCYAN of the β2AR and CYIQFF of the M2R as negative controls, since these sequences were not recognized by CRPS IgGs (see Figure 12). We calculated a cut-off (in optical density units [OD]) on the basis of the mean ± 2.5x standard deviations of the values with negative control peptides for each receptor.

We showed that 90% (18/20) of CRPS patients recognized at least one tested peptide. One peptide of each receptor was recognized by 55% of the CRPS, but none of the controls. 60% (12/20) of CRPS IgG fractions showed high affinity for β2AR sequences, 10/20 to both sequences, and one each only to HWYRAT or ATHQEAI. None of the samples showed binding to the control peptide AINCYAN (Figure 14A, Appendix 1). 90% of the CRPS IgG showed binding to one or two of the M2R-derived peptides. 13/20 CRPS IgG samples recognized the sequence VRTVED, 17/20 the sequence EDGECY, while none of them recognized the CYIQFF control peptide (Figure 14B, Appendix 1). With exception of one control binding to VRTVED, IgG of healthy controls did not show specific binding to any of the peptides tested (Figure 14).

We noticed a positive correlation between samples that recognized both peptides of β2AR (Pearson r = 0.6037; p = 0.0048; n = 20) and these with VRTVED of M2R (Pearson r = 0.7040; p = 0.0005; n = 20 with HWYRAT and Pearson r = 0.5530; p = 0.0114; n = 20 with ATHQEAI). EDGECY was recognized by the majority of CRPS patients (85% or 17/20, see Figure 14B) and therefore did not show any significant correlation with other peptides (VRTVED: Pearson r = 0.1040; p = 0.6626; n = 20. HWYRAT: Pearson r = 0.1229; p = 0.6058; n = 20. ATHQEAI: Pearson r = 0.2886; p = 0.2172; n = 20) . Indeed, only three samples of CRPS patients did not showed raised levels of IgG antibodies to the sequence EDGECY of M2R, one of them presented however raised levels to both sequences

HWYRAT and ATHQEAI of the β2AR, and other two did not recognized any of the tested sequences.

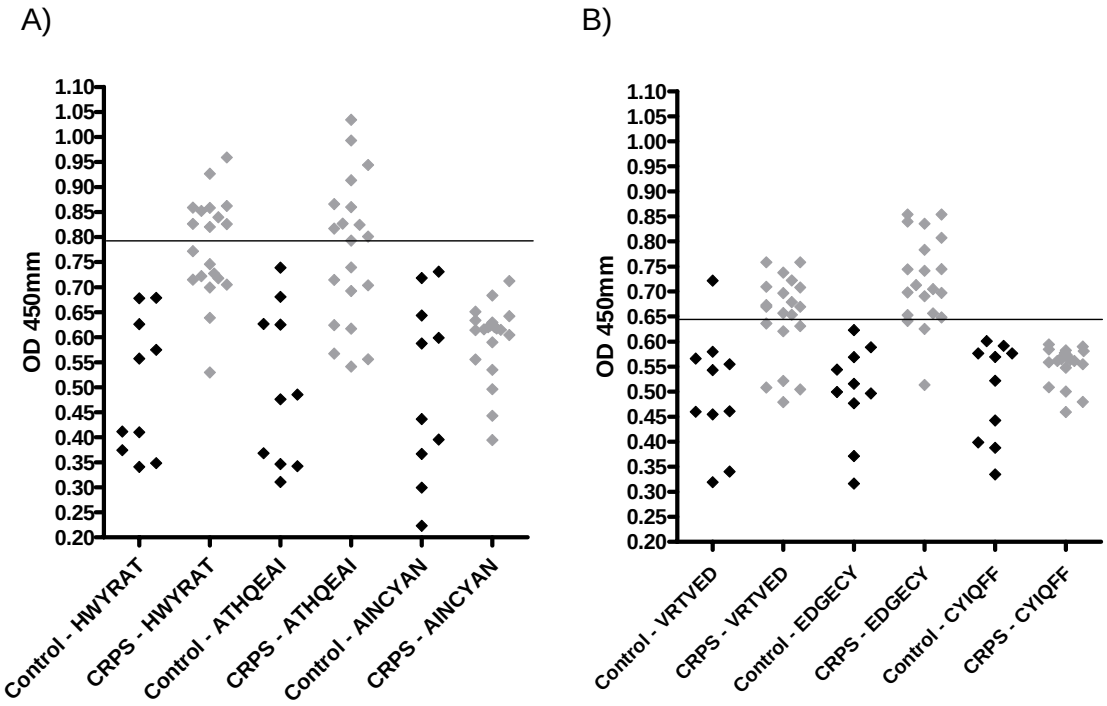


Figure 14. CRPS antibodies detected by ELISA. (A) Antibodies against β2AR peptides HWYRAT and ATHQEAI were found in 12/20 CRPS patients - 10/20 (50%) samples recognized both sequences. No CRPS IgG recognized the peptide AINCYAN. None of control samples showed affinity to any β2AR peptide. (B) 13/20 (65%) CRPS IgG samples showed affinity the sequence VRTVED and 17/20 (85%) the sequence EDGECY of the M2R, while none of them recognized the sequence CYIQFF. One control sample recognized VRTVED sequence. Cut-off (in optical density units [OD]), marked as horizontal line, on the basis of the mean + 2.5x standard deviations of the values with negative control peptides for each receptor.

4.2.5. Receptor autoantibodies in CRPS do not cross-react between M2R and β2AR

Since two functionally active autoantibodies could be detected in the majority of CRPS patients, we addressed a possible cross-reactivity between them using a pharmacological manipulation with receptor antagonists and peptides. We preincubated 6 randomly selected IgG of CRPS patients with sequences of β2AR or M2R peptides and, in parallel, incubated cardiomyocytes with either propranolol or atropine. The intention was to determine if those antibodies responsible for the activation of M2R can be blocked by peptides of the β2AR or vice versa. In the first experiment, we tested the ability of M2R antibodies to bind peptides of the β2AR. CRPS IgG fractions were preincubated with the two β2AR peptides HWYRAT and ATHQEAI, both of which were able to block the β2-agonistic effect of the CRPS IgG fractions. Another peptide without blocking effect, AINCYAN, was used as a negative control. Cardiomyocytes were treated with propranolol or atropine and then incubated with the pre-blocked CRPS IgG. In the propranolol-treated cells, the preincubated IgG induced a negative chronotropic effect, indicating (1) that the β2-agonistic effect can be removed by preincubation with the two peptides from the second extracellular loop of the β2AR and (2) that anti-M2R antibodies were still present and did not bind to the β2AR peptides (Figure 15A). Vice versa, CRPS IgG pre-blocked with the M2R peptides VRTVED and EDGECY was still able to induce a positive chronotropic effect in atropine pre-treated cardiomyocytes (Figure 15B). Both experiments indicate that no cross-reactivity occurs between the anti- β2AR and the anti-M2R antibodies.

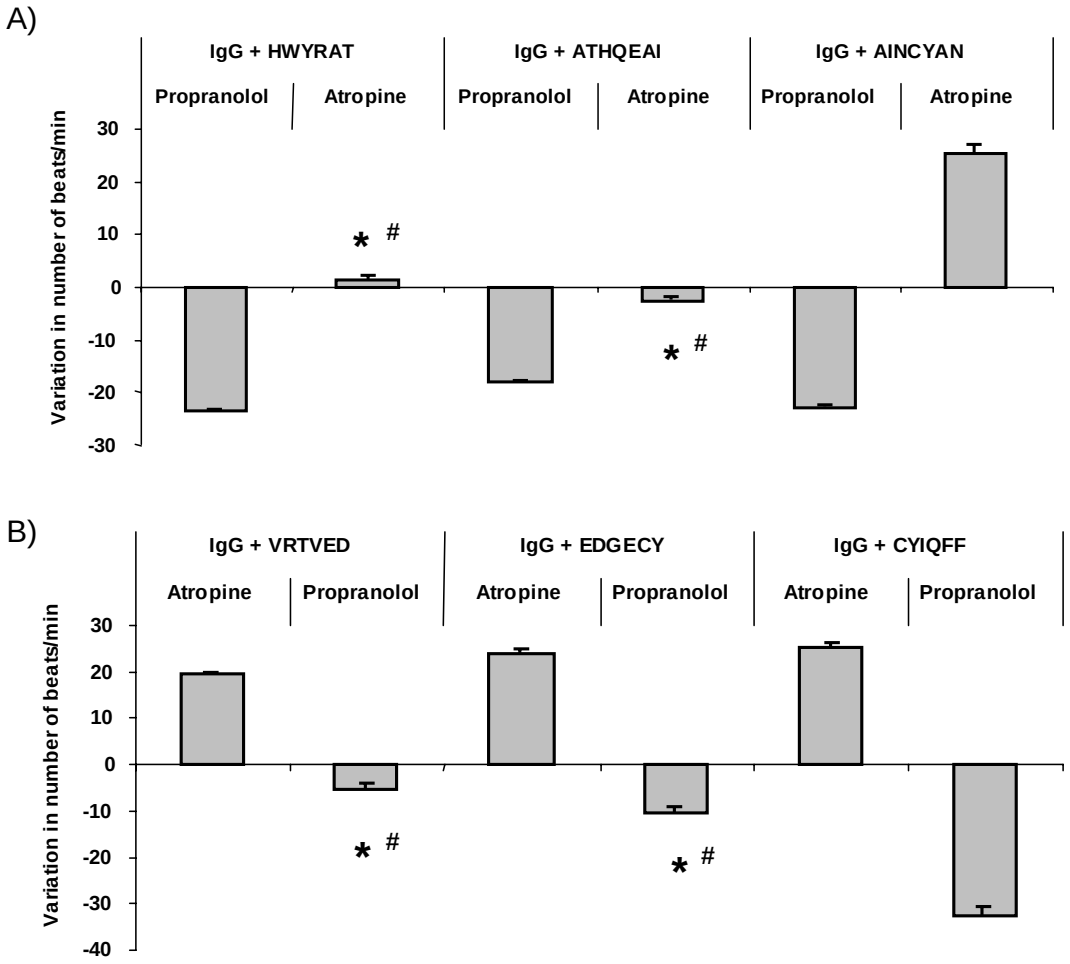


Figure 15. Evaluation of possible cross-reactivity between CRPS IgGs. No cross reactivity could be observed between M2R and β2AR antibodies. In panel (A) antibodies were preincubated with correspondent β2AR peptides while cardiomyocytes were pre-treated with propranolol (1 μM) or atropine (1 μM) as displayed. M2R autoantibodies were not blocked by peptides corresponding to residues of β2AR and showed negative chronotropic activity in propranolol-treated cells. However, β2AR antibodies were successfully blocked by HWYRAT and ATHQEAI peptides and showed no effect in atropine-pretreated cells. (B) Vice versa, CRPS IgG pre-blocked with VRTVED and EDGECY was still able to induce a positive chronotropic effect in atropine pre-treated cardiomyocytes - β2AR agonistic effect was not impaired by M2R peptides. *different from (A) IgG + AINCYAN and (B) IgG + CYIQFF (n = 6, Kruskal-Wallis test followed by Dunn's Test, $p < 0.001$). #different from the other cardiomyocyte treatment (propranolol or atropine) for the same IgG + peptide (n = 6, Kruskal-Wallis test followed by Dunn's Test, $p < 0.001$). Values are expressed as mean of samples + S.E.M.

4.3. Experiment set 3: Immunoglobulins subclasses of pathogenic antibodies

4.3.1. IgG subclasses characterization of CRPS antibodies

The autoantibodies against the β2AR and the M2R belong to the IgG class. IgG includes the subclasses IgG1-4. These subclasses differ in their immunological function, and the subtype identification of pathogenic antibodies might indicate which T helper (Th) cell subset is mostly involved in an autoimmune response, and whether the autoantibodies may be able to bind complement. Using anti-IgG1-4 to preincubate and precipitate the patients IgG subclasses. We demonstrate that pathogenic β2AR antibodies belong to IgG1 and IgG2 subtypes (Figure 16A), whereas the M2R antibodies are IgG1 and IgG3 subtypes (Figure 16B).

4.4. Experiment set 4: CRPS pathogenic antibodies bind to the surface of hM2R- and hβ2AR-transfected CHO cells

To confirm whether CRPS autoantibodies bind specifically to the β2AR and M2R, we incubated CHO cell line wild type or CHO cells expressing hβ1AR, hβ2AR, or hM2R with HC, CRPS, or PNP/NL IgGs, and assessed the surface binding against the non-permeabilized cells using flow cytometry, an established method to detect surface binding of human autoantibodies. 55% (11/20) of the CRPS patients, none of the controls, and only one patient of the PNP/NL group showed binding to either hβ2AR CHO cells (ANOVA, $p = 0.0077$) or hM2R CHO cells (ANOVA, $p = 0.0202$) or both (hβ2AR, n = 3; hM2R, n = 3; both receptors, n = 5) (Figure 17A,B). CHO cells transfected with hβ1AR were used as a control, two patients in the PNP/NL group, and one of the CRPS but none of the healthy controls showed binding to these cells (ANOVA, $p = 0.2060$) (Figure 17C). There was no binding to wild type CHO cells in any group (ANOVA, $p = 0.2854$) (Figure 17D).

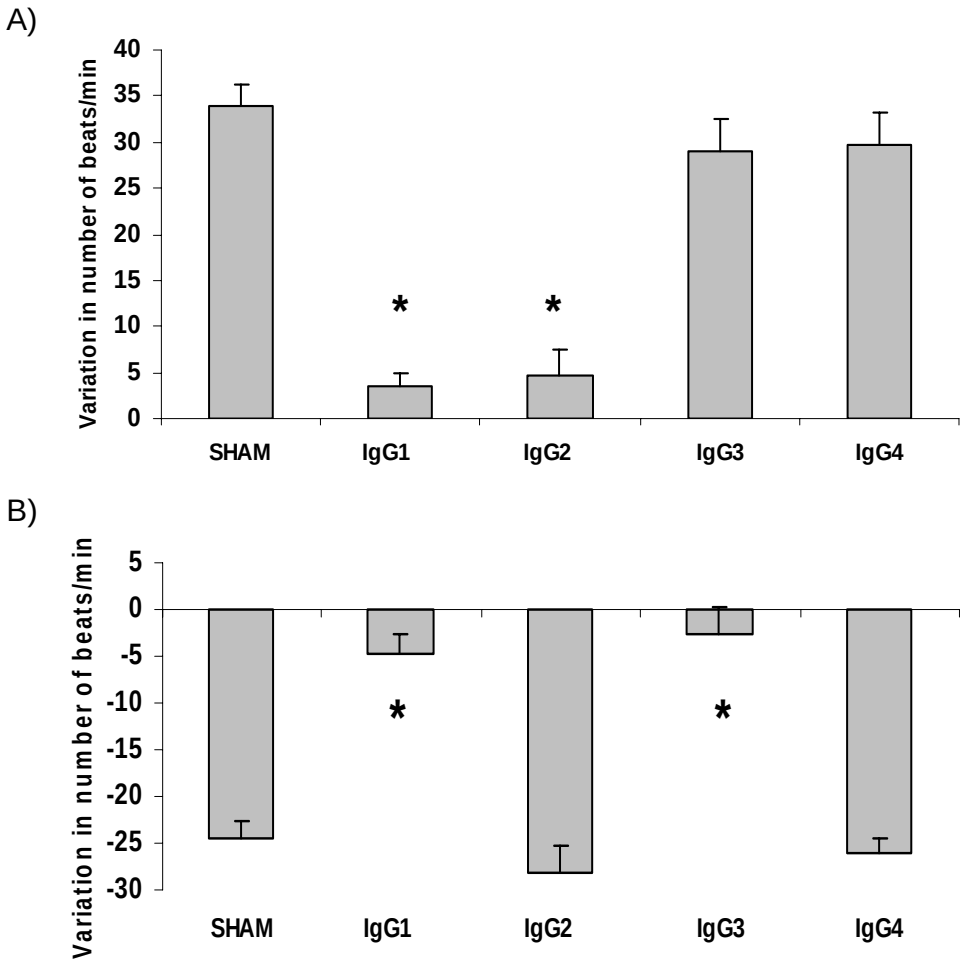


Figure 16. Identification of IgG subclass of CRPS autoantibodies. Pathogenic antibodies of CRPS patients (n = 5) were preincubated with mouse anti-human IgG1, -2, -3 and -4 antibodies before the incubation with spontaneously beating cardiomyocytes and then changes in the beating rate were assessed. (A) β2AR antibodies are mainly IgG1 and IgG2 (Kruskal-Wallis test, p = 0.0014). (B) M2R antibodies are predominantly IgG1 and IgG3 (Kruskal-Wallis test, p = 0.0012). *mean different from whole IgG effect (Kruskal-Wallis test, Dunn's Test, $p < 0.01$). Values are expressed as mean of samples + S.E.M.

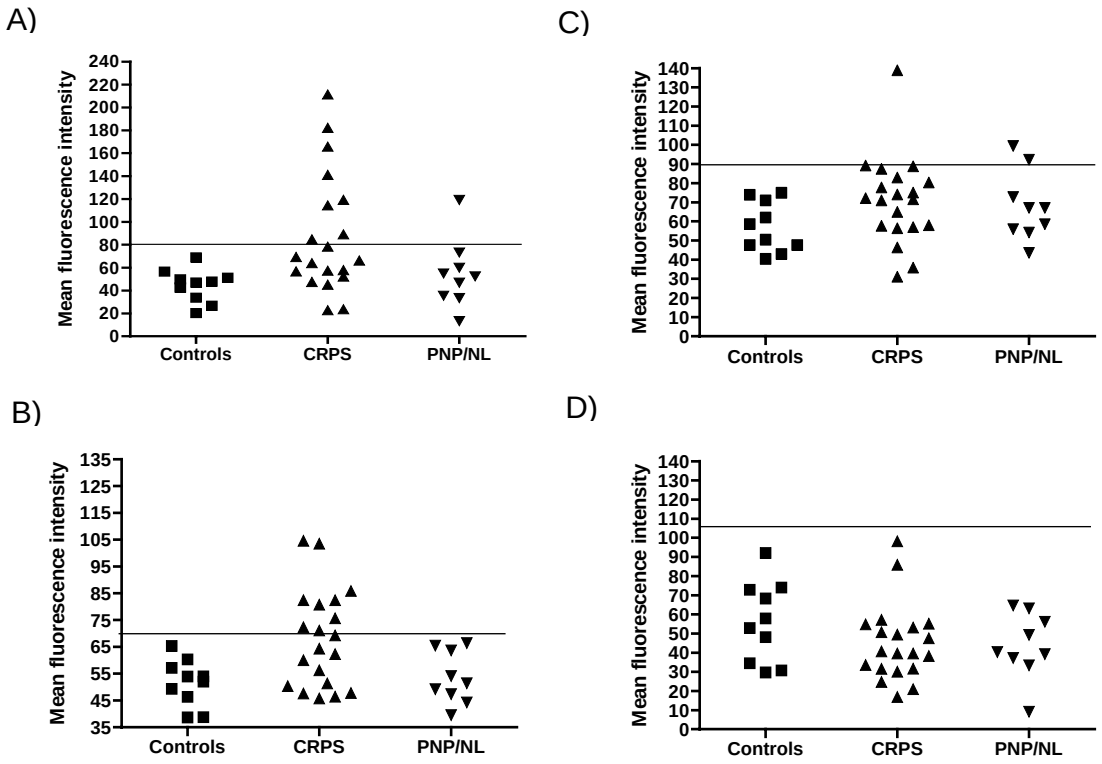


Figure 17. Binding of controls (n = 10), CRPS (n = 20), or PNP/NL (n = 9) IgG samples to CHO cell line expressing **(A)** hβ2AR, **(B)** hM2R, **(C)** hβ1AR, or **(D)** wild type. No significant difference could be observed in binding to CHO wild type cell (ANOVA, $p = 0.2854$) or hβ1AR (ANOVA, $p = 0.2060$) between controls, CRPS or PNP/NL IgGs. CRPS patients have however more and higher binding to CHO cells expressing hβ2AR (ANOVA, Tukey's test, $p = 0.0078$) or hM2R (ANOVA, Tukey's test, $p = 0.0202$) than controls, neuropathy patients or peripheral nerve lesions patients (PNP/NL). Cut-off, marked as horizontal line, was determined as mean of the controls + 2.5x standard deviations for each cell line.

Since the percentage of positive binding was similar to our previous results with differentiated autonomic cells (40% surface binding). We additionally detected upregulation of these receptors in BMP-2/RA differentiated SH-SY5Y neuroblastoma cells (Figure 18).

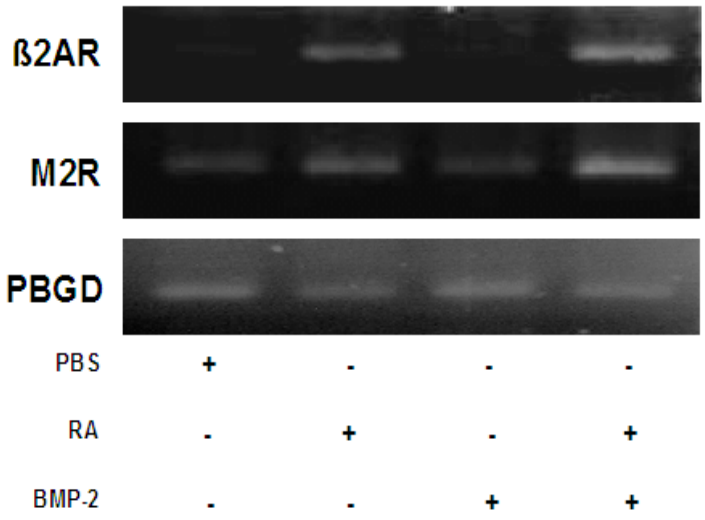


Figure 18. Expression of β2AR and M2R in SH-SY5Y cells non-differentiated and differentiated with RA and/or BMP-2. After differentiation total RNA was prepared and subjected to cDNA synthesis. This cDNA was used for PCR amplification of β2AR, M2R and PBGD. SY5Y cells differentiated with the combination of BMP-2/RA showed increased expression of β2AR and M2R.

4.5. Experiment set 5: Functional assays

4.5.1. Pathogenic antibodies show agonistic-functional effects but do not induce desensitization

Affinity purified antibodies from CRPS patients were added to the medium of cultured cardiomyocytes pre-treated either with atropine (Figure 19A) or propranolol (Figure 20A), and their effect was investigated as a function of time.

In cells pre-treated with atropine, IgG showed a positive chronotropic action. This effect was stable along the time and maintained for about 6 h (Figure 19A), in contrast with the sharp effect of clenbuterol that waned along the time (Figure 19B). CRPS IgG positive chronotropic property was completely reversed by the β2AR antagonist ICI 118.551 (0.3 μM). Further, after a washout procedure and addition of fresh medium, the stimulation of autoantibody-stimulated cardiomyocytes with agonist clenbuterol (Figure 19A) promoted in both patients an increase in beating frequencies similar to that induced by the agonist alone in the first stimulation, as displayed in the Figure 19B (response in about 45 beats/min). Thus, the desensitization phenomenon normally induced by the agonist did not occur when cells were pre-exposed to autoantibodies. The desensitization phenomenon could on the other hand be demonstrated in cardiomyocytes re-stimulated with clenbuterol, since the second stimulation with agonist induced only a mild response - four times lower than that induced by the first stimulation (see Figure 19B).

The same behaviour was observed for the M2R agonistic effect of affinity-purified CRPS IgGs in cardiomyocytes pre-treated with propranolol. In this case, CRPS IgG decreased the beating frequencies in a specific manner (Figure 20A) but not like the agonist carbachol (Figure 20B), and no longer induced desensitization - clearly observed after re-exposition to agonist carbachol (Figure 20B).

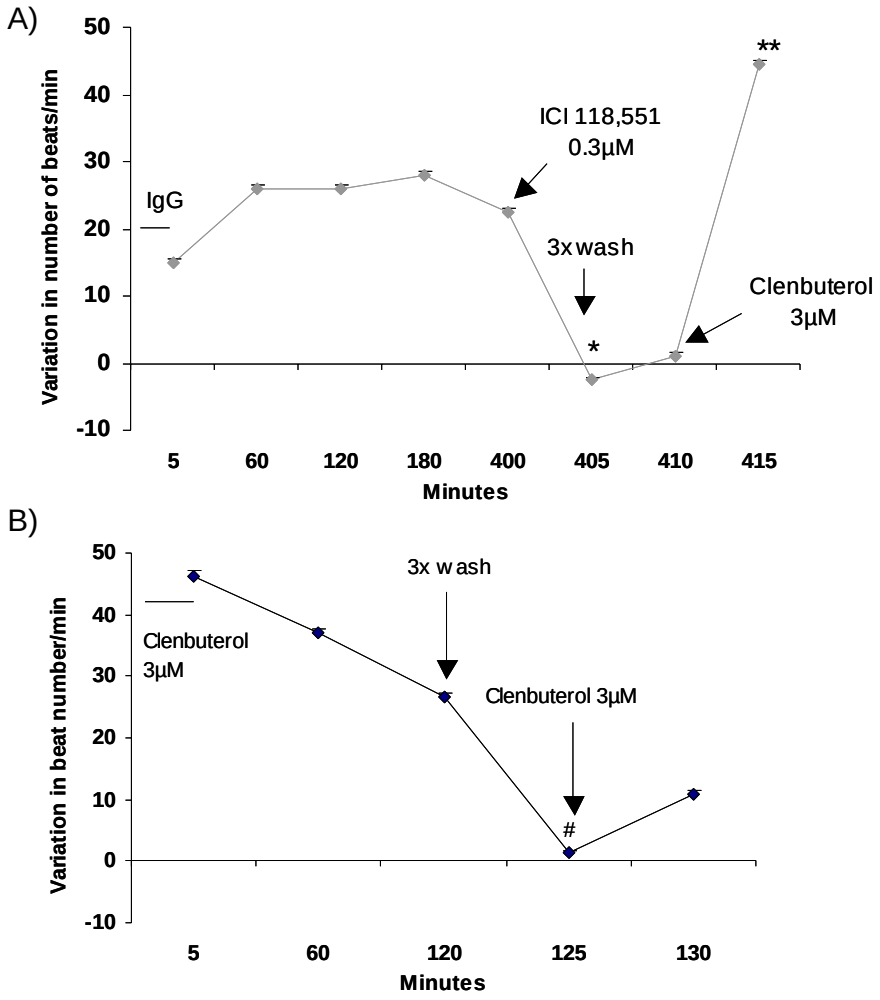


Figure 19. Time dependency of the chronotropic effect induced by pathogenic antibodies directed against the second extracellular loop of the β2AR. Cells were pre-treated with atropine to block the M2R agonistic effect of IgGs. (A) The positive chronotropic effect of autoantibodies was sustained until the addition of selective β2AR antagonist (ICI 118,551). Addition of agonist clenbuterol increased the beating rate in the same order of magnitude as that induced by the agonist alone (about 45 beats/min - see stimulation with clenbuterol in panel B), showing that no desensitization occurred after exposition to antibodies. (B) Typical receptor desensitization induced by the re-exposition to agonist clenbuterol. The agonistic effect wane along the time and was completely abolished after washing out with fresh medium. A second exposition to agonist did not induce the same changes in beating rate induced by the first administration. Values are expressed as mean of replication in different cultures + S.E.M. *different from 180 min time point (n = 8, Friedman test, Dunn's Test, $p < 0.01$). **different from 5 min time point. $^{\#}$different from 5 min time point (n = 8, Friedman test, Dunn's Test, $p < 0.01$).

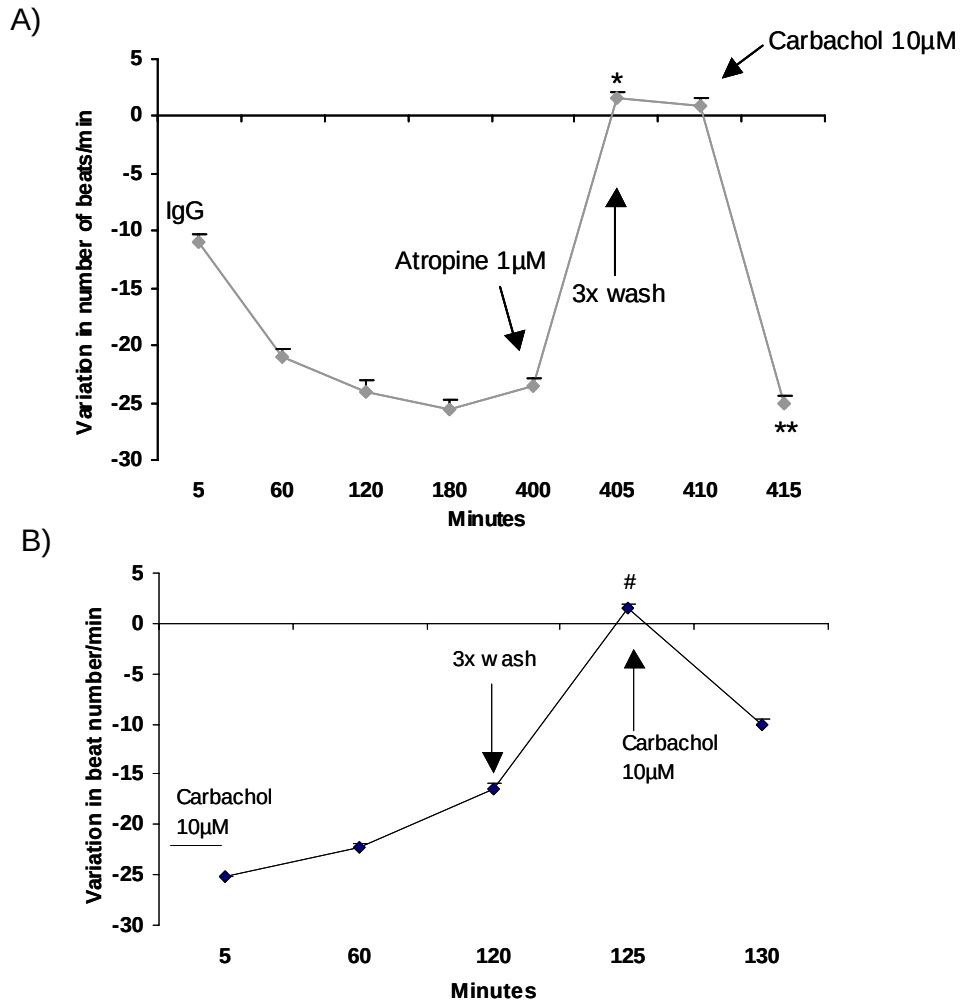


Figure 20. Time dependency of the chronotropic effect induced by M2R antibodies. Cardiomyocytes were pre-treated with propranolol. The effect of autoantibodies was sustained along the time. After 3 washes and a replace of new medium, agonist carbachol (10 µM) displayed a negative chronotropic effect similar to the first administration observed in the panel B (about -25 beats/min), showing that no desensitization occurred after treatment with antibodies. (B) Typical receptor desensitization induced by the re-exposition to agonist carbachol. The agonist effect wane along the time and was completely abolished after washing out with fresh medium. The second exposition to agonist did not induce the same decrease in beating rate induced by the first administration. Values are expressed as mean of replication in different cultures + S.E.M. *different from 180 min time point (n = 8, Friedman test, Dunn's Test, $p < 0.01$). **different from 5 min time point (n = 8, Friedman test, Dunn's Test, $p < 0.01$). $^{\#}$different from 5 min time point (n = 8, Friedman test, Dunn's Test, $p < 0.01$).

4.5.2. β2AR and M2R autoantibodies do not downregulate their corresponding receptors on the cell surface

In myasthenia gravis, binding of autoantibodies to the nicotinic acetylcholine receptor leads to a downregulation of nAChR at the neuromuscular junction (Lang and Vincent 2003). We therefore incubated β2AR- and M2R-transfected CHO cells with patient and HC IgG. After 24h (Figure 21A) and 48h (Figure 21B), there was no downregulation of the β2AR or M2R on the cell surface as detected by flow cytometry.

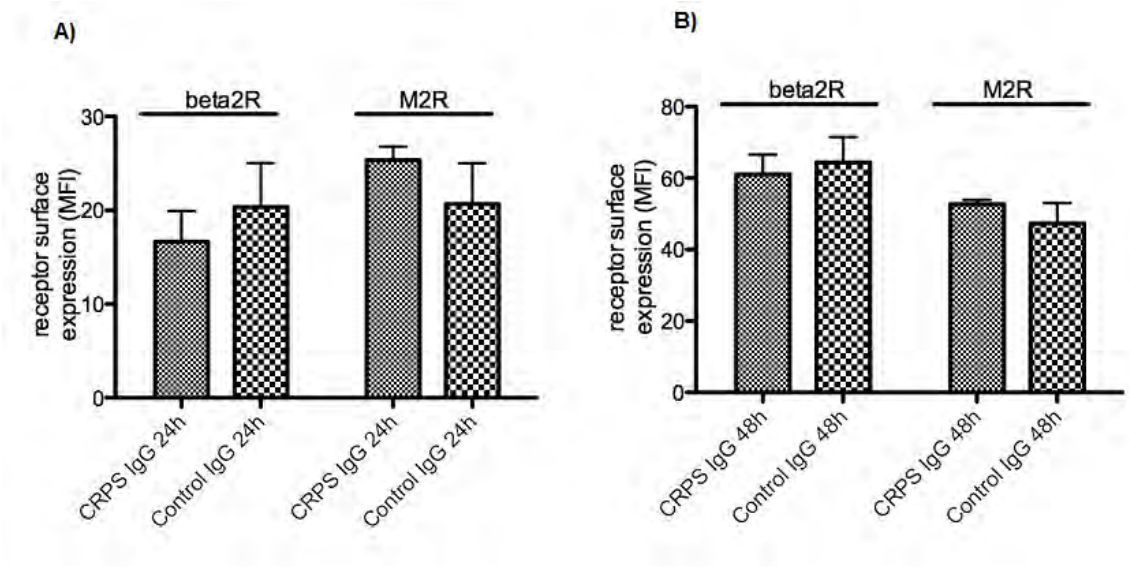


Figure 21. Receptor expression of β2AR- or M2R-transfected CHO cells after incubation with CRPS IgG or control IgG for 24 (A) or 48 (B) hours. Flow cytometric surface expression analysis of β2AR or M2R on the transfected CHO cells revealed no difference in the receptor expression after incubation with CRPS IgG compared to control IgG after 24 (A) or 48 (B) hours. Data are represented as mean of triplicates ± S.D. Mann Whitney test (n = 9; β2AR 24 h p = 0.863, M2R 24 h p = 0.90, β2AR 48 h p = 0.37, M2R 48 h p = 0.28) MFI: mean fluorescence intensity.

4.5.3. β2AR and M2R autoantibodies cause receptor dependent increase in cytosolic Ca^{2+} concentration in transfected cell lines.

Activation of both β2AR (Magne, Couchie et al. 2001) and M2R (Sorimachi, Furukawa et al. 1995) by agonists increase intracellular Ca^{2+} concentration. Muscarinic receptor-agonist bethanechol (100 µM) as well as the β2-agonist clenbuterol (30 µM) induced a prominent increase in intracellular Ca^{2+} concentration in hM2R or hβ2AR transfected cells, respectively (Figure 22A).

Further, we tested the effect of 6 randomly selected CRPS patients and 3 controls IgG in wild type or transfected CHO cells. HC did not induced any substantial changes in intracellular Ca^{2+} concentration (Figure 22B). CRPS IgG but none of HC IgG evoked a significant increase of the fura-2 ratio signal by 0.087 ± 0.056 on average (n = 6, paired Student's t-test, p = 0.0128) in CHO cells expressing hβ2AR. The IgG effect on the β2AR could be blocked by propranolol (1 µM) in 5/6 samples (Figure 23A, other patients in Appendix 2 and 3).

Only 2/6 CRPS patients and none of HC induced an increase of the fura-2 signal in hM2R CHO cells (n = 6, paired Student's t-test, p = 0.1929). The effect of these two sera was successfully blocked when cells were previously treated with atropine (1 µM) (Figure 23B, other patients in Appendix 4). No influence of the IgG fractions could be observed in wild type CHO cells (Figure 24).

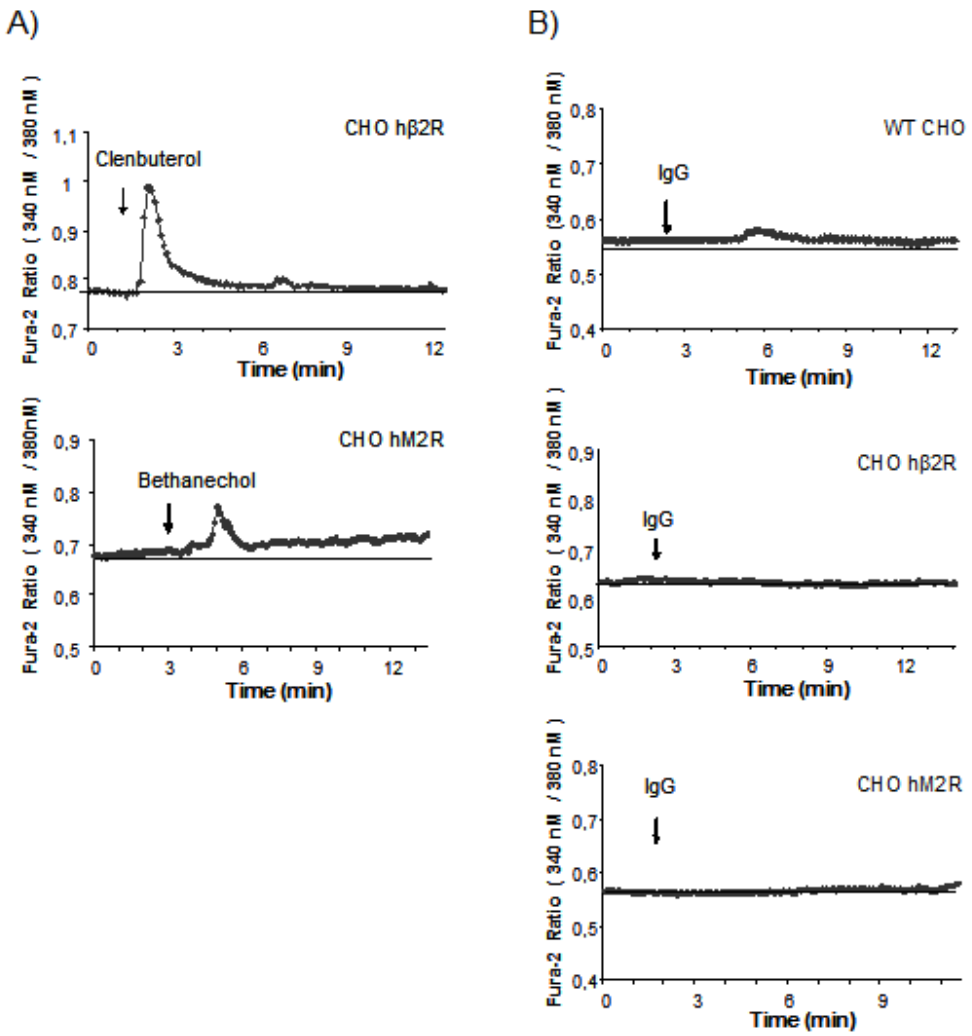


Figure 22. (A) Changes in the intracellular Ca^{2+} concentration induced by agonists. Beta2-agonist clenbuterol (30 µM) and muscarinic receptor-agonist bethanechol (100 µM) induced a prominent Ca^{2+} influx in hM2R- or hβ2AR-CHO cells, respectively. (B) Illustration of one samples of HC in WT, hβ2AR-, M2R-CHO cells. HC IgG did not induce any substantial changes in intracellular Ca^{2+} concentration.

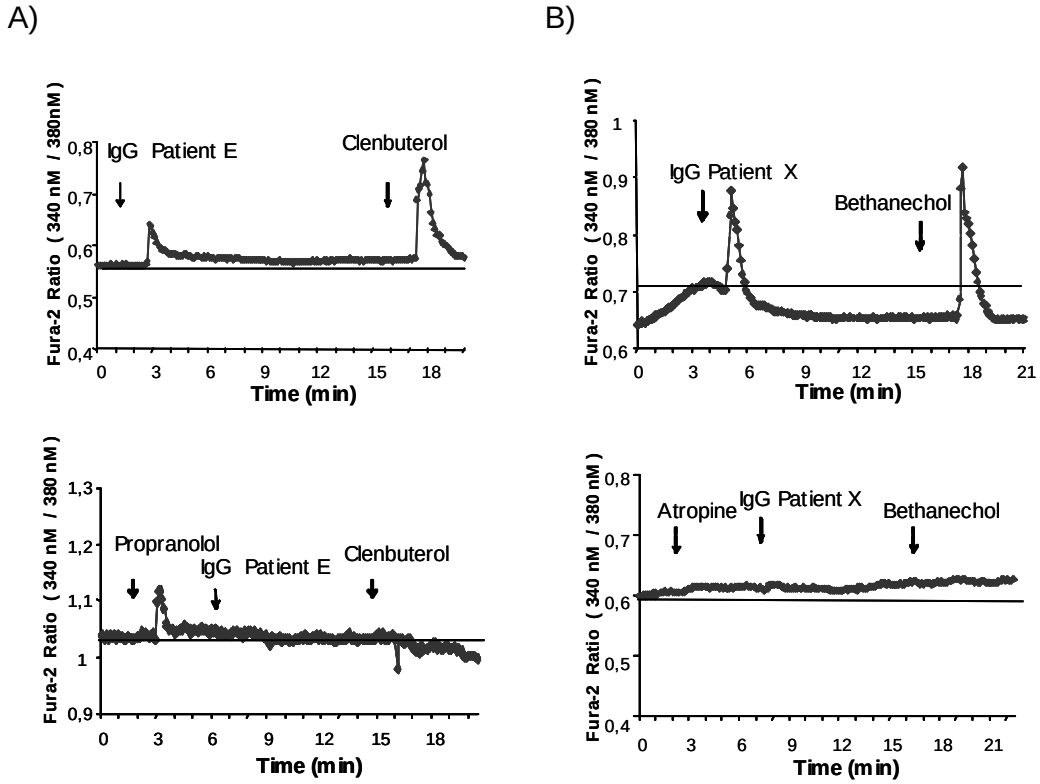


Figure 23. Changes in the intracellular Ca^{2+} concentration induced by putative autoantibodies of CRPS patients. (A) Illustration of one samples showing that CRPS IgG (60 mg/L) induced an increase in the fura-2 ratio in CHO cell line overexpressing hβ2AR. The pre-treatment with 1 µM propranolol inhibited the effect of IgG (60 mg/L) in 5/6 cases. (B) Illustration of the effect of one sample in cells overexpressing hM2R. 2/6 CRPS IgG (60 mg/L) and the agonist bethanechol (100 µM) induced an increase in the fura-2 ratio, blocked by preceding treatment with the antagonist atropine (1 µM). For statistics see text.

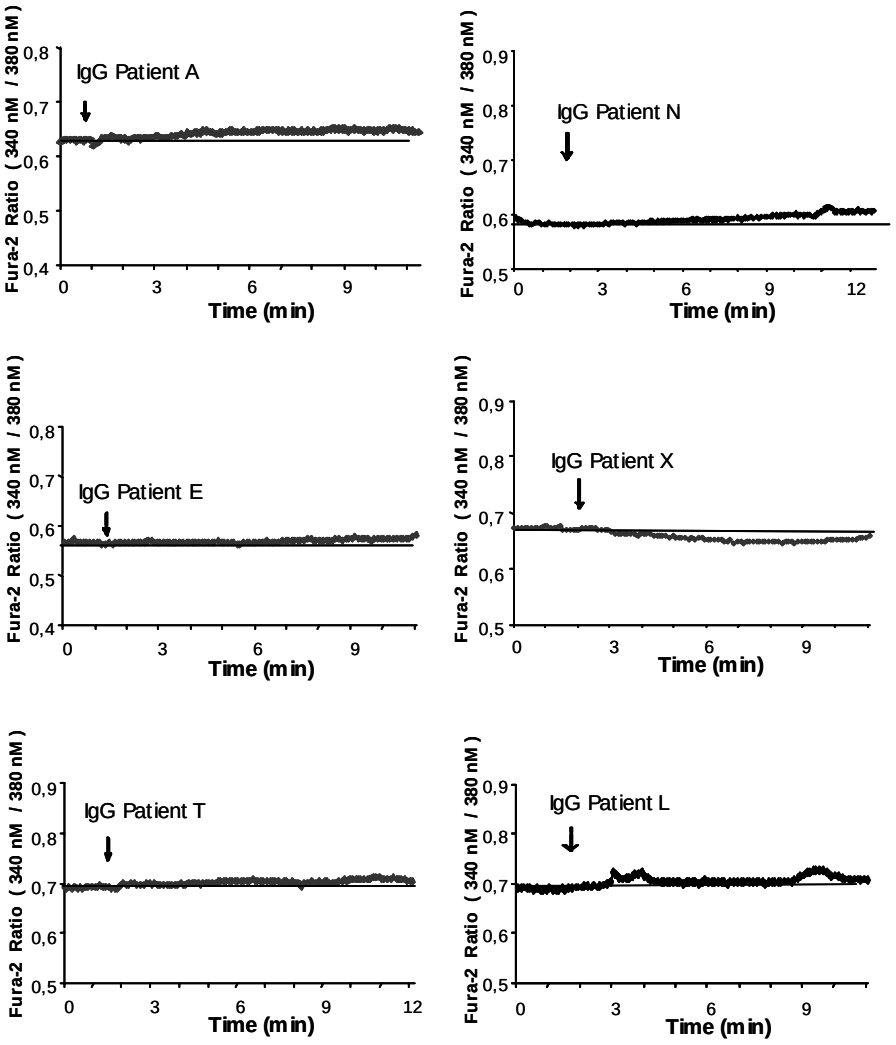


Figure 24. Effect of affinity-purified CRPS IgG in WT-CHO cells. CRPS autoantibodies did not induce any substantial change in the intracellular Ca^{2+} concentration in wild type CHO cells. Paired Student's t-test, $p = 0.0607$.

4.5.4. Association with clinical data

We did not identify any significant correlation (Spearman's correlation, $p > 0.05$) between the presence of functional active autoantibodies to one and/or two receptors, surface-binding to receptor-transfected CHO cell line or Elisa optical density values for different peptides and clinical / epidemiological data (gender, age, CRPS I or II, warm or cold, CRPS duration, inciting event, pain score, affected limb, sensory or motor impairment, vasomotor, sudomotor or trophic changes) of the analyzed CRPS patients.

4.6. Experiment set 6: CRPS antibodies do not induce cytotoxicity

The rational was to measure the cytotoxic effect of different patients' IgG on receptor-transfected CHO cell lines. Cells were first plated at different densities for 24 h in 1% FCS medium to find out the right cell density for further experiments. It has been deduced that 10,000 cells per well is the optimum cell density which has a linear function in the cytotoxicity assay. Ten thousands cells per well were then incubated with purified IgGs (120 mg/L) in 96 well plates for 24 h and later the cytotoxicity was measured using LDH cytotoxicity assay kit. There could not be shown any cytotoxic effects of CRPS IgG (ANOVA one-way, $p > 0.05$). None of the healthy controls showed any cytotoxicity. The results obtained after 24 h incubation with IgGs are summarized in percentage of cytotoxicity in the Figure 25.

Additionally, we tested CRPS, PNP/NL or HC affinity-purified IgGs in hβ2- and hM2-CHO cell lines for their ability to influence cell growth and viability or to induce cytotoxicity using cell proliferation/cell viability WST-1 assay. Cells were incubated with IgGs for 24 h, WST-1 reagent was added and then WST-1 cleavage was detected by ELISA plate reader after 1 h, 2 h and 3 h. Neither CRPS nor PNP/NL IgGs influenced cell growth or viability of any transfected CHO cells in comparison with HC samples (ANOVA one-way, $p > 0.05$). Moreover, no cytotoxic effect could be demonstrated after 24 h treatment with IgG in none of the samples tested. Positive control of cytotoxicity was the treatment with 2% Triton-100 (Figure 26).

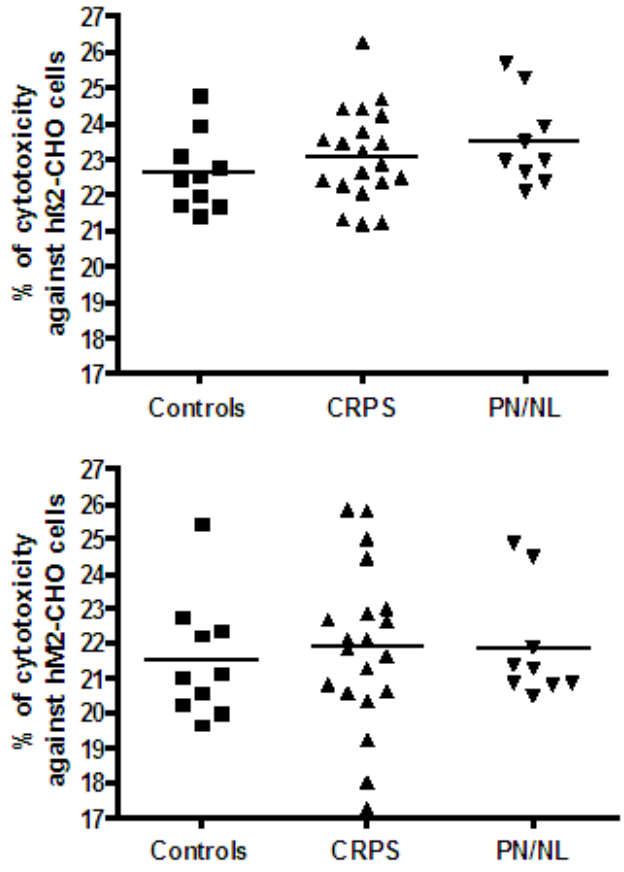
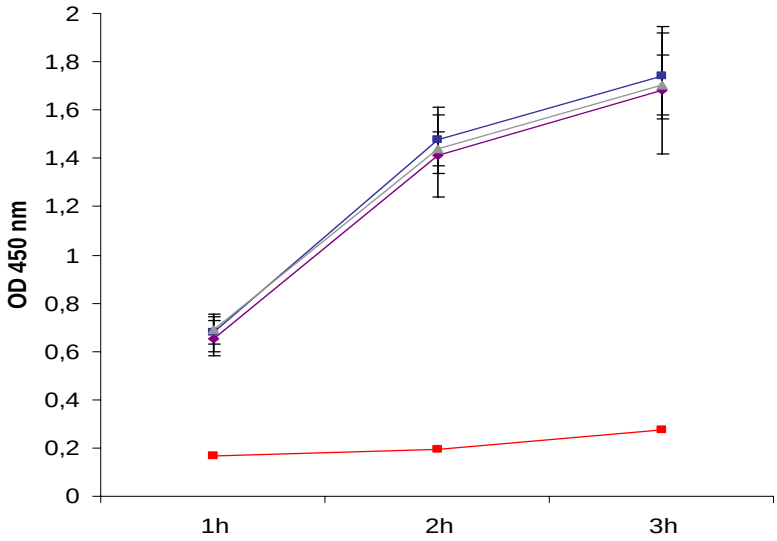


Figure 25. Purified IgGs from CRPS patients tested with hβ2AR- and hM2R-CHO cell lines for their cytotoxic effects using LDH assay. Cells were incubated with IgGs for 24 h and then supernatant was used to detect cytotoxicity using LDH cytotoxicity assay. No cytotoxicity was observed in receptor transfected cells (ANOVA one-way; hβ2-CHO, $p = 0.3491$; hM2-CHO, $p = 0.8851$).

A)



B)

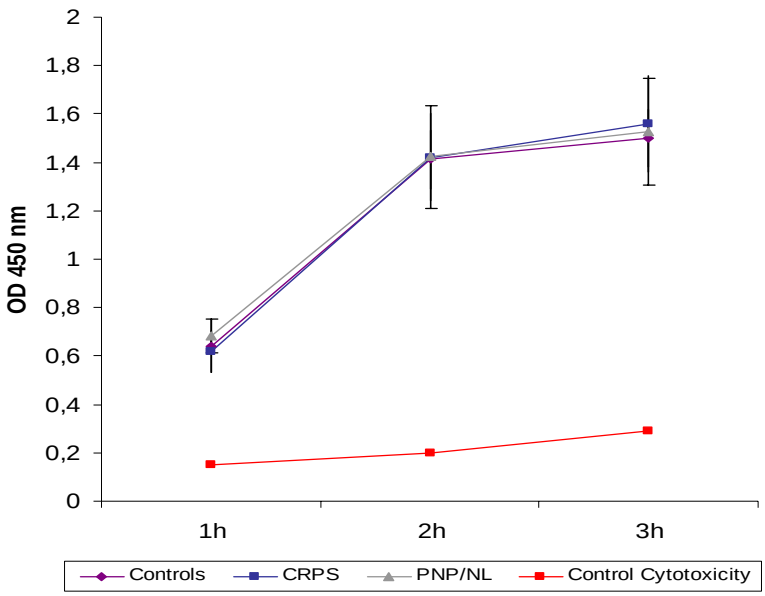


Figure 26. Purified IgGs tested in hβ2- and hM2-CHO cell lines for their cytotoxic effects using cell proliferation or cell viability WST-1 assay. Purified IgGs did not influence cell viability as well as did not shown any cytotoxic effect in hβ2-CHO (A) and hM2-CHO (B) cell lines. Positive control of cytotoxicity was the treatment with 2% Triton-100. One-way ANOVA, hβ2-CHO 1 h p = 0,5823; 2h, p = 0,4610; 3h, p = 0,7248; hM2-CHO 1 h, p = 0,2102; 2 h, p = 0,9927; 3 h, p = 0,7735.

V - DISCUSSION

A significant number of CRPS patients have autoantibodies against a surface epitope of autonomic neurons. Our results further indicate that this surface antigen is weakly expressed in undifferentiated neuroblastoma cells, but can be induced by differentiation into a distinct phenotype, preferentially cholinergic. Further, we have defined two specific target antigens for these autoantibodies - M2R and β2AR - supporting an autoimmune hypothesis in CRPS. The antibodies bind to specific epitopes of the second extracellular loop of the two receptors, and do not cross-react. Both antibodies exhibited functional effects on neonatal cardiomyocytes *in vitro*, implying that they could interfere with the function of these receptors, in the peripheral and possibly also in the central nervous system *in vivo*. Moreover, antibodies were neither able to induce receptor desensitization and/or downregulation of the target receptor nor cell cytotoxicity. All in all these results provide further evidence that autoimmunity against the autonomic nervous system may be involved in the pathogenesis of CRPS.

The complex regional pain syndrome (CRPS) may develop after peripheral limb trauma. Clinical symptoms of acute CRPS are characterized by signs of inflammation (pain, edema, red skin) and neurologically mediated disturbances (loss of vasomotor control, increased or decreased sweating, urogenital symptoms) that result from a contribution of the sympathetic and/or peptidergic innervation of the target tissue (Chancellor, Shenot et al. 1996; Wasner, Schattschneider et al. 2001; Albrecht, Hines et al. 2006), and later on in the course of chronic CRPS, abnormal central nervous system plasticity. Pain and hyperalgesia might be related to sensitization of primary and secondary nociceptive neurons by e.g. proinflammatory cytokines (Maihofner, Handwerker et al. 2005; Sabsovich, Guo et al. 2008), with increased release of neuropeptides (CGRP, SP) from these sensitized peripheral nociceptors (Weber, Birklein et al. 2001; Guo, Offley et al. 2004). The origin of the variable sympathetic disturbances is largely unknown, and both peripheral (Uceyler, Eberle et al. 2007) and central disturbances (Moseley,

Zalucki et al. 2008) have been discussed. The reason why only some patients develop CRPS after trauma, while the majority does not, remains elusive.

We postulated the immune system is involved in the development of CRPS, since antibodies have been demonstrated to recognize autonomic nervous system structures in some patients (Blaes, Schmitz et al. 2004). In the present study, we sought to identify the target autoantigen and characterize putative functional properties of these autoantibodies. We confirmed the presence of putative antibodies, and showed that these antibodies display agonistic properties on M2R and β2AR in most CRPS patients tested (90%). It is not clear whether these antibodies are already present before the injury happens or are produced after injury and the following inflammation. Indeed, the exposition of autoantigens from autonomic neurons to the immune system might induce the production of autoantibodies. Autoimmunity is though mediated by a variety of complex mechanisms, molecular and cellular events, and responses. It is worthy pointing out that even if a given autoimmune disease was to be initiated primarily by a single trigger, other events and regulating mechanisms come into play, thereby adding complexity to the process.

Predisposition to a given autoimmune response requires the requisite allele(s) that controls antigen presentation by antigen-presenting cells for T cell recognition. Indeed, CRPS has been associated to some HLA alleles - DQ1, DR13, DR15 and the centromeric regions of the HLA class 1 antigens were linked to CRPS (Mailis and Wade 1994; Kemler, van de Vusse et al. 1999; van Hilten, van de Beek et al. 2000; van de Beek, Roep et al. 2003; Meier, Alexander et al. 2006). Although association with HLA alleles does not necessarily implicate an immune etiology of a disease, yet many autoimmune diseases have distinct HLA associations. The presence or absence of appropriate MHC determines whether the potential autoantigen will be presented for immune recognition and responses or not.

Furthermore, proteins to which the immune system is ordinarily self-tolerant might, if altered, elicit autoimmune responses (Yu, Mamchak et al. 2003). Sequestered proteins normally sheltered from immune recognition once exposed to the immune

system become immunogenic and targets of immune effector functions. Tissue damage, inflammation and its mediators that precede the development of CRPS could play a pivotal role in exposing or modifying self-proteins in inflammatory sites to the immune system.

Other alterations can occur because of disruption in the levels or activity of regulatory proteins, and as a consequence disturbed activity of regulatory cells. Mutation in certain alleles can cause immune dysregulation and lead to systemic autoimmunity such as TNF-α. CRPS patients display significant increases in proinflammatory cytokines (TNF-α, interleukin-1β, -2, and -6) in local blister fluid, circulating plasma and cerebrospinal fluid, and higher plasma levels of soluble TNF-α. Interestingly, administration of a TNF-α antibody (infliximab) produced notable reductions in CRPS symptoms in some patients (Huygen, Niehof et al. 2004). Further arguments for an involvement of immune system dysregulation in CRPS are studies showing altered monocyte reactivity to interferon-γ stimulation (Hartrick 2002).

Many autoimmune responses have been shown to emerge following infection by a pathogen, whose protein(s) possess structural similarities in some of its epitopes to host proteins. Thus, antibodies developed against a pathogen might cross-react with a self-protein and act as autoantibodies. The involved autoantigen becomes then a source for persistent stimulation (Blank, Barzilai et al. 2007). There are reports about the increased prevalence of parvovirus B19 IgG in CRPS patients (van de Vusse, Goossens et al. 2001; Gross, Tschernatsch et al. 2007). Parvovirus B19 infection induces autoantibody production and might lead to autoimmune diseases (Holm, Hansen et al. 1995). A comparable molecular mimicry causing autoimmune inflammation of the nervous system has been described in campylobacter-associated polyradiculitis. Interestingly, an association of campylobacter-positive serology to CRPS has also been described (Goebel, Vogel et al. 2005; Willison 2005). Accordingly, one may assume that immunoglobulins can arise independently during an infection but only later on function as templates for the generation of pathogenic autoantibodies.

Imbalance in factors involved in immune responses to an infectious pathogen, such as cytokines and associated mediators or receptors, can contribute to enhance antigen mimicry in infections (Tsang and Valdivieso-Garcia 2003). Peripheral trauma, in particular if it is accompanied by partial peripheral nerve lesion, causes a rapid release of cytokines and neurotrophins (Sabsovich, Guo et al. 2008; Sabsovich, Wei et al. 2008), which activate and sensitize primary and probably also spinal afferents (Maihofner, Handwerker et al. 2005). Activation and sensitization of primary afferents also cause an increase of neuropeptide release into the affected body region (mainly substance P and CGRP). Chronic release of neuropeptides might not only be responsible for the clinically evident inflammatory CRPS symptoms but also contribute for immune dysregulation (Weber, Birklein et al. 2001).

The sum of these entire factors can contribute to the breakdown of self-tolerance. Autoantibodies may emerge through a process of clonal selection if self-tolerance is disturbed, as a result of inflammatory processes and tissue damage. As a consequence, it might lead to the production or spreading of detected autoantibodies. What follows is the exposition of autoantigens and/or dysregulation of the immune system, resulting in the emergence of an autoimmune disease. Indeed, no single hypothesis until now could explain all features of CRPS. Whether such syndrome could be the result of the development of autoantibodies needs still to be further investigated. However, the identification of agonistic-like autoantibodies against M2R and β2AR receptors must not be underestimated, since they showed important agonistic properties and do not induce receptor desensitization or downregulation of target receptors. One may speculate that the persistent stimulation of these receptors by functionally active autoantibodies could well lead to autonomic dysregulation observed in CRPS.

Impairment of sympathetic function has been repeatedly shown in CRPS including disturbances of vasomotor (Birklein, Riedl et al. 1998; Wasner, Schattschneider et al. 2001) and sudomotor function (Birklein, Kunzel et al. 2001). The origin of these sympathetic nervous system symptoms in posttraumatic CRPS is still widely

unclear. Human and animal studies strongly support the concept of an adrenergic supersensitivity in CRPS (Drummond, Skipworth et al. 1996; Chemali, Gorodeski et al. 2001; Gibbs, Drummond et al. 2008; Drummond 2009). Vasoconstriction in the affected extremity could be triggered by injection of lower doses of noradrenaline than in the unaffected side. Correspondingly, Jorum et al. showed that sensitized mechano-insensitive nociceptors are activated by endogenous sympathetic activity and thereby may contribute to sympathetically maintained pain observed in a subset of CRPS patients (Jorum, Orstavik et al. 2007). The adrenergic sensitivity that appears to develop in primary sensory afferents after nerve injury and inflammation is consistent with the notion that dysfunction of the sympathetic activity might be due to the effect of functionally active autoantibodies binding to β2AR receptors of peripheral autonomic neurons, target cells or immune cells in CRPS.

Though antibodies showed an agonistic effect on M2R and β2AR, the epitope sequences identified correspond to common allosteric binding sites for allosteric modulators. These have frequently the property of enhancing binding and effect of the agonists mostly stabilizing the receptor in its “active” conformation (Lebesgue, Wallukat et al. 1998; Peter, Eftekhari et al. 2003; May, Avlani et al. 2007; Hernandez, Nascimento et al. 2008). If antibodies activate receptors, in one way or another, the endogenous sympathetic discharge might trigger activity in primary sensory fibers. The fact that noradrenaline injection in the skin induces pain in CRPS patients as well as rekindle pain that had subsided after sympathectomy endorsed this hypothesis. Moreover, axon-reflex sweating induced by iontophoresis of phenylephrine was greater in the affected side than in the unaffected side, and was greater than in control subjects or resolved CRPS (Chemali, Gorodeski et al. 2001). Though phenylephrine is a well known alpha 1 adrenergic receptor agonist, it has the property of stimulating also βAR (Torp, Tschakovsky et al. 2001).

Wasner et al. suggested that sympathetic vasoconstrictor reflexes and venous levels of norepinephrine and its metabolites are diminished as a result of

decreased release of transmitter from postganglionic sympathetic fibers on the affected side (Wasner, Schattschneider et al. 2001). This is consistent with the notion that persistent agonistic stimulation of β2AR by immunoglobulins and the following supersensitivity might lead to reduction of neurotransmitter release in the affected limb. Conversely, it seems reasonable to assume that the effect of circulating antibodies would not be restricted to the affected limb. Indeed, there is evidence for a subclinical contralateral sympathetic dysfunction in CRPS, specially in the early stage (Rosen, Ostergren et al. 1988; Bej and Schwartzman 1991; Kurvers, Jacobs et al. 1996).

Additionally, autoantibodies against M2R might contribute to autonomic symptoms in CRPS. Cholinergic receptor antibodies may explain associated features of autonomic dysfunction in Sjögren's syndrome (Waterman, Gordon et al. 2000). Cholinergic neurons are all central autonomic neurons and the peripheral parasympathetic and sudomotor neurons (Low 1997). Thus, autonomic disturbances induced by anticholinergic autoantibodies might be very different in CRPS patients and include vasomotor (Wasner, Schattschneider et al. 2001), sudomotor (Birklein, Sittl et al. 1997), cardiovascular (Meier, Alexander et al. 2006) and urogenital (Chancellor, Shenot et al. 1996) symptoms.

Functional autoantibodies that act at ion channels or receptors disrupting autonomic or cardiovascular functions have been described in many autoimmune diseases, and are often pathogenic (Waterman, Lang et al. 1997; Waterman, Gordon et al. 2000; Goldblatt, Gordon et al. 2002; Gleicher, Barad et al. 2007; Jackson, Gordon et al. 2008; Maciejewska-Rodrigues, Al-Shamisi et al. 2010). In well characterized autoantibody-mediated neuroimmunological diseases, such as myasthenia gravis or Lambert-Eaton myasthenia syndrome, pathogenically relevant autoantibodies are directed against voltage- or ligand-gated ion channels and their associated proteins at the neuromuscular junction (Lang and Vincent 2003). Surface antibodies not only play a pivotal role in the pathophysiology of neuroimmunological diseases but also in cardiologic diseases like autoimmune- or Chagas-associated cardiomyopathy (Wallukat and Wollenberger 1987; Goin,

Borda et al. 1994; Lang and Vincent 2003).

Functional active autoantibodies against G-protein coupled receptors with pathophysiologic relevance have been clinically and experimentally demonstrated in patients with heart diseases such as Chagas' disease (β1-, β2AR and M2R antibodies), dilated cardiomyopathy (β1AR and M2R antibodies), pre-eclampsia (angiotensin 1 receptor antibodies) and essential hypertension (alpha 1 adrenergic receptor antibodies) (Wallukat, Fu et al. 1999; Fu, Herlitz et al. 2000; Wallukat, Nissen et al. 2000; Zhou, Zhang et al. 2008). We here identified autoantibodies against two autonomic receptors in patients with CRPS - the M2R and the β2AR, which are characterized by functional effects *in vitro*. Similar pattern of autoantibodies against the M2R and β2AR has been reported in Chagas' disease. However, the antibodies found in CRPS have different epitope specificity and also different functional properties. In contrast to the M2R antibodies in Chagas' disease, agonistic M2R antibodies in CRPS bind only to the second extracellular domain of the receptor; this is similar to the M2R autoantibodies in patients with dilated cardiomyopathy.

Autoantibodies against M2R occur in patients with dilated cardiomyopathy in combination with beta-adrenergic receptor antibodies (Stavrakis, Kem et al. 2010). Most groups failed to demonstrate the importance of β2AR reactive autoantibodies in dilated cardiomyopathy. Furthermore, beta2 blockage in dilated cardiomyopathy increased the contractility in cardiomyocytes suggesting an antagonistic effect of these antibodies. In contrast, the positive chronotropic effect of the CRPS IgG demonstrated an agonistic effect. Blocking the CRPS anti-β2AR antibodies with specific peptides revealed an additional functional epitope (ATHQEAI).

The question whether autoantibodies are simply epiphenomenon or directly linked to pathogenesis of CRPS remains unclear. In fact, 90% of CRPS IgG were detected by ELISA to bind to at least one of reported peptides from the second extracellular domain of these receptors; and, the same percentage showed functional effect on cardiomyocytes assay. Though the immunogenicity of proteins is mostly inherent to their three dimensional conformation, an ELISA displaying

small-peptides in linear sequences was very efficient in detecting CRPS antibodies. Cardiomyocyte bioassay has also been proved to be a very sensitive method for detection of receptor antibodies (Wallukat, Fu et al. 1999), and therefore showed high detection efficiency. It is very important to mention that we performed these experiment many times and the effect was entirely replicable for all patients enabling us to affirm these methods as sensitive in detecting CRPS antibodies.

One explanation why antibodies could not be demonstrated in all patients might well be the inclusion of patients according to the IASP research criteria. Although all these patients meet the IASP criteria for CRPS, it is unclear whether they all have the same physiopathology. Even by clinical criteria, there seem to be subtypes within these groups. For instance, patients with “warm” and “cold” CRPS do not only differ in the temperature of the affected limb, but also show a distinct clinical pattern including differences in sensory impairment measured by quantitative sensory testing or differences in the frequency of associated dystonia (Eberle, Doganci et al. 2009). Therefore, patients with autoantibodies might represent a special pathophysiological subgroup of CRPS. It is noteworthy that pathogenic hβ1AR autoantibodies - classically proven to play a crucial role in the pathophysiology of dilated cardiomyopathy by reducing cardiac function and inducing chronic heart failure - are also not detected in all dilated cardiomyopathy patients, but rather in less than 50% (Jahns, Boivin et al. 1999).

Approximately 40-50% of CRPS patients were demonstrated to have surface-binding antibodies detected by flow cytometry with differentiated autonomic neurons or CHO cells overexpressing hβ2AR or hM2R. One reason for the lower detection in this case might be the method used. If antibodies are low-affinity binding IgGs, our method (flow cytometry) is not able to discriminate the specific binding from background. Low-affinity binding autoantibodies have also been recently found in patients with myasthenia gravis where no anti-nAChR antibodies could be found in normal tests (Leite et al. 2008).

Neuroblastoma cells are a well-established model for the differentiation of

dedifferentiated cells into a variety of neuronal subtypes (Gomez-Santos, Ferrer et al. 2002). The high binding to differentiated autonomic neurons in comparison to neuroblastoma cells was surprising, since other autoantibodies (i.e. anti-VGCC in LEMS) show preferential binding to undifferentiated neuroblastoma cells in a high percentage (Waterman, Lang et al. 1997). We could obviously induce the autoantigen by differentiation of neuroblastoma cells into a cholinergic phenotype, whereas differentiation into a catecholaminergic phenotype by TGF-β1 decreases the surface-binding of CRPS sera. These data suggests that the underlying autoantigen is highly restricted to distinct neuronal phenotype and may be located on special cholinergic neurons. The mutual use of BMP-2 and RA in the presence of fetal calf serum has been proved to play a trophic positive role in the differentiation of immature cells like SH-SY5Y to cholinergic phenotype neurons that express choline acetyltransferase (Gomez-Santos, Ferrer et al. 2002). We could additionally show an up-regulation of VAChT expression associated with a downregulation of catecholaminergic markers in BMP-2/RA differentiated cells. Interestingly, in further experiments, we demonstrated that both β2AR and M2R were found to be upregulated in BMP-2/RA differentiated SH-SY5Y neuroblastoma cells. This might indicate that the previously detected, but unidentified, cell-surface antigen on differentiated autonomic cells must be either β2AR (expressed also in sympathetic neurons) and/or M2R.

Indeed, one problem on CRPS research is the lack of a gold standard. The demonstration of autoantibodies in most CRPS patients could be a very promising tool on developing markers to make or improve diagnosis, in a more sensitive and specific way, as well as to follow the development of the disease or the effect of interventions. For more than two decades, the detection of serum autoantibodies has been used for the diagnosis and classification of autoimmune diseases. It is now clear that many autoantibodies have the ability to predict the development of an autoimmune disease and serve as biomarkers for different disease activity. Many autoantibodies, produced during an infection far before disease's onset, are detected in the pre-clinical phase of autoimmune diseases many years before the

disease becomes apparent (Verge, Gianani et al. 1996; LaGasse, Brantley et al. 2002; Maclaren, Lan et al. 2003; Scofield 2004; Shoenfeld, Blank et al. 2008). It would be very promising identifying persons at risk to develop CRPS, especially if disease onset or progression can be prevented by avoiding environmental factor that could work as a trigger. However, further studies are required to optimize these methods for the diagnosis of CRPS.

Further research is also necessary to gain more insight into the clinical significance of autoantibodies and their pathogenic mechanisms in CRPS. The clinical picture of CRPS is clearly restricted to one (rarely two or three) limb(s), and an autoimmune process against two ubiquitously expressed receptors might not provide a unifying hypothesis for all CRPS symptoms. However, both β2AR and M2R are involved in the modulation of pain and inflammation (Hocking, Smith et al.; Diatchenko, Anderson et al. 2006; Hayashida, Bynum et al. 2006; Danielson, Andersson et al. 2007).

One prospective study has reported that haplotypes reflecting variability in eight polymorphisms in the β2AR gene were associated with risk for later development of a common chronic pain condition (Diatchenko, Anderson et al. 2006). Such β2AR polymorphisms also play a role in regulation of vascular tone, and thus may be relevant to understand the vasomotor characteristics of CRPS (Brodde and Leineweber 2005). Of note, catecholamines regulate the production and release of proinflammatory cytokines acting via β2AR on immune cells (Maestroni 2006). Activation of the β2AR possess pro-inflammatory properties and mediate increased production of proinflammatory cytokines IL-1β and IL-6 by macrophage cell lines (Tan, Nackley et al. 2007).

Recently it has been reported that the cholinergic system has an important role in the modulation of nociceptive responses (Haberberger et al, 2004; Rashid et al, 2006). Under physiological conditions M2R activation on peripheral nociceptors interferes with nerve excitability and therefore with release of neuropeptides, the major mediators of neurogenic inflammation (Bernardini, Roza et al. 2002). Thus, if the immune-mediated inflammation causes changed receptor expression in

neurons, this might explain the localized CRPS symptoms.

Activation of both G protein coupled receptors β2AR (Magne, Couchie et al. 2001) and M2R (Sorimachi, Furukawa et al. 1995) by agonists increase intracellular Ca^{2+} concentration mostly mediated by L-type Ca^{2+} channels. Calcium is an important messenger in intracellular signaling mechanisms; therefore, it has been contemplated that the agonistic antibodies might have an effect on intracellular Ca^{2+} concentration. The results showed that the purified IgG from patients indeed triggered a rapid increase in intracellular Ca^{2+} concentration in receptor transfected cells similar to the response of agonist, while HC IgG did not show any effect at all. This change in Ca^{2+} might result from the agonistic effects of antibodies on G protein coupled receptor. A change in Ca^{2+} concentration is the first feature after protein G receptor activation, which initiates and coordinates specific cellular activities after a given agonistic stimuli followed by phosphorylation of multiple proteins and alteration of gene expression. Calcium is an important intracellular messenger that is involved in modulating a vast array of cellular events.

Autoimmunity against the autonomic nervous system with functional autoantibodies against ion channels or receptors that disrupt autonomic or cardiovascular functions have been described in many autoimmune diseases including Lambert-Eaton Myasthenic syndrome (LEMS) (Waterman, Lang et al. 1997), autonomic neuropathies (Vernino, Low et al. 2000; Goldblatt, Gordon et al. 2002), Scleroderma (Goldblatt, Gordon et al. 2002), Sjögren's syndrome (Waterman, Gordon et al. 2000), and others (Gleicher, Barad et al. 2007; Jackson, Gordon et al. 2008; Maciejewska-Rodrigues, Al-Shamisi et al. 2010). LEMS IgGs impair transmitter release from parasympathetic and sympathetic neurons through downregulation of one or more subtypes of voltage-gated calcium channels (Waterman, Lang et al. 1997). Pharmacological overstimulation of beta-receptors by beta-agonists has been shown to induce both receptor desensitization and internalisation, and to lead to cytotoxicity on cardiomyocytes (Karliner, Simpson et al. 1986; Zhang, Kimura et al. 2005). Conversely, we showed CRPS autoantibodies did not possess additional properties of inducing either

desensitization or downregulation of β2AR or M2R. Besides, pharmacological overdriving of G protein receptors can induce disturbances of cell functions and lead to an increased cell death rate (Gao, Liu et al. 2006). However, CRPS antibodies did not show any cytotoxic effects against receptor transfected cells.

We addressed a possible cross-reactivity between both functionally active receptor autoantibodies. The rational behind that was to prove whether they represent two functionally independent receptor antibodies. In Chagas disease, beta1-antibodies react with an epitope on the second extracellular domain of β1AR, but interestingly cross-react with an epitope on the M2R (Elies, Ferrari et al. 1996). Nonetheless, β2AR or M2R antibodies that coexist in the serum of CRPS patients are two entirely distinct antibodies, rather than cross-reacting antibodies. They have been proved to belong to different immunoglobulin subclass - β2AR belong to IgG1 and IgG2 subtypes whereas M2R antibodies to IgG1 and IgG3 subtypes – and therefore probably present different immunological function.

One may discuss it is very unlikely a syndrome involving symptoms mostly locally restricted to the affected extremity to be antibody-mediated. Though the main clinical feature of CRPS is limited to one extremity, the involvement of other extremities or systemic disturbance in the urogenital (Chancellor, Shenot et al. 1996), cardiovascular (Meier, Alexander et al. 2006) and gastrointestinal (Goebel, Buhner et al. 2008) system are frequently observed. As likely as not, autoantibodies - an important serological feature of CRPS - might induce weak systemic but strong localized symptoms if receptor expression in neurons has changed locally after trauma.

Since its first description more than 150 years ago, the pathophysiology of CRPS is very complex and still not understood. Some components have been identified such as a neurogenic inflammation involving neuropeptides such as substance P and CGRP; a disturbance of the sympathetic nervous system, in both peripheral and central nervous system, and a cortical reorganization process leading to chronic disability. Additionally, a reduction of small-diameter axons in the affected area of the limb has also been described (Oaklander, Rissmiller et al. 2006). Here

we achieved important progress in understanding the involvement of the immune system in this complex symphony of CRPS by proving the coexistence of two functionally active autoantibodies against autonomic nervous system receptors in the majority of CRPS patients.

The immune-hypothesis of CRPS has also been supported by different treatment effects: IvIg have been shown effective in some patients with CRPS in a randomized cross-over trial and in case reports (Goebel, Baranowski et al.; Goebel, Stock et al. 2005). Most surface-binding autoantibodies in neurological diseases, such as acetylcholine receptor antibodies in myasthenia gravis or potassium channel antibodies in limbic encephalitis with obviously pathogenic effects respond well to corticosteroids, IvIg or plasmapheresis (Vincent, Buckley et al. 2004; Linker and Gold 2008). Thus, immunosuppressive or immune-modulatory therapies could represent a promising treatment option in patient with otherwise treatment-resistant chronic CRPS, if surface-binding autoantibodies are detectable, and therefore worthy to be further investigated.

We were not able to find any correlation between serological and clinical features. Although there were no differences in clinical autonomic symptoms between surface binding-antibody-positive and –negative patients; more subtle differences, which would need differentiated autonomic testing in forthcoming studies, could not be excluded. Since many polyneuropathy patients tested also had medical history of chronic pain and none of them showed binding antibodies, the presence of receptor binding antibodies seems not to be related to chronic pain, but rather a serological feature of CRPS patients.

The origin of the autoimmune process and its relevance for the pathophysiology of CRPS remains to be completely elucidated. It further remains unclear whether the autoantibodies are already present before the clinical manifestation of CRPS or appeared thereafter. We could not detect these autoantibodies in controls, NL and NP indicating that a trauma or nerve lesion alone does not induce specific autoimmunity. Regarding the signs of inflammation and the association with antimicrobial serology, one may speculate that in CRPS the autoimmune process

may be the result of a cross-reactive immunity between viral or bacterial epitopes and structures of the autonomic nervous system in genetically predisposed individuals. Taken together, the identification of receptor-binding autoantibodies directed against the muscarinic M2R and the adrenergic β2AR with functional properties provides clear evidence of an autoimmune etiology of CRPS - at least in certain patients. We supplied sufficient data to justify further exploration in order to gain more insight into the clinical significance of autoantibodies and their pathogenic role in CRPS. Thus, huge progress has been achieved in our understanding of the pathophysiology of this most complicated pain disorder and should additionally foster the rationale for an immune-based treatment approach in these patients.

VI - SUMMARY

Complex regional pain syndrome (CRPS) is a painful condition affecting one or more extremities of the body, marked by a wide variety of symptoms and sings that are often difficult to manage since pathophysiology is incompletely understood. Thus, diverse treatments might be ineffective. A recent report revealed the presence of autoantibodies against nervous system structures in CRPS patients. However it remained unclear how the antibodies act in the development of CRPS. We therefore aimed to characterize these antibodies and identify target antigens.

Sera of CRPS patients, neuropathy patients and healthy volunteers were tested for surface-binding autoantibodies to primary cultures of autonomic neurons and many differentiated neuroblastoma cell lines using flow cytometry. Further, functional properties of affinity-purified IgG of controls or CRPS patients were assessed using a cardiomyocyte bioassay. Putative receptors and target antigens were identified using antagonistic drugs and synthesized peptides sequences corresponding to segments of these receptors. Peptides were also used to establish an enzyme immunoassay. Antibodies were tested in stable transfected CHO cells with putative receptors to ensure observed binding. Further, changes in the intracellular Ca^{2+} concentration induced by agonistic IgG were measured using the Ca^{2+}-sensitive fluorescent dye fura-2 assay. Potential cytotoxicity of binding antibodies were tested using LDH and WST-1 assay.

13/30 CRPS patients, but none of 30 healthy controls and only one out of 20 neuropathy sera had specific surface-binding to autonomic neurons. The majority of the sera reacted with both sympathetic and myenteric plexus neurons. The differentiation of SH-SY5Y into a cholinergic phenotype induced a surface-antigen, which is recognized by 60% of CRPS sera (18/30), but not by controls. Moreover, ELISA and an established cardiomyocyte assay demonstrated the presence of autoantibodies in most CRPS patients with agonistic-like properties on the β2AR

and M2R. We identified these autoantibodies as immunoglobulin G directed against peptide sequences from the second extracellular loop of these receptors. Though antibodies acted on receptors and increase in intracellular Ca^{2+} concentration in transfected cells, they were not able to induce receptor downregulation, desensitization or cytotoxicity.

The recent detection of functionally active autoantibodies in serum samples from CRPS patients against cell-surface determinants of sympathetic, myenteric and differentiated autonomic nervous system neurons, and the identification of these determinants as part of β2AR or M2R expressed in these cells suggested an autoimmune mechanisms as one important pathogenic factor in the pathophysiology of CRPS. Thus, our findings contribute to the understanding of this disease, could help in the diagnosis in future, and encourage new treatment strategies focusing on the immune system.

VII - ZUSAMMENFASSUNG

Das komplexe regionale Schmerzsyndrom gehört zur Gruppe der chronisch posttraumatischen Schmerzsyndromen. Die Krankheit ist gekennzeichnet durch regionale, persistierende neuropathische Schmerzen mit sensorischen-, vaso-, sudomotorischen-, motorischen- und trophischen Veränderungen nach äußerer Einwirkung (z. B. Traumen, Operationen und Entzündungen). Da der zugrundeliegende Pathomechanismus des CRPS noch nicht eindeutig geklärt ist, sind die Symptomenkomplexe therapeutisch nur symptomatisch beeinflussbar. Unserer Gruppe gelang erstmals der Nachweis von Autoantikörpern gegen Strukturen des autonomen Nervensystems bei Patienten mit CRPS. Bis heute wurde jedoch noch nicht untersucht, in wie weit diese Antikörper eine Rolle bei der Entstehung des CRPS spielen könnten. Das Ziel der vorliegend Arbeit war die Charakterisierung dieser Antikörper, sowie die Identifizierung der zugehörigen Antigene.

In der hier vorgestellten Studie untersuchten wir Seren von gesunden Probanden, CRPS- und Polyneuropathie-Patienten auf das Vorliegen relevanter Autoantikörper gegen Oberflächenepitopen primärkultivierter autonomer Neuronen und differenzierter Neuroblastom-Zellen mittels Durchflusszytometrie. Darüber hinaus untersuchten wir mögliche funktionelle Effekte antineuronaler Autoantikörper im Bioassay der spontan pulsierenden neonatalen Rattenherzmuskelzellen. Zur Identifizierung der Bindungsrezeptoren bzw. Bindungsepitops untersuchten wir den Effekt von Rezeptoren-Antagonisten und von kurzen überlappenden Peptiden der extrazellulären Schleife verschiedener Rezeptoren. Diese Peptide der extrazellulären Schleife autonomer Rezeptoren wurden zur Etablierung eines ELISA-Assay verwendet. Zur Bestätigung der Bindungseigenschaften der Autoantikörper wurden stabil transfizierte CHO-Zellen mit Bindungsrezeptoren durch Anwendung der Durchflusszytometrie und Imaging-(Fura-2)-Technik getestet.

Bei 13/30 CRPS-Patienten aber nur bei 1/20 Patienten mit Polyneuropathie und bei keiner Kontrolle konnte die Bindung von IgG an der Oberfläche primärkultivierter autonomer Neuronen durchflusszytometrisch nachgewiesen werden. Die meisten Seren reagierten sowohl mit Neuronen aus sympathischen Ganglien, wie auch kultivierten Darmplexusneuronen. 18/30 der CRPS- aber keine Kontroll-Seren erkannten eine Epitop erzeugt durch Differenzierung von SY5Y zum cholinergischen Phänotyp. Unsere Ergebnisse zeigten, dass die Mehrheit der CRPS-Patienten stimulierende Antikörper gegen β2AR und M2R aufweisen. Der Nachweis der Autoantikörper erfolgte mittels ihres chronotropen Effekts auf die Pulsationsrate der Herzmuskelzellen sowie dessen Blockierung durch den adrenergen β2-Blocker, den M2-Antagonist und synthetische gegen die 2. extrazelluläre Schleife der beiden Rezeptoren gerichtete Peptide. Diese Daten wurden durch erhöhte Bindung patogenischer Antikörper zu der Oberfläche β2AR und M2R transfizierter Zellen bestätigt. Obwohl durch die Imaging-(Fura-2)-Technik der stimulierende Antikörpereffekt wieder bestätigt wurde, führte keine untersuchte IgG-Fraktionen von CRPS-Patienten zu zellbiologischen Veränderungen wie Zytotoxizität oder Rezeptor-Desensitisierung bzw. -Downregulation.

Die von uns nachgewiesenen funktionellen Effekte antineuronaler Autoantikörper von CRPS-Patienten gegen primärkultivierter und differenziert autonomer Neuronen und der Charakterisierung stimulierender Autoantikörper gegen Peptiden der zweiten extrazellulären Schleife der β2AR und M2R deutet darauf hin, dass Immunmechanismen in der Pathogenese des CRPS, möglicherweise durch eine Autoimmunreaktion gegen das autonome Nervensystem eine wichtige Rolle spielen könnten. Unsere Erkenntnisse bieten einen Einblick in mögliche pathophysiologische Mechanismen bei der Entstehung von CRPS. Sie könnten als Basis für neue Überlegungen über diagnostische Methoden und den Einsatz immunmodulierender Therapiestrategien dienen.

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X – PUBLICATIONS

Publications originated from this dissertation:

1) Blaes F, Tschernatsch M, Braeu ME, Matz O, Schmitz K, Nascimento D, Kaps M, Birklein F. Autoimmunity in complex regional pain syndrome. Ann N Y Acad Sci. 2007 Jun;1107:168-73. Review.

2) Kohr D, Tschernatsch M, Schmitz K, Singh P, Kaps M, Schäfer KH, Diener M, Mathies J, Matz O, Kummer W, Maihöfner C, Fritz T, Birklein F, Blaes F. Autoantibodies in complex regional pain syndrome bind to a differentiation-dependent neuronal surface autoantigen. Pain. 2009 Jun;143(3):246-51.

3) Kohr D, Singh P, Tschernatsch M, Kaps M, Pouokam E, Diener M, Kummer W, Birklein Vincent A, Goebel A, F, Wallukat G*, Blaes F*. Autoimmunity against the beta2 adrenergic receptor and muscarinic 2 acetylcholine receptor in complex regional pain syndrome (CRPS). Pain. 2011 Aug 2.

Other publications:

4) Nascimento DC, Pereira BAF, Teixeira JRM. *Mikania lindleyana D. C.* effect on the memory: experimental study in rats. Rev. Para. Med. 2003. 17(2)6-11.

5) Andersen ML, Nascimento DC, Machado RB, Ping W, Roizenblatt S, Moldofsky H, Tufik S. Substance P and Sleep: Interactions Between Tachykinins and Sleep-Wake Mechanisms. Behav Brain Res. 2006 Feb 28;167(2):212-8.

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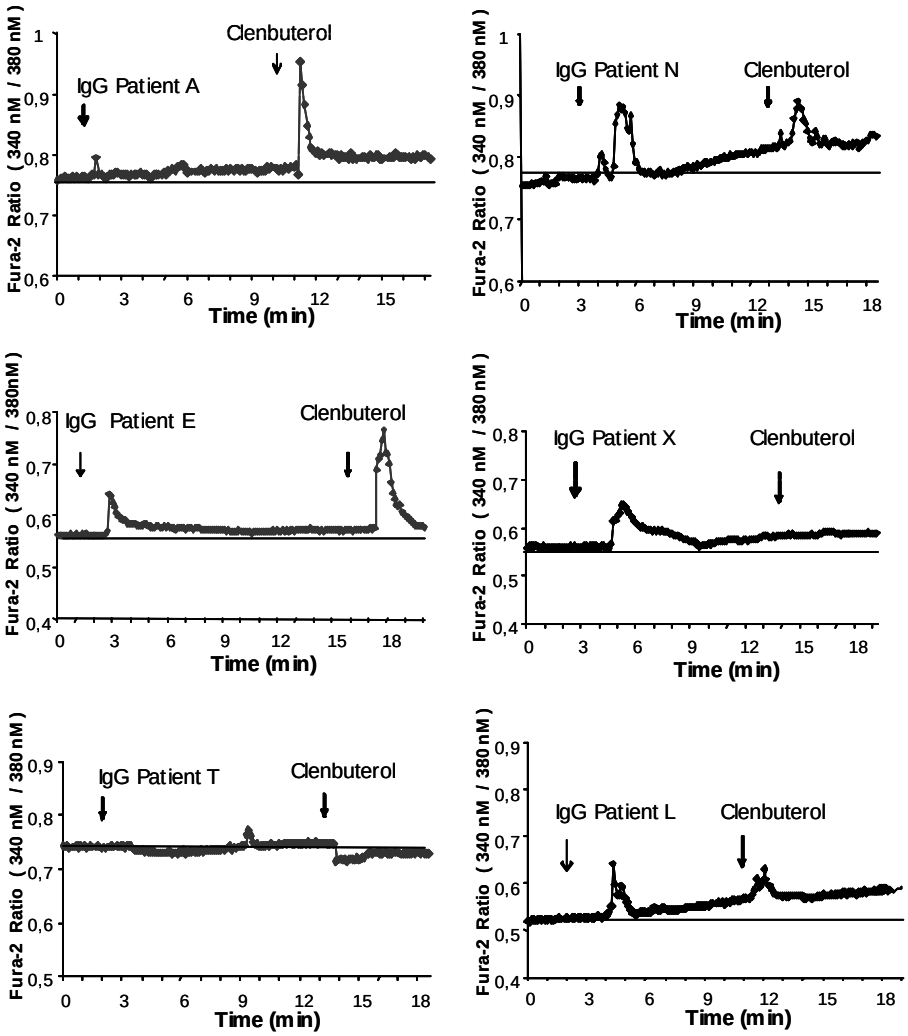
XI – APPENDIX

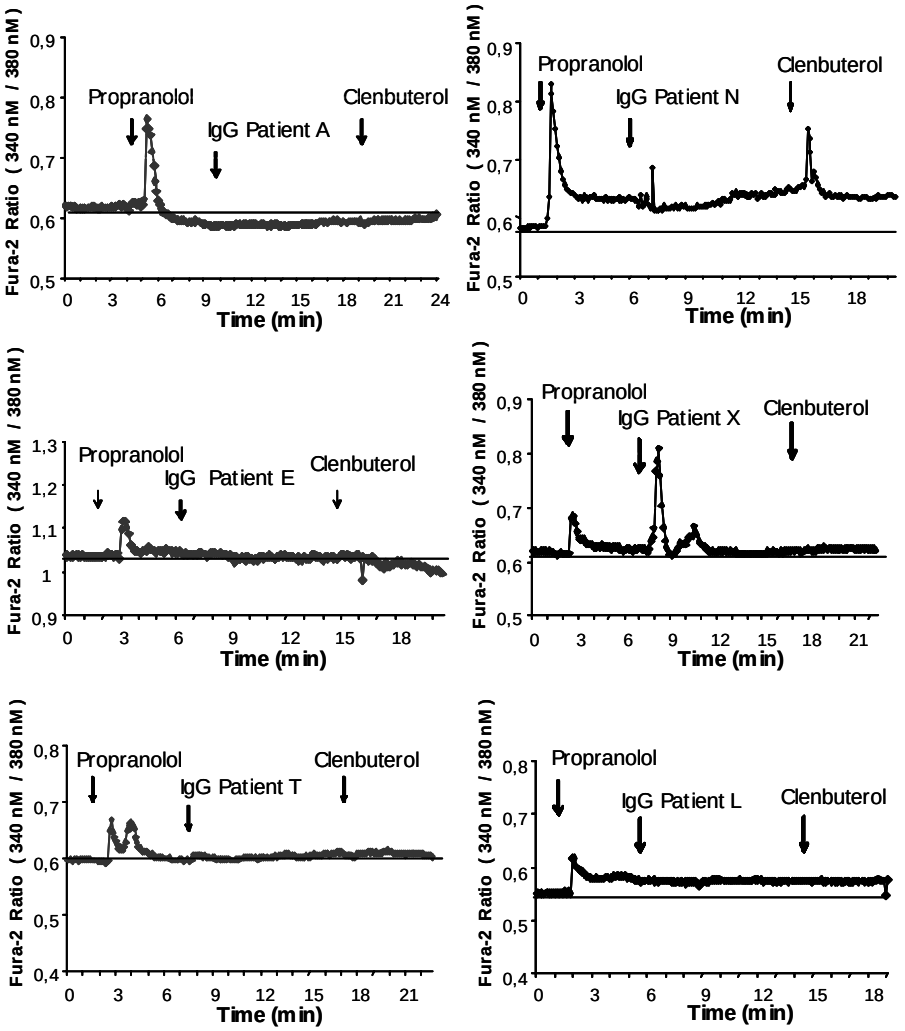
Appendix 1. Table displaying the binding of each tested patient to peptides of β2AR and/or M2R detected using ELISA.

ID	CRPS type*	CRPS Warm/Cold*	β2AR Peptides			M2R Peptides		
			HWYRAT	ATHQEAI	AINCYAN	VRTVED	EDGECY	CYIQFF
A	2	Cold	-	-	-	+	+	-
B	1	Warm	-	-	-	+	+	-
C	2	Warm	-	-	-	-	+	-
D	1	Cold	-	-	-	-	+	-
E	1	Warm	+	-	-	+	+	-
K	2	Warm	-	+	-	-	+	-
L	2	Warm	+	+	-	+	+	-
M	2	Warm	-	-	-	+	+	-
N	2	Warm	-	-	-	-	-	-
T	2	Cold	-	-	-	-	+	-
V	2	Warm	+	+	-	+	-	-
W	2	Warm	+	+	-	+	+	-
X	2	Cold	-	+	-	+	+	-
Y	2	Cold	+	+	-	-	+	-
6	1	Warm	+	+	-	+	+	-
7	1	Warm	+	+	-	+	-	-
8	1	Warm	+	+	-	+	+	-
9	1	Warm	+	+	-	+	+	-
10	1	Warm	+	+	-	+	+	-
11	1	Warm	-	-	-	-	-	-

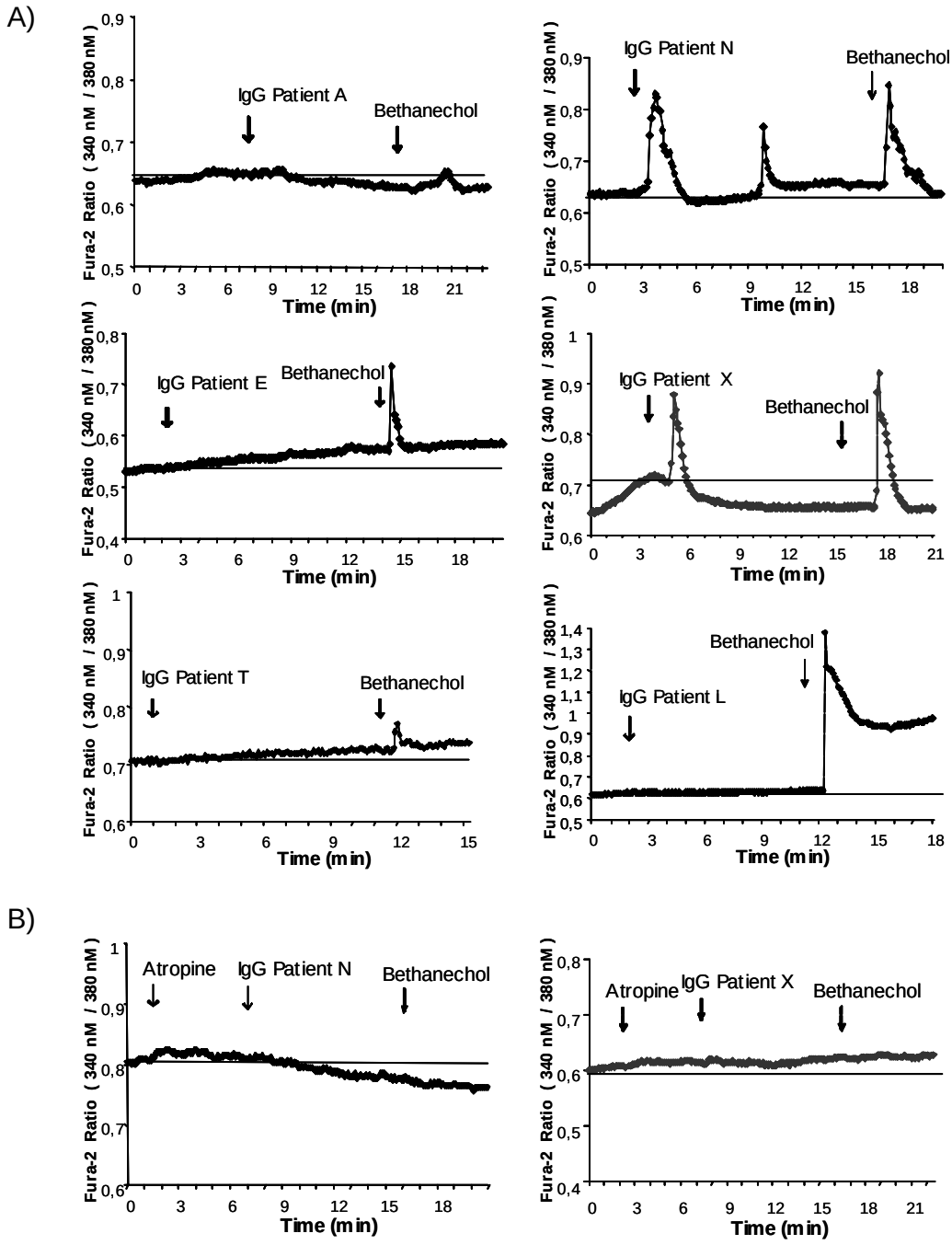
**There were no significance between CRPS type or warm/cold and positive binding to any tested peptide (Fisher's exact test, $p > 0.05$).*



Appendix 2. Changes in the intracellular Ca^{2+} concentration induced by CRPS autoantibodies or agonist in hβ2AR-CHO. CRPS autoantibodies and β2-agonist clenbuterol (30 µM) induced an increase of the fura-2 signal in hβ2AR-CHO cells. For statistics see text.

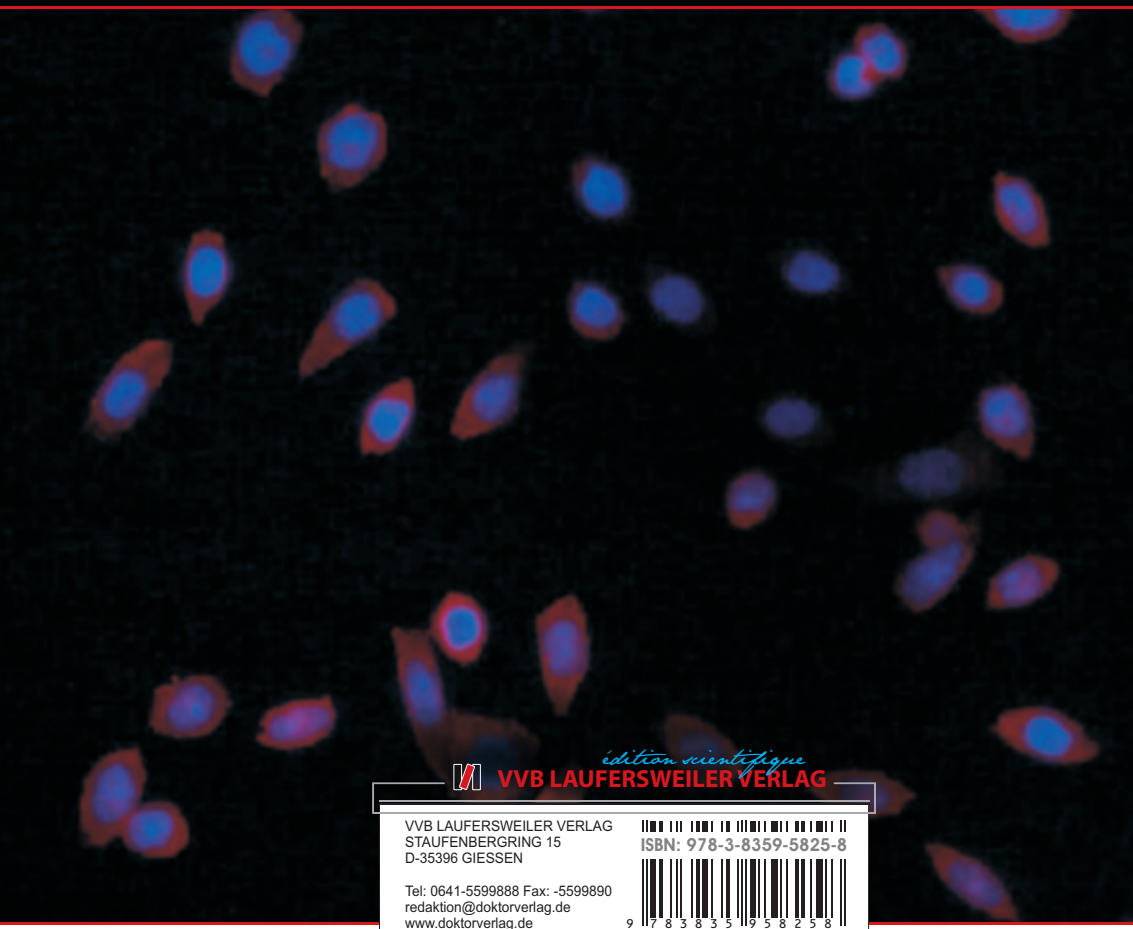


Appendix 3. Changes in the intracellular Ca^{2+} concentration induced by autoantibodies of CRPS patients in hβ2AR-CHO after pre-treatment with antagonist propranolol. Pre-treatment with 1 µM propranolol inhibited the effect of IgG (60 mg/L) and of the agonist clenbuterol (30 µM), IgG effect on the β2AR could be blocked by propranolol (1 µM) in 5/6 samples. For statistics see text.



Appendix 4. Changes in the intracellular Ca^{2+} concentration induced by CRPS autoantibodies or agonist in hM2R-CHO. (A) 2/6 CRPS IgG (60 mg/L) and the agonist bethanechol (100 µM) induced a prominent increase in the fura-2 ratio. (B) Both CRPS samples are blocked by preceding treatment with the antagonist atropine (1 µM). For statistics see text.

Over the last years an immune system dysfunction has been implicated in the physiopathology of CRPS. In this work, we characterize and discuss the presence of functional active autoantibodies against autonomic nervous system receptors in serum-samples of CRPS patients.



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