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Development, plasticity and modulation of visceral afferents

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Abstract

Visceral pain is the most common reason for doctor visits in the US. Like somatic pain, virtually all visceral pain sensations begin with the activation of primary sensory neurons innervating the viscera and/or the blood vessels associated with these structures. Visceral afferents also play a central role in tissue homeostasis. Recent studies show that in addition to monitoring the state of the viscera, they perform efferent functions through the release of small molecules (e.g. peptides like CGRP) that can drive inflammation, thereby contributing to the development of visceral pathologies (e.g. diabetes Razavi, R., Chan, Y., Afifiyan, F.N., Liu, X.J., Wan, X., Yantha, J., Tsui, H., Tang, L., Tsai, S., Santamaria, P., Driver, J.P., Serreze, D., Salter, M.W., Dosch, H.M., 2006. TRPV1+ sensory neurons control beta cell stress and islet inflammation in autoimmune diabetes, *Cell* 127 1123–1135). Visceral afferents are heterogeneous with respect to their anatomy, neurochemistry and function. They are also highly plastic in that their cellular environment continuously influences their response properties. This plasticity makes them susceptible to long-term changes that may contribute significantly to the development of persistent pain states such as those associated with irritable bowel syndrome, pancreatitis, and visceral cancers. This review examines recent insights into visceral afferent anatomy and neurochemistry and how neonatal insults can affect the function of these neurons in the adult. New approaches to the treatment of visceral pain, which focus on primary afferents, will also be discussed.

Keywords

Bladder; Colon; Jugular; Nodose; Neonatal; Pain; Pancreas; P2X; TRPV1; TRPA1; TRPV4; Vagus

1. Introduction

For somatic structures there is a relatively clear division between nociceptive afferents that detect noxious or potentially damaging stimuli and non-nociceptive afferents that are specialized to detect non-noxious stimuli such as vibration or light touch. In viscera, the distinction is not as clear because even afferents with low mechanical thresholds, which code innocuous physiological stimuli, can code stimuli in the noxious range (Sengupta and Gebhart, 1994a,b). However, both somatic and visceral nociceptors are heterogeneous and can be divided into various populations based on functional response properties, neurochemical phenotypes and anatomical structure. For example, in the epidermis, polymodal nociceptors (responding to noxious thermal, intense mechanical and/or chemical stimuli) are the most common type of nociceptor. In mouse, these afferents are overwhelmingly IB4-positive (binding the isolectin IB4) C-fibers (Lindfors et al., 2006; Lu et al., 2001; Woodbury et al., 2004) with conduction velocities below 1 m/s. These neurons can be easily distinguished from “heat only nociceptors” (mechanically insensitive neurons responding to noxious heat) that typically express neuropeptides (e.g., CGRP and/or SP), TRPV1 and have conduction velocities in the 0.5 m/s range (Lawson et al., in press). For viscera, this distinction does not appear to hold, as the majority of spinal afferents projecting to the gastrointestinal tract or pelvic organs are TRPV1, TRPA1 and/or TRPV4 positive and respond to mechanical stimulation (Cenac et al., 2007; Christianson et al., 2006a; Fasanella et al., 2008; Malin et al., in press; Zhong et al., 2007).

Sensory neurons innervating the viscera are also represented by diverse populations that differ in response to defined stimulation paradigms. For example, mucosal afferents (innervating the structures lining the lumen of the GI tract) have been found to respond to both light brushing, as well as chemical irritants (Brierley et al., 2004; Lynn and Blackshaw, 1999). Muscular afferents, with endings located presumably within one of the muscle layers that form the walls of the GI tract, respond preferentially to distension or stretch, but are also responsive to chemical stimuli (Brierley et al., 2004; Iggo, 1957; Lynn and Blackshaw, 1999). However, these distinctions are based on response profiles rather than actual identification of simple or specialized nerve endings within the mucosal or muscular layer.

In addition, the viscera are unique in that they are monitored by extrinsic sensory neurons arising from two distinct sources: cervical, thoracic and abdominal organs receive afferent projections from spinal afferents in dorsal root ganglia and cranial nerves (glossopharyngeal nerve and vagus). Pelvic structures are innervated by thoracolumbar and lumbosacral afferents. Current evidence suggests that these distinct afferent pathways differentially contribute to sensory processing. Spinal afferents transmit acute nociceptive input, while activation of vagal pathways contributes to associated sensations, such as nausea, fullness or bloating (Bielefeldt et al., 2005, 2006; Yu et al., 2005). For pelvic organs, emerging data indicate that the thoracolumbar afferents play an important role after inflammation or other sensitizing processes, but do not contribute significantly to nociception under baseline conditions (Christianson et al., 2006b; Traub, 2000; Traub and Murphy, 2002; Wang et al., 2005). Although beyond the scope of this review, it has been proposed that visceral afferents can have an efferent role through the release of small molecules like CGRP. The ablation of TRPV1 expressing pancreatic afferents in diabetes prone NOD mice, slows or prevents the development of islet inflammation and hyperglycemia (Razavi et al., 2006).

In this review we will examine recent data that attempt to explain, on a mechanistic level, how visceral afferents are specialized to detect different stimuli, how these afferents arise during development and how normal and abnormal experience can lead to long-lasting functional changes within this population. Finally, we will explore recent approaches directed at treating

persistent visceral pain by inducing long-lasting changes in gene expression in primary visceral afferents.

2. Comparison of extrinsic afferents innervating visceral structures

An excellent model for comparing the source and function of different populations of visceral afferents is the extrinsic innervation of thoracic viscera. Fast conducting afferent A-fibers and slow conducting C-fibers from the vagus nerves innervate these structures. In the respiratory tract and esophagus, the A-fibers comprise mainly low threshold mechanosensors that transmit normal physiological activity. In the lungs, these fibers are referred to as slowly and rapidly adapting receptors (SARs and RARs) (Canning et al., 2006). In the esophagus, the low threshold mechanosensors are often referred to as tension receptors (tension mechanosensors) (Yu et al., 2005; Zagorodnyuk and Brookes, 2000). The C-fibers innervating the respiratory tract and esophagus are nearly uniformly capsaicin-sensitive and have biophysical and pharmacological properties similar to capsaicin-sensitive C-fibers in the somatosensory system. They are activated by potentially noxious stimuli including capsaicin, acid, tissue inflammation and strong mechanical stimulation (stretch). In the respiratory tract, the vagal C-fibers are quiet during normal respiration.

The vagal C-fibers innervating the esophagus and respiratory tract fit Sherrington's definition of nociceptors: those sensory nerves that are reserved to provide an organism a sense of its own potential injury (Sherrington, 1906). However, activation of the respiratory nociceptors typically does not evoke the sensation of pain. Rather they elicit a panoply of protective cardiopulmonary reflexes including changes in the rate and depth of breathing, cough, increases in airway smooth muscle tone, mucus secretion, changes in heart rate and vascular resistance. In addition to these reflexes, activation of respiratory vagal C-fibers may lead to unpleasant, itchy, urge to cough sensations and sensations of dyspnea (Coleridge and Coleridge, 1984; Lee and Pifarri, 2001). The consequence of activation of vagal nociceptors in the esophagus has not yet been well described, but likely results in the initiation of protective reflexes, as well as possibly sensations of chest discomfort.

In the somatosensory system, at least two distinct sensory nociceptive C-fiber subtypes have been described based mainly on neuropeptide content and binding the isolectin B4 (IB4) (Molliver et al., 1995). The two fiber subtypes display distinct functional characteristics *in vitro* (Stucky and Lewin, 1999). Moreover, they may also serve distinct physiological functions based on the fact that their central terminals synapse on distinct layers of the dorsal horn of the spinal cord (Hunt and Rossi, 1985; Molliver et al., 1995). More recently, these nociceptor phenotypes have been distinguished based on expression of transcription factors such as Runx1, and neurotrophic factor receptors such as trk-A and ret (Woolf and Ma, 2007).

Much less is known about vagal C-fiber subtypes innervating visceral tissues. In the 1970s, the Coleridges and their colleagues concluded that two different types of capsaicin-sensitive vagal C-fibers innervate the respiratory tract and that they can be distinguished based on the vascular accessibility of the nerve endings (Coleridge and Coleridge, 1977). One C-fiber type responded immediately to capsaicin only when injected into the right atrium (pulmonary circulation), the other responded immediately only when injected into the left atrium or directly into the bronchial artery (systemic circulation). The former type was referred to as "pulmonary C-fiber" whereas the latter was referred to as "bronchial C-fibers". The bronchial C-fibers were thought to reflect those that terminated within the wall of the conducting airways. Despite observations that these two C-fiber subtypes were differentially stimulated by certain inflammatory mediators, the idea that they represent distinct phenotypes was not universally accepted (Sant'Ambrogio and Sant'Ambrogio, 1982).

Independent evidence of vagal bronchopulmonary C-fiber subtypes was obtained by considering the location of the cell body rather than the nerve endings (Undem et al., 2004). As with several cranial nerves, the vagus nerve is comprised of two sensory ganglia; namely the nodose ganglion and the jugular (or supranodose) ganglion (Fig. 1). The cell bodies of about 50% of the vagal C-fibers innervating the guinea pig lungs were found to reside in the nodose ganglion with the balance located in the jugular vagal sensory ganglia. These two ganglia are embryologically distinct, with neurons in the jugular ganglion derived from neural crest cells of the postotic hindbrain, whereas the nodose neurons arise from the epibranchial placodes (Baker, 2005). The nodose and jugular C-fibers could be readily distinguished pharmacologically. The nerve terminals in both subtypes responded directly to capsaicin and bradykinin with action potential discharge, but only the nodose C-fibers responded with action potential discharge to purinergic P2X receptor-selective agonists, adenosine receptor (A1 and A2A) agonists, and 5-HT3 receptor agonists (Chuaychoo et al., 2005, 2006; Undem et al., 2004). In addition, the jugular C-fibers were more apt to express neuropeptides than the nodose C-fibers (Undem et al., 2004).

Recently, the C-fiber phenotype, focusing specifically on purinergic receptor expression and function, of spinal (dorsal root ganglion) neurons innervating the intrapulmonary tissues was compared with that of jugular and nodose ganglion lung-specific C-fiber neurons (Kwong et al., 2008). The neurons in the more rostral DRGs are derived from the same postotic hindbrain neural crest structure as the jugular neurons. Therefore, if the embryonic environment selects the phenotype, one would predict that these neurons would resemble jugular neurons more than nodose neurons.

Patch clamp electrophysiology combined with single cell RT-PCR reveals that the reason guinea pig nodose ganglion neurons respond to ATP is due to the expression of both P2X₂ and P2X₃ receptors. Consistent with a P2X_{2/3} receptor expression pattern, these neurons respond to ATP (or α,β -methylene-ATP) with a large inward current. By contrast, the lung C-fiber terminals arising from jugular ganglia do not respond to ATP with action potential discharge. This appears to be due to the fact that the jugular neurons express P2X₃, but not P2X₂ receptors. The presumed homomeric P2X₃ receptors support only a rapidly inactivating current that is apparently insufficient to evoke action potential discharge at the nerve terminals (Fig. 1).

The lung-specific DRG neurons were found to be essentially identical to the jugular type of neurons with respect to their response to ATP (e.g., with rapid inactivating current) and express P2X₃, but not P2X₂ receptors (Kwong et al., 2008). Another similarity between lung-specific jugular and DRG C-fibers innervating the respiratory tract is that they are more apt to express neurokinin than nodose C-fiber neurons (Oh et al., 2006).

These data support the hypothesis that, with respect to purinergic responsiveness and neuropeptide content, the C-fiber phenotype may be selected by the embryological environment rather than instructed from the innervated tissue environment. To indirectly assess this hypothesis further, a similar analysis was carried out in a disparate tissue environment, namely the guinea pig esophagus. The results were similar to those observed in the lungs. That is, the esophagus was found to be innervated by vagal nodose and jugular capsaicin-sensitive C-fiber phenotypes (Yu et al., 2005, 2008). The nodose C-fibers in the esophagus had properties similar to the nodose lung C-fibers, and the esophageal jugular C-fibers (and DRG C-fibers) had properties similar to their lung counterparts (Kwong et al., 2008 and Fig. 1).

In summary, data are accumulating that indicate that the vagal capsaicin-sensitive C-fibers innervating the respiratory tract comprise two distinct phenotypes. The two phenotypes can readily be segregated by whether their cell body is situated in the jugular (neural crest-derived) or nodose (epibranchial placode-derived) ganglia. The jugular C-fibers are similar to C-fibers

arising from the DRG (neural crest-derived). The neural crest and placodal C-fiber populations maintain their phenotypes (with respect to neuropeptide content and P2X receptor expression and function) regardless of whether they innervate the respiratory tract or esophagus. Whether these two C-fiber visceral populations find analogies with the two C-fiber somatic populations remains to be seen. Recently obtained preliminary data do, however, indicate some important differences. In the somatosensory system, the two nociceptive phenotypes can be segregated based on the expression of the ret tyrosine kinase receptor (Ret) (Woolf and Ma, 2007). In the vagal innervation of the adult mouse respiratory tract, Undem and coworkers have found that Ret is expressed in both the presumed placodal-derived, non-peptidergic, purinergic responsive fibers, as well as the neural crest-derived, neuropeptide positive, purinergic unresponsive (neural crest) C-fibers (data not shown).

3. P2X receptors in visceral afferents

As noted in the previous section, visceral afferents are particularly sensitive to a number of small molecules released by the parenchyma. In the lung and gastrointestinal tract, specialized epithelial cells release such mediators in response to mechanical or chemical stimulation (Burnstock, 2001). One of the most effective of these molecules is ATP. ATP is released during cell injury and has long been known to play a role in nociception. In the viscera, ATP is released from bladder and epithelial lining the gastrointestinal tract during physiologic stimulation (e.g. stretch). Typically, the amount of ATP released is increased when tissue is inflamed. It has been suggested that epithelial cells will release ATP in order to function as intermediaries in the signaling of luminal events from subepithelial tissues to visceral sensory neuron terminals (Ferguson et al., 1997; Hanna-Mitchell and Birder, 2008), but this remains to be established.

Both bladder and colon sensory neurons in DRG express ligand-gated, ionotropic P2X receptors and respond to purinergic agonists (Fig. 2). Studies employing P2X receptor agonists (e.g., Tsuda et al., 2000), antagonists (e.g., Jarvis et al., 2002), antisense oligonucleotides (Barclay et al., 2002) and knockout mice (Cockayne et al., 2000; Vlaskovska et al., 2001) support an important role for P2X receptors in bladder function and nociception. For example, in P2X receptors are present in the bladder (e.g., Elneil et al., 2001) and changes in P2X receptor expression/function are found in bladder disorders: P2X₂ receptor expression is increased in patients with idiopathic detrusor instability (O'Reilly et al., 2002), P2X₂ and P2X₃ receptors are increased in the urothelium of interstitial cystitis patients (Andersson, 2002), and P2X₃ receptor expression is increased during stretch from urothelial cells taken from cystitis patients (Sun et al., 2001), where ATP signaling is enhanced (Sun and Chai, 2004).

Complementary, but less complete information is also available for the colon. As in urinary bladder, ATP is released from colon epithelium (Wynn et al., 2003) and has been proposed to contribute to mechanosensory transduction. ATP release from rat colon is dependent on distension pressure and is associated with activation of pelvic nerve afferent fibers (Wynn et al., 2003). In humans, P2X₃ expression is increased in inflammatory bowel disease (Yiangou et al., 2001).

To more fully characterize the role of P2X receptors in colon mechanosensitivity and colon hypersensitivity, Gebhart and coworkers examined responses of wild type, heterozygous P2X₃^{+/-} and homozygous P2X₃^{-/-} litter-mate mice to colon distension (Shinoda et al., 2008). In these studies, colorectal distention (CRD) testing and evaluation was carried out as previously described (Kamp et al., 2003; see also Christianson and Gebhart, 2007). Briefly, electromyographic (EMG) activity was recorded during constant pressure phasic balloon inflation to 15, 30, 45, and 60 mm Hg. Each distension lasted 20 s, each pressure was tested three times, and 4 min separated each distension. Immediately following baseline CRD testing, 0.1 ml of a suspension of 30 mg/ml zymosan (in 0.9% saline) or vehicle was administered

transanally and the mouse was returned to its cage for 4 days. Zymosan was used to mimic two prominent features of irritable bowel syndrome: 1) colon hypersensitivity and 2) the absence of colon inflammation (Jones et al., 2007).

Naïve P2X₃ knockout mice exhibited reduced mechanosensitivity to colon distension relative to wild type mice (Fig. 3). (Results from previous studies in TRPV1 and ASIC3 knockout mice (Jones et al., 2005) are included for comparison.) These results suggest that P2X₃ receptors are important for normal colon mechanosensitivity, but do not rule out possible contributions of P2X₃ receptors in the central nervous system or elsewhere. The potential confound of differential release of ATP in wild type and P2X₃ knockout mice was also examined. The intraluminal ATP content at the four distending pressures was not different in wild type and P2X₃ knockout mice, supporting the conclusion that the absence of P2X₃ mediated the decreased colon sensitivity in naïve P2X₃ knockout mice (Shinoda et al., 2008).

As previously reported Jones et al., 2007), intracolonic administration of zymosan did not inflame the colon, but did produce hypersensitivity to colon distension in wild type mice 4 days after instillation of zymosan. No hypersensitivity was seen in the P2X₃^{-/-} mice (Fig. 4). In related work (Jones et al., 2005), sensitization of mechanosensitive pelvic nerve afferents to stretch was absent in ASIC3 knockout mice, suggesting cooperativity between multiple ligand-gated channels with respect to both mechanosensitivity and hypersensitivity.

4. Transient potential receptors (TRP) channels in visceral afferents

In addition to the P2X and ASIC receptors, the role of the TRP channels in visceral nociception has received intense examination in the last 5 years. The TRP channel family contains 28 members in mice (27 in human) that can be divided into seven subfamilies (for a review of the nomenclature see Clapham et al., 2003; Ramsey et al., 2006). This family of six-transmembrane cation-permeable channels (Fig. 5) has received intense interest because when select members are expressed in heterologous systems they responded to different temperature ranges, in essence defining a “molecular thermometer” that was hoped to explain temperature detection in both vertebrates and invertebrates (Bandell et al., 2007). The reality has turned out to be far more complicated; e.g., whereas a subset of afferents require TRPV1 to respond noxious heat (Lawson et al., in press), other populations are perfectly capable of detecting heat in the absence of any known TRP channel (Woodbury et al., 2004).

The GI tract is innervated by primary visceral afferents that express at least three of these channels including TRPV1, TRPA1 and TRPV4. TRPV1 and TRPA1 are found in both spinal (Christianson et al., 2006a,b; Fasanella et al., 2008; Jones et al., 2005) and vagal visceral afferents (Bielefeldt and Davis, 2008; Bielefeldt et al., 2006; Zhong et al., 2007) (TRPC-expressing afferents are also found in the nodose ganglia (Elg et al., 2007)). TRPV4 has recently been shown to be expressed in colon afferents, where it appears to have a significant role in nociception and the development of hypersensitivity (see below).

4.1. TRPV1

In viscera, the most studied of the TRP channels has been TRPV1 (Transient Potential Receptor Vanilloid-like 1). TRPV1 responds to noxious heat, vanilloid compounds (e.g. capsaicin and lipids) and protons (Caterina et al., 1997, 2000; Tominaga et al., 1998). However, its role in visceral sensation may extend beyond what would be predicted by the known stimuli that directly gate current through this channel. For example, a number of recent reports have shown that mice lacking functional TRPV1 exhibit altered mechanical sensitivity of visceral afferents (Birder et al., 2002; Daly et al., 2007; Jones et al., 2005; Rong et al., 2004). These observations suggest that either TRPV1 has the ability to directly detect mechanical deformation of sensory

neuronal endings (or adjacent tissues) or that it can modulate overall sensitivity, such that detection of multiple stimuli is diminished by its absence.

Although the role of TRPV1 in mechanosensation is unclear, there are numerous reports suggesting that TRPV1 is required for acute and chronic hyperalgesia. This is true in models of both somatic and visceral pain. For example, Jones et al. (2005) used an inflammatory soup to induce acute sensitization of colonic afferents in wild type and TRPV1^{-/-} mice. Whereas significant hypersensitivity was observed in wild type mice, the changes in the TRPV1^{-/-} mice were not significant. Winston et al. (2007) used a neonatal injury model to induce long-term hypersensitivity. In this paradigm, the neonatal rat colon is irritated via instillation of acetic acid. When adult rats are tested using colorectal distension, they are hypersensitive, although the colon exhibits no overt pathology. This hypersensitivity is blocked if rats are pretreated with a TRPV1 antagonist i.p. 30 min prior to CRD. Examination of DRG in adult rats that received neonatal acid instillation revealed a significant increase in the level of TRPV1 expression, leading the authors to conclude that TRPV1 was playing a central role in initiation and maintenance of colonic hypersensitivity.

Inflammation-induced pain in bladder also appears to have a TRPV1 component. Charrua et al. (2007) induced increased sensitivity in mice by instilling LPS into the bladder. When inflamed bladders from wild type mice were distended there was an increase in the number of fos-positive cells in the spinal cord, as well as increased reflexive bladder contractions. These changes were absent in TRPV1^{-/-} mice. One caveat of TRPV1 studies in the bladder is that TRPV1 is expressed in both bladder afferents and the bladder urothelium (Birder, 2007; Birder et al., 2002), and thus the effect of TRPV1 deletion could be affecting function of the bladder lining and/or sensory neurons innervating the bladder.

4.2. TRPA1

Another member of the TRP family that has been implicated in inflammatory pain is TRPA1, however significantly less is known about this receptor. TRPA1 was originally proposed to transduce noxious cold, but knockout mice from two different labs exhibited little or no thermal behavioral phenotype (Bautista et al., 2006; Kwan et al., 2006). However, it was observed that, similar to TRPV1^{-/-} mice, TRPA1^{-/-} mice exhibit a decrease in inflammatory hyperalgesia (Bautista et al., 2006). The role of TRPA1 in inflammatory pain may be related, at least in part, to its relationship with TRPV1. Studies from multiple labs have shown that most TRPA1 expressing cells also express TRPV1 (Elitt et al., 2006; Kobayashi et al., 2005). Moreover, Akopian et al. (2007) showed that although activation of TRPV1 could desensitize TRPA1, and vice versa, TRPV1 expression was needed to prevent internalization of TRPA1 and that TRPV1 co-expression greatly reduced mustard oil (a specific TRPA1 ligand)-induced tachyphylaxis of TRPA1. Some of the cross interactions between TRPV1 and TRPA1 could be through shared second messenger systems such as PLC/PIP₂. However, it should also be noted that both of these channels have numerous ankyrin domains (Sotomayor et al., 2005) that could be linking these channels to each other, to other channels and receptors, or to common cytoskeletal components that might coordinate the function of the various membrane proteins that control sensory neuron sensitivity.

Another characteristic that both TRPV1 and TRPA1 have in common is that they are both sensitized by growth factors. In both rodents and humans, *in vivo* injection of nerve growth (NGF) produces thermal hypersensitivity within 30 min and mechanical sensitivity within hours (Dyck et al., 1997; Lewin and Mendell, 1994). *In vitro*, acute administration of NGF sensitizes TRPV1 receptor function (Shu and Mendell, 1999a; Zhu et al., 2004). Under normal conditions, repeated activation results in progressive desensitization of TRPV1. NGF potentiates TRPV1 function, antagonizing or blocking tachyphylaxis. NGF is also up-regulated in the periphery following inflammation. Together these results have led to the hypothesis that

NGF plays a major role in acute inflammatory hyperalgesia (Shu and Mendell, 1999b). Much less is known about growth factors and TRPA1 function, but a recent report shows that 1) NGF up-regulated the expression of TRPA1 in dissociated trigeminal sensory neurons, 2) systemic injections of NGF increased TRPA1 expression and 3) NGF increased mustard oil induced currents in trigeminal neurons (Diogenes et al., 2007).

4.3. TRPV4

The transient receptor potential vanilloid-4 (TRPV4) is an osmo-sensitive channel that responds to mechanical stimulation. It also can be activated by temperatures ranging from 27 to 34 °C and endogenous lipids such as anandamide, arachidonic acid or 5' 6' epoxyeicosatrienoic acid (Barbara et al., 2004; Nilius et al., 2003; Watanabe et al., 2003). A synthetic agonist, the 4- α -phorbol, 12, 13-didecanoate (4 α PDD) is also able to activate this receptor and is commonly used as a pharmacological tool to study the physiology of this receptor. TRPV4 is expressed by a number of tissues and cell types, including lung, spleen, kidney, testes, skin, and smooth muscles, as well as sensory structures such as dorsal root ganglia neurons, trigeminal ganglia neurons and cochlear hair cells (Barbara et al., 2004; Nilius et al., 2003; Watanabe et al., 2003).

Whereas the role of TRPV4 in somatic nociception, particularly in cancer pain has been investigated (Alessandri-Haber et al., 2004; Levine and Alessandri-Haber, 2007), a recent study by Vergnolle and colleagues provided convincing evidence that it plays a role in visceral nociception, and colonic hypersensitivity (Cenac et al., 2008). These investigators found that TRPV4 is expressed in the colon of mice, as well as in mouse dorsal root ganglia neurons. In the colon, brush bordered epithelial cells are TRPV4-positive, as well as submucosal and muscular layer structures that appear similar to neuronal plexi. Functional TRPV4 expression was demonstrated by calcium imaging experiments that showed strong calcium signals in cultures of dissociated retrogradely labeled colon afferents in response to the TRPV4 agonist 4 α PDD. Blockade of TRPV4 expression in DRG neurons by intrathecal treatments with TRPV4 siRNA (but not with scrambled siRNA) completely abolish calcium response and currents induced by exposure of those neurons to 4 α PDD. These experiments confirm the expression and functionality of TRPV4 in DRG neurons projecting from the mouse colon, and validate the use of *in vivo* injections of TRPV4 siRNA, to down-regulate TRPV4 expression (Cenac et al., 2008).

To test the role of TRPV4 in colon function *in vivo* the TRPV4 agonist 4 α PDD (100 μ M) was infused into mouse colon. This induced activation of fos in nuclei of the superficial laminae (nociceptive regions) of the dorsal horn grey matter at the spinal cord level L5/S1 (Cenac et al., 2008). This result suggests that TRPV4 can activate nociceptive pathways, and therefore may play a role in visceral pain. In addition, intracolonic administration of 4 α PDD provoked allodynia and hyperalgesia in response to colorectal distension, thereby confirming hypersensitivity following colonic activation of TRPV4-positive afferents. Blockade of TRPV4 by gene deletion (in TRPV4-deficient mice), or by siRNA intrathecal treatments in wild type mice, completely inhibited the pro-algesic effects of 4 α PDD (Cenac et al., 2008). Finally, in TRPV4-deficient mice or in wild type mice that received intrathecal injections of TRPV4 siRNA, visceromotor response to colorectal distension was significantly reduced for distension pressures ranging from 30 to 60 mm Hg (Cenac et al., 2008). Taken together, these data provide evidence that TRPV4 activation in the mouse colon leads to visceral hypersensitivity and that TRPV4 plays a role in visceral nociception.

4.4. TRPV4 and irritable bowel syndrome

Irritable Bowel Syndrome (IBS) is a medical condition characterized by abdominal pain, discomfort and altered bowel functions. A common feature of IBS patients is visceral

hypersensitivity in response to colorectal distension (Whitehead and Palsson, 1998). Because TRPV4 activation causes visceral hypersensitivity and appears to be implicated in nociceptive responses to colorectal distension in mice, it is legitimate to ask if TRPV4 plays a role in IBS. Several peripheral mediators, in particular mediators released from mast cells, have been proposed as contributing to IBS (Barbara et al., 2004; Collins, 2001). A recent study has shown that proteases, through the activation of the Protease-Activated Receptor-2 (PAR₂), are important mediators released by IBS patients. Biopsies from IBS patients (but not from controls) release mediators that are able to sensitize cultured mouse sensory neurons. Supernatants from cultured IBS biopsies also produce sensitization of mouse colons (Cenac et al., 2007). Other laboratories have concentrated their efforts on demonstrating a role for serotonin and histamine in IBS-associated symptoms (Bradesi and Mayer, 2007; Collins, 2004). Based on these observations, Cenac et al. hypothesized that TRPV4 is activated by PAR₂, histamine and serotonin release, thereby serving as a common mediator responsible for the generation of colonic hypersensitivity (Fig. 6). TRPV4 down-regulation in sensory neurons by intrathecal TRPV4 siRNA treatments completely inhibited hypersensitivity (allodynia and hyperalgesia) induced by SLIGRL-NH₂, a peptide agonist of PAR₂, (Cenac et al., 2008). Furthermore, PAR₂ activation was able to sensitize TRPV4 function in sensory neurons, causing increased calcium signals and currents of larger amplitude (Grant et al., 2007). *In vivo*, sub-algesic doses of PAR₂ agonists were capable of producing TRPV4 sensitization and visceral hypersensitivity in response to colorectal distension (Cenac et al., 2008). Similarly, in the mouse model of colorectal distension, allodynia and hyperalgesia induced by serotonin or histamine (both administered intracolonic) were completely inhibited by TRPV4 blockade (Vergnolle et al. unpublished data). Taken together, these results suggest that TRPV4 may be a central mediator for the induction of colonic hypersensitivity.

5. Plasticity in neonatal pain pathways

Discoveries in neonatal pain during the last 20 years have dramatically changed the perception of nociceptive processing during early neonatal periods. Prematurely born infants can spend significant time in neonatal intensive care units and undergo multiple surgical procedures, many of which induce pain and/or inflammation that last for weeks (Fitzgerald and Beggs, 2001). The long-term negative effects of inadequately treated neonatal pain have been shown to include increased morbidity, mortality, hyper- or hypoalgesia and have a negative impact on development (Zempsky and Schechter, 2003). Rodent studies on the effects of neonatal exposure to noxious stimulation have documented similar results (Fitzgerald, 1995). By using these models to determine the mechanisms underlying long-term changes in pain perception, a better understanding of nociceptive pathway development during neonatal periods can be obtained. The vast majority of this work has been performed within the somatosensory system, although several recent studies have looked at the long-term effects of neonatal injury within the viscera (Al-Chaer et al., 2000; Christianson and Davis, 2005; Lin and Al-Chaer, 2003; Randich et al., 2006). These models are associated with (or result in) significant changes in behavioral responses to visceral stimuli in adulthood, but potential mechanisms underlying these changes have yet to be fully addressed.

5.1. Postnatal development of visceral afferents

Considerable growth and differentiation of nociceptors occurs during early neonatal periods in rats. Peripheral somatic nociceptors appear physiologically mature at birth, displaying adult-like firing frequencies and response patterns to noxious mechanical, thermal or chemical stimulation of the skin (Fitzgerald, 1987). Anatomically, these afferents continue to branch and innervate specific end organs during early neonatal development (Payne et al., 1991; Reynolds et al., 1991). The central terminals of somatic nociceptors are slower to develop, as many of the chemical markers associated with C-fibers, including SP, CGRP and thiamine

monophosphatase (TMP), are not visible at birth and some require several weeks before they are expressed at adult levels (Marti et al., 1987). Also, until the second postnatal week, C-fiber evoked action potentials are not observed in the spinal cord and stimulation of the hindlimb using mustard oil does not evoke hindlimb flexion or *c-fos* expression within the dorsal horn (Fitzgerald, 1988; Fitzgerald and Gibson, 1984; Soyguder et al., 1994; Williams et al., 1990). While the neonatal development of visceral afferents has not been directly studied, their similarity to specific subpopulations of somatic afferents allows for some inferences to be made.

5.2. Long-term consequences of noxious stimulation during postnatal development

The ability of pre-term infants to perceive and process nociceptive input has long been a subject of research and debate. An infant in the neonatal intensive care unit (NICU) is subjected to an average of 14 procedures a day, one-third of which are considered noxious. However, fewer than 35% of neonates receive analgesics on a daily basis while in the NICU (Simons et al., 2003). Recently, two studies have addressed whether infants can process noxious stimulation at the cortical level. Using real-time near-infrared spectroscopy to detect changes in cortical blood flow, both studies showed that noxious stimuli produce activation of the primary somatosensory cortex in newborns (Bartocci et al., 2006; Slater et al., 2006). This was shown to occur in even pre-term infants, where the youngest tested was 25 weeks gestational age (Slater et al., 2006).

Exposure to noxious stimuli during early neonatal periods can have long-term effects on nociceptive processing (Anand, 2001; Fitzgerald, 1995; Fitzgerald and Beggs, 2001; Lidow, 2002). Several studies have shown that children exposed to excessive noxious stimulation in the NICU can later develop decreased behavioral responses and increased physiological responses to painful events (Anand, 1998; Whitfield and Grunau, 2000). Rodent models of neonatal tissue damage have been studied to investigate the long-term effects on nociceptive processing. Hindpaw skin wounds on the day of birth, modeled to mimic procedures performed in the NICU, result in hyperinnervation and mechanical hypersensitivity within the injured area (Alvares et al., 2000; Reynolds and Fitzgerald, 1992, 1995; Torsney and Fitzgerald, 2003). The receptive field size of dorsal horn neurons innervating the affected area is also significantly larger in those rats receiving neonatal tissue damage (Alvares et al., 2000; Reynolds and Fitzgerald, 1992, 1995; Torsney and Fitzgerald, 2003). The long-term effects of neonatal inflammation have also been investigated by injecting known inflammatory agents, i.e. complete Freund's adjuvant (CFA), into the hindpaw of neonatal rats. This results in a similar increase in dorsal horn input from the affected area, however behavioral responses to noxious thermal stimuli are increased in these animals only after re-inflammation of the hindpaw (Peng et al., 2003; Ruda et al., 2000; Tachibana et al., 2001; Walker et al., 2003).

The effect of neonatal organ insult on adult nociceptive processing has not been directly addressed in humans. A possible clinical correlation exists within interstitial cystitis patients, where 10–30% report having experienced childhood bladder problems, including infection (Jones and Nyberg, 1997). Data from animal studies suggest that, similar to changes within the somatosensory system, organ insult during early development can induce long-term changes in adult nociceptive processing of visceral pain. Neonatal rats that receive noxious mechanical or chemical stimulation of the colon on postnatal days 8–21 display more severe abdominal contractions in response to colorectal distension (CRD) during adulthood. The firing rates of dorsal horn neurons in response to either CRD or cutaneous stimulation are also significantly increased (Al-Chaer et al., 2000). A subsequent study revealed that the number of thoracolumbar spinal afferents responding to CRD was increased and lumbosacral spinal afferents exhibit increased background activity and decreased activation thresholds (Lin and Al-Chaer, 2003).

Recently, work from the Randich laboratory has shown that neonatal bladder inflammation also induces a long-lasting hypersensitivity (DeBerry et al., 2007; Randich et al., 2006). Intravesical instillation of zymosan, a cell wall component of yeast that produces a robust inflammatory response, was performed on postnatal days 14–16 or 28–30, to determine the consequences of bladder inflammation during neonatal or adolescent periods, respectively. When the rats were reinflamed with zymosan as adults and 24 h later tested for abdominal electromyographic (EMG) responses to urinary bladder distension (UBD), the rats that received zymosan treatments as neonates exhibited a much higher mean EMG response than their control counterparts or rats treated with zymosan as adolescents (Fig. 7). The rats that received neonatal zymosan treatments also exhibited a higher arterial blood pressure response to UBD (Randich et al., 2006). Additional studies have shown that the endogenous opioid-inhibitory system may be disrupted in these neonatally inflamed rats, as treatment with naloxone did not enhance EMG responses to UBD, unlike its effect in acute bladder inflammation in naïve rats (DeBerry et al., 2007). It is important to remark on the fact that the neonatal injuries sustained in these experiments occurred after the critical period that was established for somatic afferents to induce long-term changes in nociceptive processing (Fitzgerald and Beggs, 2001; Lidow et al., 2001; Ruda et al., 2000). However, when injury occurred after PN21, no changes in visceral sensitivity were observed in the adult rats, indicating that visceral and somatic afferents may have different periods of vulnerability to alterations in nociceptive processing.

It is clear that early perturbation of either the somatosensory or viscerosensory systems can cause long-lasting changes in pain transmission and perception lasting well into adulthood. As the issue of neonatal analgesia gains importance, we will continue to uncover the mechanisms underlying these dramatic changes in nociceptive processing.

6. New treatments for visceral pain

Visceral pain is a common problem with a significant burden for the individual due to suffering, and for society due to healthcare-related costs and loss of productivity. In the United States, abdominal pain accounts for more than 12 million consultations each year (Anand, 1998; Russo et al., 2004). The challenge goes beyond mere numbers, as common treatment strategies, such as non-steroidal anti-inflammatory drugs or opioids often adversely affect gastrointestinal function or even integrity, thus limiting their clinical utility and prompting a search for alternatives. The more detailed understanding of visceral sensation has led to the development of candidates for 'visceral-specific' analgesics, which, as of yet, have not met expectations. Above we described the potential role of purinergic signaling in visceral sensation and pain. The proposed role of ATP as a signaling molecule is similar to that of serotonin (5-HT), which is released from enteroendocrine cells and activates sensory neurons. Thus, 5-HT₃ receptor antagonists seemed to hold promise and are indeed quite useful in treating nausea (Gershon and Tack, 2007). However, a relatively high incidence of ischemic colitis led to a withdrawal of alosetron (a 5-HT₃ receptor antagonist) as an agent used for irritable bowel syndrome, a functional disorder characterized by pain and altered bowel habits. Another strategy focused on alternatives to conventional opioid therapy. To limit the potential of adverse effects of opioids, peripherally acting agents with high affinity to the κ -opioid receptor were developed that showed analgesic effects in preclinical studies (Joshi et al., 2000). Asimadoline, a peripherally acting κ -opioid receptor agonist showed limited or no efficacy in patients with irritable bowel syndrome (Mangel et al., 2008; Szarka et al., 2007). Thus, alternative strategies are still needed to more effectively treat visceral pain.

The preceding sections primarily focused on hollow organs as a cause of visceral pain. With the high prevalence of irritable bowel syndrome and functional dyspepsia, understanding these pain syndromes is certainly very important. However, progress may be hampered by the

complexity of these disorders, which manifest with a plethora of symptoms in the absence of morphological or consistent physiological changes. It may thus be easier to develop and test new concepts in other disorders associated with pain and defined pathology, which may facilitate the identification of novel treatment targets. Recent studies on animal models of pancreatic diseases illustrate the potential utility of this strategy. Chronic pancreatitis and pancreatic cancer both manifest with pain as the dominant symptom in up to 80% of patients (Ammann and Muellhaupt, 1999; Mitsunaga et al., 2007). Thus, pain management plays a central role in the care for patients with these pancreatic diseases. In both cases, the pain can become intractable and unresponsive to morphine, necessitating additional research efforts to stop pain in these patient populations.

Acute and chronic animal pancreatitis models have been developed in the Westlund-High lab for studies of clinically relevant visceral pain and for study of visceral pain mechanisms (Fig. 8). The acute pancreatitis model is induced by tail vein injection with the caustic industrial plasticizer, dibutyltin dichloride, found in trace amounts in polyvinyl chloride (PVC) pipes (Vera-Portocarrero et al., 2003). Rats with the chemically induced pancreatitis develop histological and immunological responses resembling severe acute bouts of human pancreatitis as well as pain-related behaviors measurable in open-field testing. Beta cell function apparently remains intact with stable blood glucose levels. Chronic pancreatitis can be induced by feeding young Lewis rats a liquid alcohol diet for 3 weeks (Yang et al., 2008). Hotplate sensitivity develops in these animals along with histological features of chronic pancreatitis, such as fibrosis and acinar cell necrosis.

A novel approach to eliminating pancreatic pain in animal models of painful human pancreatitis employs a replication defective Herpes Simplex Virus-1 (HSV-1) viral vector encoding a human pre-proenkephalin transgene (HSV-ENK) that is applied to the surface of the pancreas (Lu et al., 2007; Yang et al., 2008). In addition to significantly reducing nociceptive behavioral measures, application of HSV-ENK to pancreatic tissue results in tissue protection from the insult itself. This is accomplished by the increased expression of met-enkephalin in the spinal cord and pancreatic tissue. Tissue sparing was seen in both acute and chronic models of pancreatic inflammation, observed as an absence of fibrosis seen in rats receiving the alcohol and high fat diet-induced chronic pancreatitis model (Fig. 9). Histology of the pancreas in these models receiving the neutral control vector shows evidence of edema, steatosis, inflammatory cell infiltration and acinar necrosis, whereas the pancreas of animals receiving the enkephalin gene therapy more closely resembles the tissue from control animals (Fig. 9).

It has been proposed that physiologically available met-enkephalin can protect the pancreas and reduce inflammatory infiltration. *In vitro* and *in vivo* studies are under way to determine the mechanism of the protective effect of enkephalin overexpression. Supportive experiments to determine the safety and efficacy of HSV-based vector-mediated delivery of met-enkephalin have shown that abundant enkephalin is expressed after targeting the pancreas with small quantities of the viral vector (2×10^6 plaque-forming-units, pfu). Studies by High and coworkers (Yang et al., 2008) show that pancreatic delivery of human proenkephalin-encoding, replication defective HSV-1 viral vector (HSV-Enk, DPE) results in increased met-enkephalin expression in primary afferent endings in the spinal cord and in pancreatic acinar cells. In these studies, no viral protein is evident in liver, kidney, colon, bladder or cervical dorsal root ganglia, supporting the known characteristic of restricted spread of replication defective vectors as an important safety feature of this gene therapy. Human HSV protein is found within the dorsal root ganglia, but only at vertebral levels providing innervation of the pancreas. The limited expression of viral vector and the transfected gene provides effective reduction of inflammation in both the acute chemically-induced pancreatitis and the chronic alcohol and high fat diet-induced pancreatitis without evidence of significant adverse effects. The met-enkephalin present in acinar cells most likely originates from axonal terminals of the same primary

afferents within the pancreas since no human HSV protein is found in the pancreas (Lu et al., 2007; Yang et al., 2008). It is likely that met-enkephalin is taken up and internalized by the abundant number of mu-opioid receptors seen on or in the acinar cells in animals with the acute pancreatitis after injection of the vector (Fig. 10).

In another safety test with a conditioned temperature stimulus, HSV vectors or vehicle were provided to normal animals by two sequential injections of the pancreas at week 3 and week 5 (Fig. 11). Weekly hotplate testing was done at 52.5 °C to condition the animals to the procedure. Response times were identical for all groups indicating normal sensory responses among the groups. However, at weeks 9 and 10 the hotplate temperature was increased to 55 °C followed by testing 30 min later at the usual 52.5 °C. The higher temperature provided a startle response, reducing response times by 2 s in all groups of animals. The repeated testing session 30 min later with the usual hotplate temperature revealed a significantly increased hypoalgesic response time for the group injected twice with the HSV-ENK vector indicating the potential for the vector to provide rapid opioid analgesia on demand within 30 min (Yang et al., 2008).

6.1. Innovative aspects and advantages of HSV gene therapy

This novel gene therapy approach has inherent advantages for delivery of an endogenous opioid peptide or related mediators that target the desired spinal cord level and pancreatic tissue for effective alleviation of pain in two pancreatitis models. A number of labs have explored the use of HSV for pain relief and these studies have shown:

- Multidisciplinary assessment of HSV-1 enkephalin gene therapy can be done in animals with persistent pancreatitis with translational potential
- HSV-1 viral constructs used in the animal models are replication deficient/defective and have already been tested for patient safety and efficacy in Phase I/II clinical trials in cancer (using unrelated transgenes/encoded peptides) (see NIH clinical trials)
- HSV-1 viral vector delivery of proenkephalin expression products by the peripheral nerves
  - Is selective for peripheral nerves and does not enter the central nervous system
  - Optimizes pain reduction without the development of tolerance
  - Provides more effective and prolonged alleviation of inflammatory and nociceptive responses
  - Normalizes pancreatic inflammatory parameters and nociception to near baseline levels
  - Preserves the target tissue architecture and function
  - Focuses delivery of met-enkephalin to the target organ using a low viral titer with a vector beginning Phase I/II clinical trials
  - Is a novel palliative strategy for alleviating the unremitting pain of pancreatic cancer and chronic pancreatitis, and ultimately, may protect from ongoing damage

Focused block of nociceptive and inflammatory symptoms could potentially alleviate the associated devastating pain, as well as protect from loss of function and potential tissue destruction that accompanies chronic clinical conditions of the visceral organs, including idiopathic pancreatitis, alcohol induced pancreatitis and pancreatic cancer. The anti-

inflammatory properties of this viral vector greatly enhance the therapeutic potential of this viral vector for novel application in pain therapy and tissue protection.

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Fig. 1.

(A) Microphotograph of the dissected neural tissues. J, vagal jugular ganglion, N, vagal nodose ganglion, IX, glossopharyngeal nerve, X, vagus nerve, XI, accessory nerve, XII, hypoglossal nerve, SCG, superior cervical ganglion. (B) Nodose C-fibers but not jugular C-fibers innervating the lung and esophagus are effectively stimulated by the P2X receptor agonist α, β -methylene-ATP. Representative traces of the single unit extracellular recordings made from nodose and jugular C-fibers projecting into lung and esophagus *ex vivo*; data from (Undem et al., 2004; Yu et al., 2005) (C) mRNA for P2X₂ receptor was detected in individual nodose C-fiber neurons but not in the jugular C-fiber neurons. Single cell RT-PCR was made from the vagal neurons retrogradely labeled from lungs or esophagus. Putative C-fiber neurons were identified by detection of the mRNA for TRPV1 receptor. L-N1 to N4 and L-J1 to J4, representative nodose and jugular neurons, respectively, retrogradely labeled from the lung. E-N1 to N4 and E-J1 to J4, representative nodose and jugular neurons, respectively, retrogradely labeled from the esophagus. For detailed quantitative analysis see (Kwong et al., 2008).

Fig. 2. Both bladder (top) and colon (bottom) sensory neurons in dorsal root ganglia express P2X3 receptors. Cells were retrogradely labeled from the organ and sections co-stained for P2X3 receptors; double labeled cells are circled.

Fig. 3. Mechanosensitivity as assessed by responses to colorectal distension is significantly attenuated in TRPV1, ASIC3 and P2X3 knockout mice.

Fig. 4. P2X3^{+/-} mice, but not P2X3^{-/-} mice, develop colon hypersensitivity to colorectal distension following intracolonic instillation of zymosan. For purposes of presentation, the baseline responses of wild type littermates for genetically manipulated mice have been grouped and responses are illustrated as percentage of baseline response at 60 mm Hg.

TRPV1

TRPA1

Fig. 5.

Example of two members of the TRP channel family. TRPV1 and TRPA1 have both been shown to play central roles in the development of inflammatory hyperalgesia. TRPV1 channel can be opened by vanilloid substances like capsaicin (found in chili peppers), protons and noxious heat. TRPA1 responds to number of environmental irritants (e.g., acrolein, a product of auto exhaust) and pungent plant compounds including isothiocyanate (found in mustard seed) and cinnamaldehyde (found in cinnamon). TRPA1 is also stimulated by endogenous alkenyl aldehydes (Andersson et al., 2008).

Fig. 6. Model suggesting that TRPV4 is part of a final common pathway for changes in PAR2, histamine and 5-HT changes that lead to IBS. TRPV4 has been shown to be downstream of PAR2 activation and blockade of TRPV4 can prevent PAR2-induced colonic hypersensitivity.

Fig. 7.

Group mean electromyographic (EMG) responses to urinary bladder distension (UBD; 10–80 mm Hg) for each of the groups receiving an early-in-life treatment (zymosan [zy], saline [sal], or anesthesia [an]) as either neonates (top panels) or adolescents (bottom panels). Rats were administered either zymosan (left panels) or saline (right panels) 24 h before UBD testing as adults. The asterisks indicate $P < 0.05$, neonatal zy treatment vs. sal or an. The inset in the bottom left panel reproduces control data for naive adult rats that received either saline, zymosan or no treatment 24 h before testing.

Persistent Pancreatitis Models

Acute Pancreatitis

DBTC i.v.
Chemical Insult
1 week
 Lu et al., Mol Therapy, 2007

Chronic Pancreatitis

High Fat /Ethanol Diet
10 weeks
 Yang et al., Mol Pain, 2008

Pancreas Histology
Hotplate Testing
HPLC Enkephalin
Serum Alcohol
Levels (120mg/dL)

Gene Therapy with Replication Defective
HSV-ENK 50μl 2x10⁵ pfu

Fig. 8.

Two rat models of persistent pancreatitis pain have been used to test met-enkephalin gene therapy. One model utilizes tail vein injection of the caustic chemical agent, dibutyltin dichloride (DBTC). The chemically induced model alters open-field behaviors and persists for 1 week. A chronic model producing hyperalgesic hotplate responses persisting through at least 10 weeks, is induced with alcohol and high fat diet. Gene therapy with replication defective viral vectors (HSV-ENK) is effective in both models. The reversal of hyperalgesic responses persists for 4–6 weeks in the chronic model.

Fig. 9.

Pancreatic histological assessment in naïve animals (A), and in animals with pancreatitis induced with alcohol and high fat (B and C). The alcohol diet produces significant tissue disruption and inflammation evident in tissue sections taken from control animals with pancreatitis, including animals given a control vector overexpressing the neutral protein, beta-galactosidase (B). The overexpression of met-enkephalin provided tissue protection/repair (C). The cells shown are acinar cells of the pancreas, with the exception of the small islet of Langerhans pictured at the top right corner in A. Insulin production is unaltered in this model as evidenced histologically by the preservation of the islet cells (not shown) and absence of change in blood sugar levels through 10 weeks on the diet. Tissue sections are stained with hematoxylin and eosin.

Fig. 10.

Immunocytochemical staining reveals high levels of mu-opioid receptor expression at 1 week in animals with chemically induced pancreatitis given the met-enkephalin gene therapy. The mu-opioid receptor staining gives the pancreatic acinar cells a granular appearance.

Fig. 11.

Behavioral responses to hotplate testing in control animals without pancreatitis, i.e. animals receiving low levels of alcohol in their diet ($n=4$), and given two doses of viral vectors at 3 and 5 weeks. Animals were conditioned weekly to hotplate testing at 52.5°. There was no difference in responses among groups. In week 9 and 10, rats were subjected to an aversive stimulus with hotplate testing at 55°, followed 30 min later by re-test at 52.5°. The animals that had received the met-enkephalin gene therapy were hypoalgesic on re-test.