Final report

Modulation of nociceptor function by membrane gangliosides:
implication for pain mechanisms
K 101873

A critical re-evaluation of the mechanisms of the capsaicin-induced „sprouting" phenomenon

A single topical application of capsaicin onto peripheral nerves produces a long-lasting regional analgesia, which we term *nociceptor analgesia.* This antinociceptive effect is confined to the peripheral innervation territory of the capsaicin-treated nerve and is characterized by a selective loss of chemogenic pain, reduction in heat pain sensitivity and abolition of the neurogenic inflammatory response. Morphological, neurochemical and immunohistochemical investigations have revealed many facets of this unique antinociceptive action including the depletion of sensory neuropeptides, such as substance P and CGRP, and the sensory neuron specific enzyme, thiamine monophosphatase. Previous studies have also revealed a marked decrease in the sensitivity of C-fiber primary afferent neurons toward capsaicin following perineural capsaicin treatment (Jancsó and Lawson, 1990). More recent studies indicate that this may be explained by massive reductions in the mRNA and protein expressions of the TRPV1 receptor, which confers capsaicin sensitivity on primary sensory neurons (Caterina et al., 1997) after perineural treatment with capsaicin (Szigeti et al., 2012). Neuronal tract tracing and histochemical studies involving use of the choleratoxin B subunit (CTB) conjugated with horseradish peroxidase (HRP) revealed that, unlike in control animals, injection of this tracer into a previously capsaicin-treated peripheral nerve resulted in marked transganglionic labeling not only of the deeper layers of the spinal dorsal horn, the termination sites of myelinated primary afferents, but also of the substantia gelatinosa, the main termination site of unmyelinated primary afferents. Since CTB and its conjugates are taken up and transported selectively only by myelinated primary afferent fibers in intact animals (Robertson and Grant, 1985), the labeling of the substantia gelatinosa after perineural treatment with capsaicin was interpreted in terms of a sprouting response of A-fiber afferents (Mannion et al., 1996). We confirmed and extended these findings and showed that, unlike in control/intact rats, intraneurally injected CTB-HRP appears in the substantia gelatinosa after perineural treatment with capsaicin, or its ultrapotent vanilloid analogue, resiniferatoxin. However, our further experiments furnished strong evidence against the sprouting hypothesis. Indeed, we have demonstrated a very marked increase in the proportion of CTB-HRP-positive small DRG neurons and, importantly, a massive ~ 20-fold increase in the proportion of CTB-HRP-transporting C-fiber dorsal root axons following perineural vanilloid application (Oszlács et al., 2015). On the basis of these findings we challenged the sprouting hypothesis and suggested that the increased CTB-HRP labelling of the substantia gelatinosa after perineural capsaicin may be accounted for by a phenotypic switch of C-fiber primary afferent neurons, rather than a sprouting response of A-fiber spinal afferents as assumed by Mannion et al. (1996).

Our observations put the spinal neuroplastic phenomena which ensue after perineural capsaicin and resinsiferatoxin treatments into an entirely new perspective. Hence,

previous findings emphasized the importance of structural changes of A-fiber primary afferents in the development of neuroplastic phenomena associated with physical or chemical injury and/or defunctionalization of primary sensory neurons. It should be noted that this (mis)belief reached a status of solid textbook knowledge to explain neuropathic changes which commence after nerve injuries. However, our findings unequivocally proved that, instead of changes in A-fibre afferents, phenotypic change of C-fiber primary afferents play a pivotal role in these phenomena (Sántha and Jancsó, 2003; Jancsó and Sántha, 2004; Oszlács et al., 2015).

Studies on physical (axotomy) and chemical (capsaicin/resiniferatoxin) injuries of peripheral nerves utilizing the sensitive neuronal tracer, CTB-HRP have led us to consider the possible role of GM1 ganglioside, the molecular target of CTB, in the function of nociceptive primary sensory neurons (cf. Sántha et al, 2010). In the frame of the present project we performed further experiments in this line.

Activation of the nociceptive ion channel TRPV1 by endogenous vanilloids is inhibited by inhibition of glucosylceramide synthase

In previous studies we demonstrated that activation of the TRPV1 receptor, by its archetypal agonist capsaicin, is inhibited by pretreatment with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of glucosylceramide synthase (GLCS), the key enzyme in neuronal ganglioside synthesis (Sántha et al., 2010). In the frame of the present project we examined, using a similar experimental paradigm utilizing the cobalt uptake method to assess TRPV1 receptor activation and administration of D-PDMP to inhibit GLCS, the possible role of gangliosides in the activation of the TRPV1 channel by high temperature and low pH, and endogenous vanilloid compounds (endovanilloids).

The TRPV1 receptor, a molecular integrator of nociceptive stimuli, is exquisitely sensitive to temperatures ›43 ^{o}C and low pH (≤5.3). The cobalt assay proved appropriate to test the activation of the TRPV1 receptor by high temperature (≥43 ^{o}C) and low pH. Inhibition of GLCS significantly reduced the proportion of heat- and acidic pH-activated neurons in DRG cultures. Importantly, these findings indicate that activation of the TRPV1 receptor by heat and acid is also dependent on neuronal ganglioside status.

Endogenous vanilloid compounds have been identified recently with a functional profile resembling that of capsaicin and resiniferatoxin. Therefore, we examined whether the activation by endovanilloids of the TRPV1 receptor expressed by DRG neurons is also dependent on gangliosides, in particular GM1 ganglioside. First we showed that endovanillod compounds similar to capsaicin, produce cobalt accumulation in cultured DRG neurons. The endovanilloid compounds N-arachidonoyldopamine (NADA), N-oleoyldopamine (OLDA) and anandamide were added to cultured DRG neurons at concentrations of 20-100 μM for 10 min. Evaluation of TRPV1 activation using the cobalt uptake assay revealed that 28.06±3.63, 27.04±2.3 and 32.27±2.76 per cent of DRG neurons were activated by NADA, OLDA and anandamide, respectively (Sántha et al., 2013). Cobalt accumulation elicited by endogenous vanilloid compounds was inhibited by capsazepine, a TRPV1 antagonist, indicating that these agents elicited cobalt accumulation through the activation of the TRPV1 receptor.

Inhibition of GLCS by D-PDMP (30 μM) resulted in a marked decrease in proportions of neurons activated by NADA, OLDA and anandamide to 8.44±3.55, 8.37±1.86 and 11.27 per cent, respectively (Sántha et al., 2013). These experiments provided evidence for the activation of the TRPV1 nociceptive ion channel by endovanilloids and, importantly, disclosed that this activation is dependent on the integrity of the ganglioside status of DRG neurons. Since endovanilloids are potential endogenous pain-producing molecules, pharmacological perturbation of their signalling pathway may be of relevance to antinociception.

Inhibition of GLCS resulted in a significant decrease in the proportion of TRPV1-immunoreactive DRG neurons, too. Quantitative assessment revealed a strong and significant decrease in membrane GM1 ganglioside level after chronic D-PDMP treatment. Importantly, the number of cobalt-accumulating neurons approached that obtained in control cultures following supplementation with GM1 (10-100 μM). These findings indicate that GM1 may be the major ganglioside which is involved in the mechanism of activation of the TRPV1 receptor by endovanilloids. The data suggest that the D-PDMP-induced decreases in sensitivity to vanilloid compounds and TRPV1 expression are associated with and probably consequent to a reduction in membrane gangliosides, in particular GM1.

The effects of GLCS inhibition may be explained by an action on the signalling pathway(s) of nerve growth factor (NGF; Mutoh et al., 1995) which is critically important for the expression and also for the maintenance of the sensitivity of the TRPV1 receptor. GM1 ganglioside participates in the mediation of the trophic effects of NGF by an enhancement of trkA dimerization and subsequent signalling. Inhibition of ganglioside synthesis may interfere with NGF action also through the organization of membrane lipid microdomains (rafts), an important component of which is GM1 ganglioside. Indeed, depletion of membrane cholesterol, another important component of membrane microdomains, has been shown to reduce the sensitivity of cultured DRG neurons to capsaicin (Liu et al., 2006).

Ganglioside GM1 is essential for the activation of the nociceptive ion channel TRPA1

Allyl-isothiocyanate was applied to activate the TRPA1 nociceptive ion channel in cultured DRG neurons. Whereas 31.97±4.28% of the neurons showed cobalt accumulation in control cultures, only 12.7±4.3% of the neurons were labelled after pretreatment with D-PDMP. This suggests a role for gangliosides in the mechanism of activation of the TRPA1 ion channel (Sántha et al. 2013; Oszlács et al., 2015). The most likely ganglioside species involved is GM1, since supplementation of the culture medium with this ganglioside largely restored the sensitivity of DRG neurons to allyl-isothiocyanate. Although GM1 seems to be the most likely ganglioside species involved in the mechanism of activation of the TRPV1 and TRPA1 receptors, other ganglioside species cannot be excluded at present. The possible cellular mechanisms involved in this process are discussed above.

Role of an endogenous vanilloid, anandamide in the modulation of nociceptive mechanisms

In collaboration with Dr. I. Nagy's group at Imperial College London, we characterized DRG neurons which synthesize the endogenous vanilloid anandamide in a Ca^{2+}-sensitive manner through a single pathway which involves the activity of N-acylphosphatidyl-ethanolamine phospholipase D (NAPE-PLD) under pathological conditions (Sousa-Valente et al., 2016). We showed that neurons which express NAPE-PLD, also express TRPV1, cannabinoid receptor 1 (CB1), and the main anandamide-hydrolyzing enzyme fatty acid amide hydrolase (FAAH). We also showed that the expression patterns of TRPV1, CB1, NAPE-PLD and FAAH are differentially regulated under pathological conditions (spinal nerve ligation and tissue inflammation), but both sets of changes are likely to produce alterations resulting in a reduced inhibition of the excitability of primary afferent neurons. These findings support the notion that, in primary sensory neurons, TRPV1, NAPE-PLD, the CB1 receptor, and FAAH are part of an autocrine regulatory system that modulates the excitability of nociceptive primary sensory neurons and, consequently the development of pain. Since activation of the TRPV1 receptor by anandamide is dependent on the ganglioside status of sensory ganglion neurons (*vide supra*), it is conceivable that gangliosides, in particular GM1, may play a role in the modulation of an autocrine signalling mechanism involving TRPV1, NAPE-PLD, the CB1 receptor, and FAAH in primary sensory neurons (Bari et al., 2005; Rimmerman et al., 2008). The possible interplay between the neuronal ganglioside status and the regulation of that autocrine regulatory system remains to be elucidated.

Discovery of a new molecular marker of nociceptive stimulation induced in spinal superficial dorsal horn neurons (SSDHNs) by stimulation of TRPV1 receptor-expressing nociceptive afferents

Morphological identification of activated neurons has been a long-pursued aim of neuroscience research, including pain research. Demonstration of specific markers, such as c-fos and p-ERK1/2 of neurons activated by noxious stimulation proved to be powerful research tools in the study of pain mechanisms. However, noxious stimulation-induced post-translational modification by histone phosphorylation of gene expression in SSDHNs has not been demonstrated previously.

In collaboration with Dr. I. Nagy's group at Imperial College London, in *in vitro* pharmacological studies we have shown that activation by capsaicin of TRPV1 receptor-expressing primary afferents resulted in the phosphorylation of serine 10 (S10) in histone 3 (H3) in SSDHNs (Torres-Pérez et al., 2017). This effect was mediated by activation of NMDA receptors, since blocking N-methyl-D-aspartate receptors inhibited upregulation of p-S10H3. Further, we have shown that signalling pathways involving extracellular signal-regulated kinases 1 and 2, and the mitogen- and stress-activated kinases 1 and 2 (MSK1/2), which phosphorylate S10 in H3, are critically implicated in the upregulation of phosphorylated p-S10H3. In further experiments we showed that upregulation of p-S10H3 in SSDHNs may play a pivotal role in the development of prolonged pain and inflammatory heat hyperalgesia.
Phosphorylation of S10H3 in SSDHNs is the first stimulus-specific epigenetic mechanism identified in spinal nociceptive neurons.

Altered neuronal ganglioside metabolism in primary afferents may result in changes in the activation of spinal dorsal horn neurons which are involved in the mediation of nociceptive information and development of pain. Preliminary findings in our laboratory suggest that gangliosides may play a significant role in post-translational modification of S10H3 and, in turn, expression of genes associated with pain mechanisms. Ongoing studies are aimed to further elucidate the role of neural gangliosides in the epigenetic modulation of pain.

Possible impact of the findings on pain research

The suggestion that gangliosides, and in particular GM1 ganglioside may play a significant role in the function of the archetypal nociceptive ion channel TRPV1, the capsaicin receptor was born out of our findings on the mechanism of the increased transganglionic labelling by choleratoxin B (CTB) and its conjugates of the spinal dorsal horn, in particular the substantia gelatinosa. Hence, we showed for the first time that this increased labelling of the substantia gelatinosa by CTB is a result of a phenotypic switch of C-fiber primary sensory neurons and not a sprouting response of A-fiber primary afferents (Sántha and Jancsó, 2003; Jancsó and Sántha, 2004). Similarly, we demonstrated, using quantitative light and electron microscopic histochemistry, that the increased labelling of the substantia gelatinosa by CTB following perineural capsaicin treatment resulted from a phenotypic switch of the affected C-fiber primary sensory neurons (Oszlács et al., 2016). These findings prompted us to consider the possible role of GM1 ganglioside, the molecular target of CTB, in the function of nociceptive primary afferent neurons and, in particular the TRPV1 receptor (Sántha et al., 2010). GM1 ganglioside seems to be an integral and important key molecule of TRPV1 function and, in addition, in a (compensatory) neuronal response to nerve injury. Our findings demonstrating the dependence on neuronal ganglioside status of the activation, by capsaicin, endovanilloids, high temperature and low pH, of the TRPV1 receptor may bear of relevance in the understanding of pain mechanisms and, in particular, TRPV1 nociceptor function.
Further, neuronal gangliosides may play a role in the regulation, in primary sensory neurons, of an autocrine regulatory system involving TRPV1, NAPE-PLD, the CB1 receptor, and FAAH that modulates the excitability of nociceptive primary sensory neurons and, consequently the development of pain. Hence, we showed that the action of anandamide, a key element in that signalling system, on the TRPV1 receptor is dependent on neural ganglioside, in particular GM1 level. Finally, our discovery of a new signature molecule of activation of nociceptive SSDHNs is expected to promote investigations into the mechanisms of pain.

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