INVESTIGATION OF THE ROLE OF SOMATOSTATIN 4 RECEPTOR AND CAPSAICIN-SENSITIVE NEURONS IN MOUSE MODELS OF STRESS AND PAIN

PhD thesis

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INTRODUCTION

1. Stress-related diseases

Stress is the non-specific response of the body to various (psychological, physical, biological, etc.) demands (Selye, 1936) which can be a physiological, adaptive phenomenon, but the long-lasting, chronic stress can result in exhaustion and activation of pathological processes. Stress-related psychiatric diseases, such as depression and anxiety disorders significantly deteriorate the quality of life and mean a severe problem on both the individual and social levels. Although, clinical studies and animal models of stress provided several data regarding the underlying mechanisms of these diseases, the exact neurobiological processes are still elusive (Krishnan and Nestler, 2008). The currently available anxiolytic and antidepressant drugs exert their effect through the GABA and monoamine systems, but since their effectivity is frequently unsatisfactory, the identification of new drug targets is basically important. Neuropeptidergic systems of the brain are particularly promising from this point of view (Kormos and Gaszner, 2013).

Through its effect exerted on the nociceptive system, stress plays an important role in chronic pain states which also mean a huge clinical problem. In some disorders (e.g. neuropathy, rheumatoid arthritis), chronic stress can deteriorate the symptoms and the course of the disease, while in others, such as the fibromyalgia (FM), it can be regarded as an important etiological factor (Diatchenko et al., 2013). Regarding the interactions of stress and nociception, especially the involvement of the abnormal pain processing, the central sensitization has been examined so far (Yunus, 2008). However, recent clinical studies in FM demonstrated pathological changes of the peripheral nervous system. Paradoxically the functional and morphological deficit of nociceptive small fibres was described (Üçeyler et al., 2013). The importance of various peripheral nociceptor populations became increasingly evident recently in chronic pain (Minett et al., 2014). Based on the abovementioned data, this approach may be interesting regarding the interactions of stress and nociceptive systems.

2. The somatostatin and its receptors

Somatostatin is a cyclic neuropeptide expressed both in the central and peripheral nervous systems and involved in the regulation of several physiological and pathological processes. For example, it inhibits the release of various hormones and exerts analgesic effect. Increasing attention has been payed to the importance of somatostatin in the central nervous system recently. In several cerebral structures, a population of GABA-ergic neurons expresses the

peptide affecting both physiological brain functions (e.g. sleep, motor activity, pain, memory) and pathological (e.g. neurodegeneration) processes as a neuromodulator (Martel et al., 2012). Several clinical and experimental data suggest that somatostatin is involved in the emotional regulation and also in the development of mood disorders (Guilloux et al., 2012). The peptide exerts anxiolytic and antidepressant-like effects in acute stress situations (Engin et al., 2008) and inhibits the maladaptive functional, endocrine and neurobiological changes in the animal models of chronic stress (Lin and Sibille, 2013).

Five G-protein coupled, heptahelical receptors (sst_1-sst_5) of the somatostatin have been described which are particularly interesting in terms of drug development and there are also some data regarding their importance in the central nervous system. The sst_2 receptor, which is the most abundantly expressed in the brain, has been shown to exert an "antiepileptic" effect and play a role in the regulation of sensory and motor functions and memory. The sst_2 is also involved in the stress-regulation (Viollet at al., 2000) and mediates the anxiolytic and antidepressant-like effects of somatostatin (Engin and Treit, 2009). The significance of the sst_4 receptor, which is present in the second largest amount in the brain, has been investigated primarily in memory regulation and in the neurodegenerative processes occurring in an animal model of Alzheimer's disease (Sandoval et al., 2011). Although, the sst_4 can be detected in several brain areas (e.g. hippocampus, amygdala) involved in stress-regulation and mood disorders (Schreff et al., 2000), there are no data regarding its role in these processes.

3. Capsaicin-sensitive neurons

Peripheral sensory neurons expressing the transient receptor potential vanilloid 1 (TRPV1), the receptor of capsaicin which is the pungent ingredient of chilli pepper, play an important role in the nociception and also in the regulation of pain sensation. The TRPV1 can be activated by various painful stimuli, such as physical (e.g. heat), chemical (e.g. low pH), etc. impacts, so the capsaicin-sensitive neurons are polymodal nociceptors. The sensation of painful stimuli and their transmission towards the central nervous system are the classical afferent functions of these neurons. Moreover, during the activation of the peptidergic capsaicin-sensitive afferents, pro-nociceptive and pro-inflammatory neuropeptides (e.g. tachykinins) are also released from them which trigger a local neurogenic inflammation (local efferent function). Besides the pro-inflammatory substances, analgesic and anti-inflammatory peptides (e.g. somatostatin and opioid peptides) are also released, get into the systemic circulation and exert their effects in the whole body (systemic efferent or "sensocrine" function). In summary, the capsaicin-sensitive

neurons have a unique triple function involving both the enhancement and attenuation of nociception besides the pain sensation (Pintér et al., 2006).

Based on the available data, it is unequivocal that the capsaicin-sensitive neurons has a crucial importance in the physiological and pathological nociception such as in chronic pain (Immke and Gavva, 2006). Considering the small fibre pathology demonstrated in stress-related chronic pain states, such as FM (Üçeyler et al., 2013), and the experimental results proving the effect of stress mediators on the peripheral TRPV1 expression (Hong et al., 2011), the examination of the interactions of capsaicin-sensitive neurons and chronic stress may provide valuable new data contributing to the understanding of some aspects of chronic pain.

AIMS

Stress plays an important role in the development and course of several diseases as a pathophysiological factor. However, the exact mechanisms are largely unknown and the currently available pharmacotherapeutic opportunities are unsatisfactory in case of both stress-related psychiatric diseases and chronic pain states. Therefore, the investigation of molecules involved in these processes and the identification of new drug targets are basically important.

We aimed to examine the role of the sst_4 receptor and capsaicin-sensitive neurons in stress-related processes in the works described here. Our aims were the followings:

I. Investigation of the effect of sst_4 activation on the anxiety and depression-like behaviour shown in acute stress situations and on the neuronal response to acute stress.
II. Examination of the role of sst_4 receptor in the behavioural, endocrine and neuronal responses to chronic stress.
III. The complex investigation of the role of capsaicin-sensitive neurons in the nociceptive as well as the related behavioural, neuronal and peripheral immunological changes.

MATERIALS AND METHODS

1. Experimental animals

We used wildtype (*Sstr4*$^{+/+}$) and *Sstr4* gene-deleted (*Sstr4*$^{-/-}$) mice (Helyes et al., 2009) to reveal the role of sst_4 activation in acute stress-related processes, while the effect of the sst_4 agonist J-2156 was examined using C57Bl/6J and CD1 mice. In the chronic stress models, the importance of the sst_4 receptor was examined using *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ animals, while the role of capsaicin-sensitive neurons was investigated in CD1 mice.

The animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy UP-MS, in standard cages, at 24-25°C, under 12-12-hour light-dark cycle, provided with normal rodent chow and water *ad libitum*.

2. Experimental designs and groups

2.1. Investigation of the role of sst4 receptor in anxiety and depression-like behaviour

The effects of *Sstr4* gene-deletion and the sst_4 agonist were examined in acute behavioural tests. Considering the potential role of strain differences (Cryan and Mombereau, 2004), the effect of J-2156 was examined in both C57Bl/6J and CD1 mice. The following groups were used:

- *Sstr4*$^{+/+}$ *vs. Sstr4*$^{-/-}$ mice (n=7-12),
- saline-treated (NaCl) vs. J-2156-treated C57Bl/6J mice (n=6-13),
- NaCl vs. J-2156-treated CD1 mice (n=8-14).

The underlying mechanisms of the effect exerted by J-2156 in the tail suspension test (TST) was examined with Fos (acute neuronal activation marker) immunohistochemistry in several stress-related brain areas. Therefore, after the TST, 7-8 randomly selected CD1 mice from the NaCl and J-2156 treated groups were anesthetized and perfused with paraformaldehyde (PFA). Then, their brains were cut into coronal slices. As control groups, we used NaCl and J-2156-treated CD1 mice not exposed to TST (n=6/group).

The mechanism of the functional difference found between *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ mice in the forced swim test (FST) was also investigated with Fos immunohistochemistry (n=5-6/group).

The presence of the sst_4 receptor in the brain was demonstrated using sst_4^{LacZ} immunohistochemistry based on the detection of the β-galactosidase protein expressed from the *LacZ* gene used for the deletion of the *Sstr4* gene. Therefore, we perfused *Sstr4*$^{-/-}$ mice (n=3) with Zamboni fixative and cut their brains at the level of the amygdala and dorsal raphe nuclei.

2.2. The chronic variable stress (CVS)

The role of sst_4 receptor in the chronic stress-sensitivity was investigated in a CVS paradigm. We used four groups: non-stressed and chronically stressed *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ mice (n=9-11/group). In the tests performed during the first week ("1st trial") of the 4-week-long experiment, we determined the baseline behavioural parameters. In the next 3 weeks, we performed the CVS (Sterrenburg et al., 2011) which consisted of 6 various stressors (shaking, overnight illumination, etc.) from which we used 2 per day. Behavioural tests were repeated in the 3rd week of the CVS ("2nd trial"). Besides the absolute values of the "1st and 2nd trial" parameters, we also compared their differences or changes ["1st trial" value – "2nd trial" value] during the statistical analysis. Finally, mice were perfused with PFA solution.

The activity of the hypothalamo-pituitary-adrenal (HPA)-axis was determined by the measurement of relative adrenal and thymus weights [organ weights (mg)/body weight (g)] and the plasma corticosterone levels (RIA), while the activity of stress-related brain structures was characterized with FosB (chronic neuronal activation marker) immunohistochemistry.

2.3. Chronic restraint stress (CRS)

The role of capsaicin-sensitive neurons in the chronic stress-induced hyperalgesia was investigated using the desensitization of these neurons with the ultrapotent capsaicin analogue resiniferatoxin (RTX) and a CRS model. The examined 4 groups were the followings: non-stressed and chronically stressed non-pretreated and RTX-pretreated mice (n=9-11/group). The RTX desensitization was performed 3 weeks before the start of the CRS. Following the control nociceptive measurements, the animals were placed into well ventilated, 50 ml plastic tubes with holes, in which their movements were restricted, for 6 hours every day during a 4-week-long period (Ihne et al., 2012). The nociceptive measurements were repeated weekly during the CRS-period, while the behavioural tests were performed on the 4th week.

Then, the mice were anesthetized, their hindpaws were removed for cytokine measurements and they were perfused, the relative adrenal and thymus weights were assessed and FosB immunohistochemistry was performed in their brains and lumbar spinal cords.

3. Pharmacological methods

3.1. J-2156 treatment

J-2156 is a small molecular peptidomimetic, potent and selective sst_4 agonist (Engström et al., 2005) which was administered i.p. 15 minutes before the behavioural tests (dose: 100 μg/kg).

3.2. Resiniferatoxin (RTX) desensitization

The long-lasting desensitization of the capsaicin-sensitive neurons was performed using systemic RTX (Sigma Aldrich) treatment (s.c. injection of 10, 20, 70 and 100 μg/kg RTX on 4 consecutive days). The defunctionalisation of these neurons was verified by the lack of eye-wiping following 10 μl 0.1% capsaicin drop into the eyes (Borbély et al., 2015).

4. Experimental methods

4.1. Behavioural tests

4.1.1. Elevated plus maze test (EPM)

The EPM was used to assess the anxiety. Firstly, mice were placed onto the central platform of the EPM with 4 arms (2 open and 2 surrounded by walls). During the 5 minutes of the test, we measured the time spent in the open arms which is reciprocally proportional to the anxiety.

4.1.2. Light-dark box test (LDB)

The LDB is also used to measure anxiety. The box contains a dark and a lit compartment, between which there is a hole at the level of the floor. The time spent in the light and the number of entries and peeks into the light were analysed during the 5-minute-long experiment (the light preference is reciprocally proportional to the anxiety according to the classical interpretation).

4.1.3. Open field test (OFT)

The mice were individually placed into a brightly lit arena, of which the floor was divided into equal areas, and then their behaviour was examined for 5 minutes. We assessed the parameters of locomotor activity (number of crossed fields and time spent with moving) and the time spent in the central fields which is reciprocally proportional to the anxiety.

4.1.4. Forced swim test (FST)

In this test, mice were placed into transparent plastic cylinders containing water and in the last 4 minutes of the 6-minute-long test, we measured the time spent with immobility (passive floating) showing the depression-like behaviour.

4.1.5. Tail suspension test (TST)

The TST was also used to examine depression-like behaviour. Mice were suspended by their tail 50 cm above the floor for 6 minutes and the immobility was measured in the last 4 minutes.

4.1.6. Sucrose preference test (SPT)

In the SPT, which is used to assess anhedonia, the animals were placed into cages in which they could choose between a bottle containing tap water and another containing 1% sucrose solution for 48 hours. After the test, the quantity of consumed tap water and sucrose solution was measured and the sucrose preference index was calculated: [weight of consumed sucrose solution/(weight of consumed tap water+weight of consumed sucrose solution)*100].

4.2. Nociceptive tests

4.2.1. Dynamic plantar aesthesiometry (DPA)

The mechanonociceptive threshold of the hind limbs was assessed using DPA (Ugo Basile, Italy). An increasing force was applied on the plantar surface with a metal filament and the force measured when the limb was withdrawn was the mechanonociceptive threshold.

4.2.2. Cold tolerance test

The cold tolerance was analysed on the hind limbs by the measurement of the paw withdrawal latency from 0°C ice-cold water.

4.2.3. Increasing temperature hot plate test

The noxious heat threshold was measured in the increasing temperature hot plate test (Life Sciences, USA). Mice were placed onto a metal plate warming gradually until the appearance of an evident nocifensive reaction.

4.3. Immunohistochemical methods

4.3.1. Fos and FosB immunohistochemistry

The neuronal responses to the TST and FST as acute stressors were assessed using Fos immunohistochemistry, while the chronic neuronal activation was examined with FosB immunohistochemistry in the CVS and CRS models in several brain areas involved in stress and pain regulation.

The primary antibody was rabbit polyclonal antiserum raised against Fos or FosB (1:500; Santa Cruz Biotechnology Inc., USA), while the secondary antibody was a biotinylated goat anti-rabbit IgG (1:200) (Vectastain Elite ABC Kit, Vector Laboratories, USA, Sterrenburg és mtsai., 2011). The numbers of Fos or FosB immunopositive nuclei were determined in the examined central nervous system structures.

4.3.2. Sst_4^{LacZ} immunohistochemistry

To detect tissue β-galactosidase, we used chicken polyclonal anti-β-galactosidase primary antibody (1:20000, Abcam), horseradish peroxidase-conjugated polyclonal anti-chicken secondary antibody (1:200, Jackson Immunoresearch) and a kit based on the tyramide signal amplification (TSA^{TM}-Plus Fluorescein System, PerkinElmer) (Fu et al., 2010).

4.4. Corticosterone radioimmunoassay (RIA)

After the CVS experiment, corticosterone RIA was performed from the plasma samples of *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ animals using ^{3}H-corticosterone (12000 cpm; NEN, NET-399, 90-120 Ci/mmol) and CS-RCS-57 (Józsa et al., 2005) corticosterone antiserum. A two-phase liquid scintillation system was used to measure radioactivity. The inter- and intra-assay variation coefficients were 9.0% and 6.1%.

4.5. Cytokine concentration measurements from the hindpaw tissues

After the CRS experiment, we made protein extracts from the hindpaw tissues of non-pretreated and RTX-pretreated animals, from which we performed cytokine measurements using BD^{TM} CBA Flex Sets (USA) (Dénes et al., 2015).

5. Statistical analysis

To compare the data of two groups, we used unpaired t-test, while the data of four groups were compared using two-way analysis of variance (ANOVA) and Fisher's *post hoc* test. The results of the repeated behavioural tests in the CVS model were analysed with repeated measures two-way ANOVA and Fisher's *post hoc* test, while the data from the nociceptive tests were analysed using repeated measures two-way ANOVA and Bonferroni's *post hoc* test. The statistical power was considered to be significant for p values below 0.05.

6. Ethical statement

All experiments were in accordance with the 1998/XXVIII. Act of the Hungarian Parliament on Animal Protection and the recommendations of the International Association for the Study of Pain (IASP). The experimental procedures were approved by the Ethics Committee on Animal Research of the University of Pécs (licence no. BA02/2000-25/2011 and BA 02/2000-2/2012).

RESULTS, DISCUSSION

The role of sst_4 receptor in the regulation of anxiety and depression-like behaviour in mouse models

Results

1. The *Sstr4* gene deletion and sst_4 activation affects the behaviour shown in acute stress situations

In the EPM, the *Sstr4* gene-deleted mice spent significantly, almost 80% less time in the open arms than the $Sstr4^{+/+}$ animals. In the FST, $Sstr4^{-/-}$ mice spent significantly, up to 50% more time with immobility, but there was no difference between these groups in the TST. In the OFT, there was no difference between $Sstr4^{+/+}$ and $Sstr4^{-/-}$ mice in any parameters.

The sst_4 agonist J-2156 increased the time spent in the open arms in C57Bl/6J mice in the EPM and although it did not affect the immobility in the FST, significantly decreased this parameter in the TST. In CD1 mice, the J-2156 did not exert any effect in the EPM and the FST, but had a significant antidepressant-like effect in the TST. Locomotor activity shown in the OFT was not affected by the J-2156 treatment in C57Bl/6J or in CD1 mice.

2. Changes of stress-induced activation pattern of stress-related brain areas in response to sst_4 activation and *Sstr4* gene deletion

To reveal the underlying mechanisms of the antidepressant-like effect of J-2156 in TST, we examined the changes of neuronal activation pattern in response to TST and/or J-2156 treatment using Fos immunohistochemistry in CD1 mice.

In the dorsal raphe nucleus (dRN), a significant, more than 3.5-fold increase of the number of Fos positive nuclei was found in the J-2156-treated mice exposed to TST, while the TST and J-2156 did not exert any effect by themselves. Similarly, in the centrally projecting Edinger-Westphal nucleus (EWcp) only the J-2156-treated mice showed significant stress-induced elevation of Fos expression. The significant Fos response to stress was further increased by the agonist in the lateral (lPAG) and dorsal (dPAG) periaqueductal grey matter.

In the parvocellular part of the paraventricular nucleus of the hypothalamus (pPVN), the number of Fos immunopositive nuclei was increased following the TST in both the NaCl and J-2156-treated groups, while this response was only detectable in the J-2156-treated animals in the magnocellular part (mPVN). The TST alone did not have any effect on the Fos

immunopositive cell number in the dorsal (dLS) and ventral lateral septum (vLS), while the response was strong in both parts of the LS in J-2156-treated animals.

The stress-induced Fos response was strongly increased by the agonist treatment in the oval (ovBST) and ventral (vBST) subnuclei of the bed nucleus of the stria terminalis, while the increase was only detectable in the J-2156-treated group in the dorsolateral (dlBST) and dorsomedial (dmBST) subnuclei. The Fos response to TST was significantly increased by the J-2156 in the central (CeA) and basolateral (BLA), but not in the medial (MeA) amygdala.

Since the deletion of the *Sstr4* gene led to the significant increase of depression-like behaviour in the FST, we also examined the changes of FST-induced Fos expression in knockout animals. We found the significant increase of Fos immunoreactivity following FST in the subnuclei of the amygdala and BST, in the PVN, in the dRN and in the vLS in *Sstr4*$^{+/+}$ mice. In the *Sstr4*$^{-/-}$ group, the stress-induced elevation of Fos expression in the CeA was significantly lower than in the wildtypes, while there was no difference in the other examined structures.

3. Sst$_4$ expression in stress-related brain areas

Sst$_4$^{LacZ} immunohistochemistry was used to visualize sst$_4$ expressing neurons in different rostro-caudal levels around the amygdala and dRN. We found strong sst$_4$^{LacZ} immunoreactivity in a compact cluster of neurons in the rostral CeA and moderate-strong immunopositivity in scattered neurons in the basolateral/basomedial amygdala, in the MeA and piriform cortex. In contrast, there was no detectable sst$_4$^{LacZ} immunoreactivity in the PVN, in the dRN and in the PAG.

Summary, discussion, conclusions

These experiments provided the first evidence demonstrating the role of the sst$_4$ receptor in the regulation of acute stress-related processes. The increased anxiety of *Sstr4* gene-deleted mice and the strain-dependent anxiolytic effect of J-2156 in the EPM suggest the anxiolytic effect of sst$_4$ receptor activation. The immobility of *Sstr4*$^{-/-}$ animals was elevated in the FST but did not differ from the immobility of wildtypes in the TST, while the J-2156 exerted antidepressant-like effect in the TST, but not in the FST. Based on these, the behaviour shown in the FST is influenced by the endogenous activation of the sst$_4$ receptor, but the stimulation with the agonist cannot exert further effect. In contrast, in the TST, the acute exogenous stimulation of sst$_4$ was effective, while the chronic lack of endogenous sst$_4$ did not affect the immobility. These differences may originate from the special pharmacological features of the J-2156, distinct mechanisms of the behaviour shown in FST and TST (Cryan et al., 2005) or the fundamental

differences between the gene-deletion and acute pharmacological stimulation (e.g. reorganisation of complex neuronal networks, compensatory mechanisms). The negative results from the OFT suggest that the observed differences do not result from the changes of locomotor activity. In summary, these are the first functional data providing evidence for the role of sst_4 in stress, anxiety and depression-like behaviour besides the previously described involvement of sst_2 (Engin et al., 2008; Engin and Treit, 2009).

Interestingly, the J-2156 increased the Fos response to the TST in most of the examined brain areas. The somatostatin receptors mediate inhibitory neuronal responses directly, so the described phenomenon can probably be related to disinhibitory mechanisms, the inhibition of GABAergic neurons, which was already described in case of the sst_2 (Bassant et al., 2005). It may seem to be surprising that the antidepressant-like effect was accompanied by increased Fos response, but similar effects were described with standard antidepressant and anxiolytic drugs earlier (Choi et al., 2013; Lkhagvasuren et al., 2014). The decreased Fos response to FST, which was expected in the *Sstr4* gene-deleted mice based on the experiments with the agonist, was only detected in the CeA. We found sst_4^{LacZ} immunoreactivity in the amygdala in accordance with earlier rat studies (Schreff et al., 2000). Based on these results, the special importance of sst_4 receptors of the amygdala can be presumed. Projections originating from the amygdala regulate the activity of several other brain areas (Drevets et al., 2008), so it is possible that the sst_4 activation modulates the activity of the other structures (e.g. dRN, PAG) indirectly, through the influence on the stress response of the amygdala. It requires further studies to reveal the exact mechanisms and to precisely characterize the neurons involved in the described processes.

Higher susceptibility of *Sstr4* gene-deleted mice to chronic stress-induced behavioural and neuroendocrine alterations

Results

1. Altered behavioural responses to the CVS in *Sstr4*$^{-/-}$ mice

There was no difference between the *Sstr4*$^{-/-}$ and wildtype mice in the "1st trial" in the control behavioural parameters in the LDB. In the "2nd trial", the time spent in the light and the number of entries into the light of the *Sstr4*$^{+/+}$ animals exposed to the CVS did not differ from the non-stressed wildtypes, but significantly increased compared to the respective control ("1st trial") data. The parameters of *Sstr4*$^{-/-}$ animals were lower than in *Sstr4*$^{+/+}$ mice. When the changes of the time spent in the light and the number of entries into the light were compared, significant differences were found between the non-stressed and stressed wildtype groups, an increase was

detected in the latter. In contrast, the CVS did not exert any effect on the changes of these behavioural parameters in the *Sstr4*$^{-/-}$ group. The number of peeks into the light decreased to the same extent in all groups to the "2nd trial".

There was no difference between the *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ groups in the TST before the CVS ("1st trial"). After the chronic stress ("2nd trial"), the depression-like behaviour increased in the stressed knockouts compared to the "1st trial" data of the same group and also to the stressed *Sstr4*$^{+/+}$ and non-stressed *Sstr4*$^{-/-}$ groups within the "2nd trial". The change of immobility was significantly larger in the *Sstr4*$^{-/-}$ mice exposed to CVS compared to the stressed wildtype and also to the non-stressed knockouts.

In the "1st trial", the immobility measured in the FST was significantly larger in the *Sstr4*$^{-/-}$ animals than in the wildtypes. After the CVS, the immobility of the chronically stressed knockouts decreased and became similar to the immobility of wildtypes, in which the stress did not exert any effect, so the decrease of this parameter was significant only in the stressed *Sstr4*$^{-/-}$ animals.

In the OFT, the genotype did not affect the behavioural parameters in the "1st trial". In this test, the CVS did not influence the behaviour significantly, while a decreasing tendency of locomotor activity and time spent in the central fields was detected in the "2nd trial" in all groups compared to the "1st trial" data (so the repetition of the test had an effect). This was stronger in the *Sstr4*$^{-/-}$ animals, but regarding the changes of behaviours, there was no difference between the groups.

In the SPT, there was no significant difference between the groups in the sucrose preference or its change.

2. Changes of neuroendocrine and somatic parameters in response to *Sstr4* gene-deletion and CVS

Although, there was no difference between the *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ mice in the relative adrenal weight, the increase of this parameter in response to the CVS was significantly larger in the knockouts. The relative thymus weight of the gene-deleted animals was significantly larger than in the wildtypes, but it decreased to the same extent in both *Sstr4*$^{+/+}$ and *Sstr4*$^{-/-}$ mice. The basal plasma corticosterone concentration was significantly increased in the *Sstr4*$^{-/-}$ animals, but in the applied experimental design, it was not affected by the CVS. The body weight of *Sstr4*$^{-/-}$ mice was lower than in the respective *Sstr4*$^{+/+}$ animals, but it was decreased by the CVS independently of the genotype.

3. The effect of *Sstr4* gene deletion and CVS on the activation pattern of the stress-related brain areas

The FosB immunoreactivity was not affected by the CVS in any of the examined brain areas (amygdala, BST, hippocampus, LS, PVN, dRN, EWcp, PAG) in *Sstr4*$^{+/+}$ mice, while it increased significantly in the CeA and BLA of the chronically stressed *Sstr4*$^{-/-}$ animals.

Summary, discussion, conclusions

We described here for the first time, that the lack of the *Sstr4* gene significantly alters the behavioural, endocrine and neuronal responses to chronic stress. The only behavioural effect of the CVS was a mild increase of the light preference in the LDB in *Sstr4*$^{+/+}$ mice, which was not detected in the *Sstr4*$^{-/-}$ animals. Consequently, the sst_4 receptor plays a role as a mediator of this "paradox anxiolytic effect" which has also been described by others (Ihne et al., 2012) and can be considered as a maladaptive response in chronic stress models. Although, the CVS did not affect the immobility of wildtype mice in the TST, it significantly increased the depression-like behaviour in *Sstr4*$^{-/-}$ animals demonstrating their increased susceptibility to the "depressogenic" effect of the chronic stress. In contrast, the CVS decreased the elevated baseline immobility of *Sstr4*$^{-/-}$ mice, which therefore became similar to the immobility of wildtypes, in which the chronic stress did not exert any effect. According to the classical interpretation of the FST, the decrease of the immobility would mean an antidepressant-like effect of the CVS, but it can also be considered as a reactive or impulsive behaviour and maladaptive response (Harro et al., 2001). The changes found in the TST and FST were in the opposite direction suggesting the significantly distinct mechanisms of these two tests used to assess depression-like behaviour (Cryan et al., 2005). Based on the OFT and SPT results, the interaction between the CVS and *Sstr4* gene-deletion did not influence the locomotor activity and the anhedonia.

The slightly, but significantly increased basal corticosterone concentration of *Sstr4* gene-deleted mice suggests the inhibitory activity of the receptor in the regulation of the HPA-axis. Although, the CVS did not influence the plasma corticosterone level with the applied protocol (plasma sampling occurred 24 hours following the last stressor), the larger stress-induced increase of relative adrenal weight in *Sstr4*$^{-/-}$ mice suggests that the sst_4 receptor is also involved in the endocrine stress-response. Since the sst_4 receptor is not expressed in the components of the HPA axis (Schreff et al., 2000), these effects are presumably exerted indirectly, through an influence on the activity of higher regulatory structures. Based on the greater baseline relative thymus weight of gene-deleted animals, the sst_4 receptor may be involved in the thymus weight and thymocyte number reducing effects of somatostatin (Petrovic-Dergovic et al., 2004). In the

Sstr4 gene-deleted mice, the CVS increased FosB immunoreactivity selectively in the CeA and BLA, which was not detected in wildtype animals. Plastic changes of the CeA and BLA are well known to play a role in the regulation of chronic stress-related procedures (Rau et al., 2015). Therefore, the sst_4 receptor in the amygdala may play a role in the inhibition of neuronal plasticity changes involved in the chronic stress-induced behavioural and endocrine responses as a mediator of the effects of somatostatin.

Investigation of the role of capsaicin-sensitive neurons in the nociceptive responses to chronic stress

Results

1. Changes of the mechano- and thermonociception in response to CRS and RTX desensitization

The CRS resulted in an approximately 20% stable decrease of the mechanonociceptive threshold (hyperalgesia) of the hind paws. The RTX pretreatment did not influence the mechanosensitivity in non-stressed animals, but the stress-induced mechanical hyperalgesia was increased in the desensitized animals (30% in the first week and then around 40%). The cold tolerance decreased after the control measurements, even in the non-stressed groups, but to a greater extent in the animals exposed to CRS. The effect of the CRS on cold sensitivity was not influenced by the desensitization. The noxious heat threshold of RTX-pretreated animals was about 3°C higher than in the non-pretreated mice, which was detected in the control measurements and then maintained throughout the whole experiment. However, the CRS did not exert any effect on the noxious cold sensitivity.

2. Behavioural changes in response to CRS and RTX desensitization

The time spent in the lit compartment in the LDB was significantly elevated by the CRS in non-pretreated animals, but not in the RTX-pretreated group. The chronic stress significantly increased the number of entries and decreased the number of peeks into the light, but these alterations were not detected in the desensitized animals. In the OFT, the number of crossed fields and the time spent with moving were lower in the RTX-pretreated animals exposed to the CRS than in the respective non-pretreated group. None of the factors influenced the time spent in the central fields. The immobility in the TST was not affected by the CRS or the RTX pretreatment.

3. Changes of somatic parameters in response to CRS and RTX desensitization

The CRS increased the relative adrenal weight, while it decreased the relative thymus weight. The body weight of non-stressed animals did not change during the experiment, while the stressed groups showed a 7-8% reduction. These alterations were not influenced by the RTX.

4. Effects of CRS and RTX desensitization on the activation pattern of stress-related brain areas

In the insular cortex (InsC), the CRS increased the number of FosB positive cells in non-pretreated animals, but this effect was not observed after the RTX desensitization. The RTX pretreatment raised the FosB immunoreactivity in the somatosensory cortex (SsC), which was not affected by the CRS. In the dorsomedial nucleus of the hypothalamus and in the dRN, the chronic stress elevated the number of immunopositive nuclei in both non-pretreated and desensitized animals. The FosB expression of other examined stress- and pain-related brain areas, the medial prefrontal cortex, the anterior cingulate cortex, the NIST, the PVN, the amygdala, the hippocampus, the PAG and the rostral ventromedial medulla did not change in response to the RTX or CRS. Similarly, neither of the factors influenced the FosB immunopositivity in the superficial dorsal horn (SDH) of the spinal cord.

5. The effects of CRS and RTX desensitization on peripheral cytokine concentrations

The RTX pretreatment increased the concentrations of interleukin (IL)-1α and RANTES (regulated on T cell expressed and secreted, or chemokine CCL5) in non-stressed animals, which was not observed in mice exposed to the chronic stress, though the stress did not exert any effect by itself. The CRS significantly decreased the KC concentration in non-pretreated animals. In case of the interferon γ, the IL-1β, the IL-4, the IL-10, the monocyte chemoattractant protein-1, the tumour necrosis factor α and the IL-6, we did not find any difference between the groups.

Summary, discussion, conclusions

This is the first experimental study providing evidence for the interaction of a specific nociceptor population and chronic stress and its effect on nociception. We demonstrated for the first time that the stress-induced mechanical hyperalgesia specifically increases following the desensitization of capsaicin-sensitive neurons, which is virtually in contrast with their important role in pain sensation. However, the role of this neuron population in chronic pain-related mechanical hyperalgesia in questionable and depends on the mechanism of the examined pain

state (Xu et al., 2015). The cold hyperalgesia developed in response to the CRS (Bardin et al., 2009) was not affected by the RTX. The noxious heat threshold was not influenced by the CRS, but it was significantly elevated by the desensitization which is in accordance with the crucial importance of capsaicin-sensitive neurons in thermonociception (Bölcskei et al., 2010). The CRS-induced increase of light preference in the LDB was not detected following the RTX desensitization suggesting the long-lasting effect of RTX on the behavioural response to chronic stress. In summary, the RTX pretreatment selectively increased the CRS-induced mechanical hypersensitivity. Since it exerted opposite effects on the other nociceptive, behavioural and endocrine parameters or did not influence them, the enhancement of mechanical hyperalgesia was a specific effect of RTX and not the consequence of a general behavioural or endocrine disturbance.

In the InsC, the CRS-induced increase of FosB expression was not detected in RTX-pretreated mice in which the mechanical hyperalgesia was enhanced suggesting an anti-nociceptive mechanism in this structure (Imbe and Kimura, 2015). In the SsC, the RTX pretreatment increased the FosB expression by itself, which may be related to the alteration of the somatosensory input (Nussbaumer and Wall, 1985) and possibly played a role in the enhancement of mechanical hyperalgesia. Among the subcortical nuclei, the increased activity of DMH and dRN could also contribute to the CRS-induced hyperalgesia. In the SDH of the spinal cord, neither the CRS nor the RTX exerted any effect, so the activity of the spinothalamic tract is not enhanced from the peripheral fibres in our model. The capsaicin-sensitive sensory neurons play an inhibitory role in the regulation of the basal ("housekeeping") production of the IL-1α and RANTES. However, these peripheral immunological alterations are probably not important in terms of the CRS-induced hyperalgesia considering the negative effects of the stress. Based on these data, primarily the neuronal plasticity changes of the brain can be presumed to be relevant in the described nociceptive changes. Our experimental data may help to reveal the complex interactions between the central and peripheral sensory systems and stress and contribute to the understanding of those surprising clinical observations demonstrating small fibre deficit accompanied by hyperalgesia in FM (Üçeyler et al., 2013).

SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

1. We showed for the first time that the activation of sst_4 receptors mediates the anxiolytic and antidepressant-like effects in acute stress-situations in mouse models. These effects originate from the modulation of acute neuronal responses to stress in the stress-related brain areas, in which the role of the sst_4 receptors of the amygdala seems to be especially important. Although, the crucial role of somatostatin expressing neurons in the emotional and stress-regulation are supported by several data in the literature, these are the first results demonstrating the significance of the sst_4 receptor in these processes.

2. We also demonstrated the role of sst_4 receptor in the responses to CVS which has a huge relevance in terms of psychiatric diseases. The lack of the receptor influences the stress-sensitivity in a complex way, induces mainly maladaptive behavioural responses and modifies the activity of the HPA-axis. These phenomena can also be related to the modulation of the response of the amygdala to stress. Our data suggest that the sst_4 receptor can be a promising drug target in stress-related psychiatric diseases. From this point of view, it is important to note the analgesic and anti-inflammatory actions of sst_4 activation, considering the frequent comorbidity of chronic pain and depression. In addition, the sst_4 is not involved in the diverse endocrine effects of somatostatin (unlike the sst_2), which is beneficial in terms of the side effects.

3. We described for the first time, that the desensitization of capsaicin-sensitive neurons enhances the mechanical hyperalgesia in response to chronic stress. This effect is modality-specific, not the consequence of a robust behavioural or endocrine disturbance. This phenomenon may be related to the modification of the activity of brain structures involved in pain-processing, while the enhanced activity of peripheral nociceptors and immunological alterations are not likely to play a role. These conclusions may contribute to the understanding of the interactions of stress and nociceptive systems. The relevance of this question is highlighted by the role of psychological factors in chronic pain states. Our results will promote the comprehension of the pathomechanism of stress-related pain states, such as FM considering the small fibre pathology described in the latter.

Our studies revealed some new factors in the complex processes of stress, of which the further precise exploration can contribute to the development of new psychopharmacological agents and drugs against chronic pain.

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PUBLICATIONS

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Scheich B, Gaszner B, Kormos V, László K, Adori Cs, Borbély É, Hajna Zs, Tékus V, Bölcskei K, Ábrahám I, Pintér E, Szolcsányi J, Helyes Zs (2016). Somatostatin receptor subtype 4 activation is involved in anxiety and depression-like behavior in mouse models. *Neuropharmacology*. 101:204-215. (**IF: 5,106**)

Scheich B, Csekő K, Borbély É, Ábrahám I, Csernus V, Gaszner B, Helyes Zs. Higher susceptibility of somatostatin 4 receptor gene-deleted mice to chronic stress-induced behavioral and neuroendocrine alterations. *Manuscript submitted to the Brain Structure and Function, under review*.

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