Instituto Juan March
de Estudios e Investigaciones

105 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Molecules of Pain: Molecular Approaches to Pain Research

Organized by

F. Cervero and S. P. Hunt

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The lectures summarized in this publication were presented by their authors at a workshop held on the 28th of February through the 1st of March, 2000, at the Instituto Juan March.

Depósito legal: M-13.045/2000
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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Introduction

F. Cervero and S. P. Hunt

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Pain research holds a halfway position between the pure and the applied neurosciences. Pain is a distinct sensation that shares properties and mechanisms with other sensations but that preserves peculiarities of its own. Some of these peculiarities, particularly the sensory amplification phenomenon known as hyperalgesia, separate pain from the other sensations and offer an exciting insight into the workings of the brain. However, pain is also the most common symptom of disease and a frequent cause for patients to seek medical attention. The applied aspects of the study of pain mechanisms have become, understandably, an essential concern of the pharmaceutical industry.

Pain researchers are frequently called upon to suggest targets for the development of novel analgesics. There are those who favour the peripheral sensory nerve as the most accessible target for exploration and those, with perhaps more respect for the plastic qualities of pain, who point to the central nervous system as the most likely site for effective long term control of pain. Whichever option is chosen it seems from past experience that certain pain conditions yield to peripheral intervention while others require drugs that act predominantly within the central nervous system. However, the success of these therapeutic approaches tended to be somewhat serendipitous. The arrival of molecular biological approaches to the pain field promises rational approaches to the alleviation of suffering.

The objective of the Workshop whose abstracts are presented in this booklet was to discuss the cellular and molecular aspects of pain mechanisms, an area of research where considerable progress has been made in the last few years. In essence the approach has been to identify a molecule that is potentially involved in the signalling of pain and then to manipulate the gene by genetic 'knockout' or some other approach. Even allowing that these approaches can be compromised by developmental and other compensations, the results have been very revealing. For the most part gene knockout has given, at best, partial confirmation of our previous hypotheses regarding the function of the encoded protein or polypeptide. More important have been the insights into pain processing that have been gained from these molecular approaches causing us to rethink and modify some of our previously held views.

Our understanding of the physiology of the sensory nerve and sensory transduction has been accelerated by the cloning of a number of sensory receptors and ion channels that are

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specific to the peripheral sensory nerve. Selective knockout of these genes has begun to reveal modest phenotypes. Deletion of genes, such as those that code for the substance P and opiate receptors that are expressed within the brain and spinal cord and that have previously been assumed to play an important role in pain processing, has proved more complicated. This is because we have had to confront not only changes in sensory processing but also in the emotional and motivational dimensions of the pain response. Ablation of bio chemically specific subpopulations of neurons was also discussed in the Workshop and the results have forced us to reconsider the interrelationships between the spinal cord and the brain through ascending and descending pathways.

The Workshop was organised into sessions on nociceptors and sensory transduction, followed by sessions built around substance P, opiate and glutamate receptors. Each topic served as a starting point for the exploration of pain control from both peripheral and central perspectives.

The Workshop underscored the conclusion that ultimately, effective pain control, particularly of long term chronic pain, will demand an understanding of pain mechanisms at all levels of the neuraxis. Different pain conditions will require different therapeutic interventions each tailored to the particular biological signature of the condition.

F. Cervero and S.P. Hunt

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Session 1: Nociceptors and nociception

Chair: Stephen P. Hunt

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Pain: molecules, cells and sensations

Fernando Cervero

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Research into pain mechanisms is often led by the need to develop novel therapeutic strategies for pain relief. Currently the emphasis has shifted from "analgesics" (substances or procedures that eliminate pain) to "anti-hyperalgesics" (substances or procedures that reduce pain hypersensitivity without altering normal pain perception). This change has been motivated by the study of the mechanisms of pain plasticity, particularly of those that mediate peripheral and central sensitization of nociceptive neurones.

The new emphasis on "anti-hyperalgesia" and the need to find "anti-hyperalgesic" agents have been paralleled with a change of focus from nociceptive systems and neuronal networks to the cellular and molecular mechanisms that mediate peripheral and central sensitization. In this talk I would like to discuss the proposal that the actions of individual molecules (transmitters or messengers) can only make sense if we also know the properties of the nociceptive network in which they operate. The cellular actions can be different, or even opposite, for the same molecule depending on the characteristics of the pain system in question. Two pieces of evidence will be discussed:

1. Role of descending influences on sensitization of nociceptive neurones in the spinal cord. The process known as "central sensitization" describes the increase in the excitability of nociceptive neurones in the spinal cord that follows a period of intense noxious stimulation. This process is believed to cause or mediate hyperalgesic states. Sometime ago we proposed (Cervero & Wolstencroft, 1984) that positive feedback loops between the spinal cord and the brain stem could mediate increased spinal excitability via a descending excitatory mechanism. This view was further supported with data using visceral pain models (Tattersall *et al*, 1986). We have also shown that persisting nociceptive input from inflamed joints evokes an increased amount of descending inhibition from supraspinal structures (Schaible *et al*, 1991). Very recently it has been shown that tactile allodynia in rats with neuropathic pain depends on descending influences from supraspinal structures (Kovelowski *et al*, 1999). All these data reveal a prominent role of a spinal-supraspinal nociceptive network in the generation of central hypersensitivity states.

2. Touch-evoked pain and presynaptic interactions between mechanoreceptors and nociceptors in the spinal cord. We have proposed a model of allodynia based on the activation of nociceptive endings in the spinal cord by afferent impulses in low threshold mechanoreceptors (Cervero & Laird, 1996a). The mechanism involves primary afferent depolarization (dorsal root reflexes) of nociceptive endings via a presynaptic mechanism mediated by a GABA-ergic interneurone. We have provided experimental support from human experiments, showing the presence of touch-evoked flare in hyperalgesic zones (Cervero & Laird, 1996b), and from animal work in which we demonstrated A-□ evoked wind-up in arthritic rats (Weng *et al*, 1998). Currently we are exploring further this model and have shown local changes in blood flow (mediated by dorsal root reflexes) following stimulation of A-□ afferents from hyperalgesic zones. This model exemplifies the close link

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between the cellular actions of a given transmitter and the functional characteristics of the neuronal network involved.

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Molecular identification of heat-activated ion channels in the pain pathway

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Pain-producing heat is detected by several classes of nociceptive sensory neurons that differ with respect to thermal response threshold, conduction velocities, and anatomical characteristics (i.e. size and myelination) (1-6). We have shown that the cloned capsaicin, or vanilloid receptor, VR1, can be activated when ambient temperatures exceed ~ 43°C. VR1 is expressed predominantly among the small-diameter sensory neurons (both IB4+ and IB4- subclasses), making this channel a candidate heat sensor for unmyelinated C-fibers that respond to moderate-threshold noxious thermal stimuli (7). VR1 is also modulated by protons and lipids (the endocannabinoid, anandamide), suggesting that this receptor plays a role in the detection of both noxious thermal and chemical stimuli in vivo (8, 9). We have also identified a capsaicin receptor homologue (VRL-1) that does not respond to vanilloids or acid, but which does respond to heat with a high temperature threshold of ~ 52°C (10). Within sensory ganglia VRL-1 is expressed within medium- to large-diameter neurons, many of which also express markers for the Aδ subclass of nociceptors, such as CGRP and neurofilament proteins. We therefore propose that VRL-1 is a candidate receptor for the transduction of high-threshold noxious heat responses by this class of afferent sensory neurons. VRL-1 transcripts are also found in a variety of neural and non-neural tissues, suggesting that this channel is activated by stimuli other than heat. Taken together, our findings suggest that responses to noxious heat involve related, but distinct ion channel subtypes whose activation thresholds are tuned to different thermal intensities (11). The identification of VR1 and VRL-1 will enable us to probe the function of these ion channels in thermal nociception and other physiological processes.

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Using mouse genetics to unravel pain pathways

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Specialised sensory neurons that respond to tissue damage (nociceptors) play a pivotal role in the induction of pain. Genes that are exclusively expressed in these neurons are likely to play a significant role in pain pathways. A variety of molecular genetic strategies have been used to identify such genes. Difference cloning led to the identification of an ATP-gated cation channel P2X3, and a voltage-gated sodium channel named SNS. Expression cloning identified the noxious heat/capsaicin receptor VR-1, and homology cloning defined channels activated by protons (ASIC-b) as well as a further voltage-gated sodium channel (NaN), all of which are selectively expressed on sensory neurons. Gene ablation studies in transgenic mice, followed by behavioural analysis support a role for some of these proteins in pain pathways. Other more broadly expressed genes are also implicated in the detection of tissue damage, but the interpretation of the role of these genes using mouse knock-outs is complicated by the effects on other physiological systems. Using tissue specific promoters to drive the bacterial recombinase Cre, genes flanked by lox-p sites can be selectively deleted in damage sensing neurons, allowing a less ambiguous interpretation of the role of such genes in pain induction. The application of tissue-specific and inducible knock-outs to manipulate gene expression in sensory neurons, but not other cells, is of potential importance in dissecting the role in pain pathways of commonly expressed genes. The information gleaned from such genetic studies, combined with pharmacological developments using high throughput screening in cell-based assays and medicinal chemistry, suggests that we may be able to develop analgesic agents of a distinct mechanism of action to opioids or aspirin like drugs in the immediate future, and address the problems of many inappropriate pain states with novel therapeutic agents.

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Transduction in nociceptors

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Nociceptor nerve endings respond with a discharge of nerve impulses to various forms of stimulating energy (mechanical, thermal, chemical, electrical) acting on peripheral tissues. Several functional types of nociceptors have been distinguished based upon their prefered activation by a particular form of energy. However, the cellular and molecular processes involved in the transformation of high intensity stimuli into a change of membrane potential at the nerve ending, are incompletely understood. This is due in part to the small size of nociceptor nerve terminals, that make it difficult to apply intracellular recording techniques.

Our group has used two strategies to analyze electrical changes associated with the activation of nociceptors: 1- Extracellular recording from single corneal nerve terminals of the potential changes evoked by natural stimuli. 2- Intracellular recording and calcium imaging in trigeminal ganglion neurons 'in vitro', subjected to mechanical and chemical stimulation of the soma.

The cornea is densely supplied with mechanosensory, polymodal and cold sensitive nerve endings. Using a 50 μ suction electrode applied to the surface of the cornea, nerve impulses can be recorded and identified as originating in single sensory nerve terminals. The configuration of nerve terminal impulses in the different types of endings and the local application of lignocaine and TTX indicate that nerve terminals possess TTX-resistant Na^+ channels and that polymodal nerve terminals are able to support regenerative action potentials whereas cold receptor terminals are passively invaded from a point more proximal in the axon where the action potential fails or is initiated.

Application of hypotonic solutions (15 % - 45 %) to cultured trigeminal ganglion neurons evoked a rise of $[Ca^{2+}]_i$ in about 80% of the neurons with either a slow (73%) or a fast (23%) rise time due to an entrance of extracellular Ca^{2+}, that was roughly proportional to the osmolality reduction and was supressed by application of the stretch-activated channel blocker gadolinium (20 – 100 μM). Whole cell patch-clamp recording of these neurons showed that hypotonic swelling produced membrane depolarization and impulse firing. Voltage clamping of the neuron did not prevent the Ca^{2+} entrance caused by hypotonic solutions. Application of these solutions caused a conductance increase, suggesting the opening by membrane stretch of non-selective cationic channels. About 60% of the mechanosensitive neurons responded also to 0.5 μM capsaicin. About a half of the neurons that were insensitive to hypotonic solutions also showed a $[Ca^{2+}]_i$ increase caused by capsaicin.

Based upon active and passive membrane properties of trigeminal ganglion neurons 'in situ' of new born and young mice, two groups of neurons were distinguished, F neurons showing a short duration spike and S neurons with long duration spike and a hump in the falling phase. The same electrophysiological types of

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neurons were found in tissue culture. No obvious relationship appears to exist between action potential characteristics and responses to hypotonic solutions or capsaicin, except that F neurons showed always a fast $[Ca^{2+}]_i$ rise under hypotonic stimulation.

The proportion of trigeminal primary sensory neurons displaying mechano- and/or chemosensitivity in the soma is roughly coincident with that of mechanosensory, polymodal and 'silent' nociceptor fibers in peripheral axons. Therefore, the soma of the neuron may serve as a model to unveil some of the mechanisms involved in the transduction of low- and high-threshold stimuli by peripheral sensory nerve terminals.

(The data presented here were obtained in collaboration with MC Acosta, J. Brock, C. Cabanes, E.de la Peña, J. Gallar, M. Lopez de Armentia, B. Pecson, RF Schmidt and F. Viana)

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Session 2: Nociceptor sensitization and other peripheral mechanisms

Chair: Allan I. Basbaum

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Nociceptor excitation by thermal sensitization

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Discovery of heat-activated ion channels and of the vanilloid receptors VR1[1] and VRL1[2] in sensory neurons of the spinal ganglion has shed essentially new light on the transduction mechanisms by which inflammatory mediators and chemical irritants excite nociceptors and contribute to pain. Prostaglandin E_2, histamine and, most potently, bradykinin and low pH as well as capsaicin, mustard oil, phorbol esters and formalin (in low concentration) induce a prominent sensitization to heat of nociceptors which includes recruitment of previously unresponsive terminals. Various membrane bound receptors and alternative second-messenger pathways, including PKC[3], cAMP[4] and calcium influx[5,6], are involved in the transduction of the sensitizing effect. Different heat-sensitive ion channel entities, including the vanilloid receptors, are the target of the sensitizing action which is probably mediated by protein phosphorylation.

With bradykinin and low pH application it can already be shown that the nociceptor thresholds, which normally exceed 40°C, rapidly drop into the range of room temperatures (19°-28°C) which enables the actual tissue or body temperature to drive a vivid discharge with a temperature coefficient Q_{10}<6 in primary afferent nerve fibers[7]. This apparently chemically but actually thermally induced activity is then subject to classical nociceptor adaptation and to the more or less slow inactivation or desensitization of the transduction pathway. However, even with bradykinin whose apparent excitatory effect fades within minutes, nociceptor thresholds stay well below body temperature in a very sustained manner which can be shown to depend on secondary prostaglandin formation induced by bradykinin. By that nociceptor sensitization and the resulting hyperalgesia are maintained for as long as the mediators are present in the inflamed tissue.

Excitation by thermal sensitization may also be a mechanism to drive deep visceral nociceptors and ectopic discharge in C-fiber axons. As a prerequisite these nerve fibers (in the sciatic nerve) are established with a well graded responsiveness to capsaicin[8], low pH and noxious heat which results in a calcium-dependent release of calcitonin-gene related peptide (CGRP). As with cutaneous nerve endings, heat and pH sensitivity are not blocked by capsazepine and ruthenium red which excludes VR1 and VRL1 as transduction mechansism.

The novel unifying theory of previously diverse and multiple nociceptive mechanisms may provide new targets for pharmaceutical development as soon as the molecular elements, heat-sensitive ion channels, will be identified.

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Changed expression of bradykinin sensitivity in nociceptive primary afferents in arthritic model rats

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Bradykinin (BK) is the most potent inflammatory mediators in sensitizing the thermal as well as mechanical response of nociceptors, namely it sensitizes the heat response of canine testicular polymodal receptors at 0.1 nM [1], and the mechanical response at 10 nM (our recent observation). These effects are mediated through the B2 receptor in normal condition. Reports of induction of B1 receptor in some inflammatory conditions have suggested that sensitivity of nociceptors themselves can also be changed in inflamed condition. To see whether primary afferent sensitivity to BK is changed in inflamed conditions, we studied BK sensitivity of single cutaneous C-fiber receptors and cultured dorsal root ganglion (DRG) neurons of adjuvant-inflamed (AI) rats. In addition, we studied implication of nerve growth factor (NGF) to changed sensitivity.

Methods: Adjuvant inflammation was induced in rats by intradermal injection of complete Freund's adjuvant (1 mg/0.1 ml) into the distal third of the tail. Rats with signs of inflammation were used for physiological experiments 2-4 weeks after inoculation. Single C-fiber activities were recorded from skin-nerve preparations. Receptors with slowly adapting mechanical response (possible C-fiber polymodal receptor (CPR)) were used for the study. DRG neurons were dissociated, and cultured in the presence or absence of NGF (100 ng/ml) or in the presence of anti-NGF (10 μg/ml,) for 2 days without serum. Intracellular recording was obtained from small neurons (<30 μm) except otherwise noted. NGF contents of DRGs were measured by ELISA.

Results and discussion : BK induced small excitation in CPRs of intact rats from 0.1 μM, while it induced a clear response in about 40 % of CPRs of AI rats. At 1 μM BK excited all CPRs in AI rats but only 30 % in intact rats, and the magnitude of the response was four times greater in AI rats. BK response was blocked by B2 antagonist. B1 agonist (des Arg^{10}-Kallidin, 10 μM) never induced excitation in intact rats, while it induced excitation in about 80 % of CPRs. However, B1 agonist-induced excitation was much smaller than that by BK [2].

None of acutely dissociated DRG neurons from intact rats responded to 0.1 μM BK. After 2-days cultivation without NGF, BK induced small depolarization without action potentials in only 13% neurons from intact rats. When cultured with NGF the percentage of neurons responding to BK increased to 54% [3]. This effect of NGF was not observed in large neurons (>35 μm). B1 agonist failed to excite neurons and B1 antagonist failed to block the BK response. Absence of B1 sensitivity was confirmed also by Ca imaging. In contrast, about 10 % of acutely dissociated neurons from AI rats responded with a small depolarization, and after 2 day-cultivation without NGF BK induced a larger depolarization, accompanied with action potentials in some cases, in 53% neurons of AI rats, a significantly higher percentage than intact rats. Addition of NGF had no effects on BK sensitivity of DRG neurons from AI rats, suggesting that NGF effect had been saturated. To confirm that increased BK sensitivity

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in AI rats was due to endogenous NGF, neurons of AI rats were cultivated in the presence of anti-NGF antibody [4]. With this treatment, the percentage of BK sensitive neurons decreased to 17%, a level near that of the neurons cultured without NGF in intact rats. We did not observe any B1-mediated BK response so far in AI rats, either. These results suggest that the expression of sensitivity to BK of DRG neurons in AI rats is increased due to an action of endogenous NGF, and that B1 receptor is not expressed in DRG neurons. The latter result also suggests that the response to B1 agonist observed in the skin-nerve preparation might be indirect.

High content of NGF in DRGs was confirmed by ELISA: Contents of NGF in DRGs (L4-L6) of AI rats was measured at 0 (control), 2, 7, 14 and 21 days after inoculation of adjuvant. NGF was increased to about 5 times of the control at 2nd day when sing of inflammation was detectable only at the injected site. Because L4-L6 DRGs do not innervate the injected site (tail), increased NGF could not be transported in the axons from the periphery. NGF content reached a peak (more than 30 times of the control) at 14th day when hind paw swelling became apparent, and declined, although still higher than the control, at 21st day. This result suggests that inflamed peripheral tissue is not the sole source of NGF in DRGs.

These results suggest that expression of B2 receptor-mediated sensitivity to BK of nociceptive afferents is increased in inflamed condition, and NGF plays an important role in this modification.

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The role of neuropeptides and neuropeptide receptors in arthritic pain

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Peptides such as bradykinin, substance P and calcitonin gene-related peptide (CGRP) play an important role in the generation and maintenance of arthritic pain. In the present contribution the interaction of CGRP and excitatory amino acids in the spinal cord and the expression of bradykinin and neurokinin 1 receptors in dorsal root ganglion cells will be addressed.

Interaction between CGRP and excitatory amino acids. CGRP is involved in the spinal processing of nociceptive input from the knee joint and in the generation and maintenance of joint inflammation-evoked hyperexcitability of spinal cord neurons (Neugebauer et al., 1996). Now we examined whether CGRP influences the excitation of nociceptive spinal cord neurons by agonists at the N-methyl-D-aspartate (NMDA) and the non-NMDA (AMPA/kainate) receptors both of which are essential for the excitation and hyperexcitability of spinal cord neurons. In anesthetized rats extracellular recordings were made from dorsal horn neurons with knee input, and compounds were administered ionophoretically close to the neurons recorded. When CGRP was administered the responses of the neurons to the application of both NMDA and AMPA were increased. The coadministration of the antagonist CGRP 8-37 had no effect on the responses to NMDA, but it prevented the enhancement of the responses to NMDA by CGRP. By contrast, the administration of CGRP 8-37 enhanced the responses of the neurons to AMPA, and it did not antagonize but rather increased the effects of CGRP on these responses. The data suggest that the facilitatory role of calcitonin gene-related peptide on the development and maintenance of inflammation-evoked hyperexcitability is caused at least in part by the modulation of the activation of the dorsal horn neurons through their NMDA and non-NMDA receptors. The different effects of CGRP 8-37 on the responses to NMDA and AMPA suggest that different intracellular pathways may facilitate the activation of NMDA and ionotropic non-NMDA receptors (Ebersberger et al., submitted).

The expression of bradykinin and neurokinin 1 receptors in dorsal root ganglion neurons. We assessed the expression of neurokinin 1 receptors (activated by substance P) and bradykinin receptors in sensory neurons of the lumbar dorsal root ganglion (DRG) in normal rats and in rats with antigen-induced arthritis (AIA) in the right knee joint. DRG neurons were removed from normal rats and AIA rats at different time points and cultured for 18 hours. Then the expression of substance P and bradykinin binding sites was determined with gold-labelled substance P and bradykinin. Substance P-gold binding sites were identified in 9% of the DRG neurons in normal rats, in up to 50% of the DRG neurons ipsi- and contralateral to the inflamed knee at days 1 and 3 of AIA and in about 7% of the DRG neurons at days 21 and 42 of AIA. Bradykinin binding sites were expressed in about 40% of the DRG neurons in normal rats and in up to 80% of the DRG neurons ipsilateral to the injected knee at days 1, 3, 10, 21 and 42 of AIA. The expression of bradykinin binding sites in the contralateral DRGs was only elevated (up to 80%) at days 1 and 3 of AIA. Thus monoarticular AIA leads to a marked bilateral upregulation of receptors for bradykinin and substance P in DRG neurons. Since bradykinin and substance P produce hyperalgesia and pain, the enhanced expression of these receptors

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could be an important factor in the process of long-term sensitization of primary afferent neurons (Segond von Banchet et al., submitted).

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MOLECULAR DETERMINANTS OF THE PORE-FORMING REGION OF VR-1 CHANNELS

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The cloned vanilloid receptor (VR1) is a cation channel with significant permeability to Ca^{2+}, and high sensitivity to block by rutenium red, Ca^{2+} and Ga^{3+}. The molecular determinants that define these permeation properties are still elusive. VR1 subunits display homology to the store-operated calcium channels having a membrane domain composed of six transmembrane segments and a pore-loop (P-loop) between the fifth and sixth membrane-spanning regions. The P-loop contains four strategically positioned negatively-charged residues (E636, D646, E648, E651) that could determine the permeation properties of homomeric VR1 channels. We have investigated this question and evaluated the functional relevance of neutralizing these negative charges. Neutralization of D646 decreased 10-fold the sensitivity of the channel to block by rutenium red, Ca^{2+} and Ga^{3+}, and modulated the divalent cation permeability of homomeric VR1 channels. Mutation of amino acids at the other positions had minor effects on the permeation properties. Accordingly, our results indicate that D646 is an important molecular determinant of the VR1 pore-forming region, and suggest that this residue forms a ring of negative charges at the vestibule or near the entrance of the aqueous pore that structures a high affinity Ca^{2+}-binding site.

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Mechanosensitive and acid-sensing DEG/ENaC channels in touch and pain

Jaime Garcia-Añoveros and David Corey

The molecular receptors that detect nociceptive and other somatic sensations are thought to include mechanosensitive channels, which gate when a mechanical force is applied to them, and acid-sensing channels, which open when the pH of the extracellular medium decreases, as occurs during inflammation, infection, and muscle ischemia. Members of the recently discovered DEG/ENaC superfamily of amiloride-sensitive sodium channels have been implicated in somatosensory and other related sensory functions, including touch in nematodes, and acid-induced pain and taste in mammals.

All channel subunits of the DEG/ENaC superfamily are characterized by two membrane spanning domains and a large extracellular loop between them. The extracellular portion has been implicated in gating the channel since some members need part of it to keep closed and others are activated by a variety of extracellular stimuli. These ion channels are permeable to sodium, sometimes in addition to other cations, and all are blocked by amiloride. The first identified members of the DEG/ENaC superfamily, the nematode degenerins, participate in several forms of mechanosensation. MEC-4 and MEC-10 are necessary for the detection of touch by sensory neurons, and UNC-105 is believed to mediate stretch sensitivity in muscle. We have proposed a model for mechanotransduction whereby extracellular structures connect to degenerin channels and convey tension to open them.

To demonstrate that degenerins indeed form ion channels we expressed the *unc-105* gene in two heterologous systems: *Xenopus* oocytes and human embryonic kidney cells. As expected, wild type UNC-105 did not elicit any currents, presumably because it was not coexpressed with the macromolecular machinery essential for its mechanical opening. We then engineered gain-of-function mutations that leave the channel open most of the time, thus generating amiloride-sensitive currents permeable to monovalent cations. These currents lead to cellular depolarization and eventually death. It remains to be demonstrated that degenerins are indeed mechanically gated.

Another branch of the DEG/ENaC superfamily is formed by the acid-sensing brain sodium channels of mammals (BNaCs or ASIC), which can be activated by low extracellular pH. We and others identified two members of this family: BNaC1 and BNaC2. BNaC2 activates with a drop of pH below 6.9 and inactivates quickly and completely, whereas BNaC1 activates with a drop below pH 5.5 and inactivates slowly. Both BNaC1 and BNaC2 are expressed in most neurons of the brain. However, in peripheral nervous tissue, the BNaCs have been detected in subsets of DRG neurons. Thus the BNaCs might mediate acid induced pain and sensitization in somatosensory neurons. This pattern of expression might also be expected of a mechanosensitive channel, suggesting that these channels might also be activated by mechanical forces.

We have also detected BNaC1 in a small subset of neurons of the cochlear spiral ganglion, the type II afferents, which innervate outer, but not inner, hair cells. Unlike the sound-responsive type I afferents, type II afferents have small diameters, are unmyelinated, and do not appear to signal sound. Their function is unknown, but we suspect they might detect noxious effects produced by extreme noises.

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Session 3: Substance P

Chair: Peter W. Reeh

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The substance P receptor knockout mouse. What has it told us about substance P and pain?

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Substance P receptor (NK1) and the preprotachykinin (PPT) knockout mice have been studied in considerable detail. It has been concluded that the peptide is involved in pain behaviour, noxious chemical signalling, anxiety and depression, epilepsy, stress and addiction to opiates. We reasoned that many of these behaviours could be grouped together as responses to danger in the environment and that animals were, in this respect, at a severe disadvantage when the SP pathway had been disabled. Obviously, pain represents one class of sensory information that signals danger and deficits have been found in noxious visceral stimulation (see Laird et al., this volume) stress induced analgesia, aggressive behaviour and so forth. But how many of these changes in mutant mice could be put down to compensatory changes during development and how useful are these mice for pharmacological discovery? There are few good NK1 antagonists that are effective in mice and rats. However, there are several excellent antagonists that work in other species including man and there is now evidence that many of the behaviours disrupted in the knockout mouse can also be altered with NK1 antagonists in a variety of non-murine species. Therefore, I will argue that the NK1 knockout mouse and many other neurotransmitter receptor gene disrupted mice can, particularly in the absence of good antagonists, tell us a great deal about the behavioural contribution of different neurotransmitter pathways. While neurochemical compensation may occur, the NK1 gene knockout mouse is largely just that, a single gene deletion and is proving to be a valuable pharmacological model.

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Mouse genetics as a tool to study nociceptive signaling

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The structural analysis of the human genome has advanced at a staggering pace since the discovery of the DNA structure by Watson and Crick in 1953[1]. Soon we will have a complete blueprint of the human and mouse genomes. A draft sequence of the human genome (98% complete) will become available in a few months (summer 2000) and the mouse sequence will follow within 36 months. The challenge for the next decades will be the comprehensive functional analysis of this sequence information. Undoubtedly, this analysis will contribute significantly to our basic understanding of the development and function of the mammalian organism.

We are using genetics to study the molecular mechanisms involved in nociceptive signaling. In a reverse genetics approach, we start with a gene that is thought to be involved in the transmission of noxious signals. Mice with targeted mutations in these genes are generated and the physiological consequences of these gene defects are analyzed. With this approach, we have generated mouse mutants which cannot produce the neuropeptides enkephalin[2], substance K or substance P[3], or the neuromodulatory cannabinoid receptor CB1[4,5]. These knockout mouse strains are viable, although CB1 knockouts show a dramatically increased mortality. All knockout mouse strains show distinct and characteristic behavioral alterations: Substance P knockout mice are hypoalgesic, while enkephalin knockout mice are hyperalgesic. These behavioral alterations reflect the antagonistic roles of these neuropeptides in the transmission of nociceptive signals. Enkephalin knockout mice also display altered 'emotional' behaviors, suggesting the possibility of an imbalance of limbic functions. Indeed, we found striking molecular changes in limbic structures of enkephalin knockout mice, including an up-regulation of opioid receptor gene expression. CB1 knockout mice were hypoalgesic and hypoactive. Because CB1 cannabinoid receptors are expressed at high levels in the basal ganglia, a brain structure critical for sensorimotor and motivational aspects of behavior, we studied the expression of various neuropeptides and transmitter-related enzymes in basal ganglia neurons. CB1 mutants display significantly increased expression of substance P, dynorphin, enkephalin, and GAD 67 in neurons of the two output pathways of the striatum that project to the substantia nigra and the globus pallidus, thus indicating a critical role of the CB1 receptor in normal basal ganglia function.

The analysis of congenic C57BL/6 and DBA/2 mouse strains with the enkephalin mutation showed that the effect of the mutation on nociceptive signaling are strongly influenced by the genetic background. Interestingly, these strain effects are specific for the nociceptive test. Mutant C57BL/6 congenic mice are hyperalgesic in the hotplate test and more sensitive in tests for visceral pain. Mutant DBA/2 congenics display increased stress-induced analgesia. It should be possible to utilize these strain difference to identify modifier genes that modulate the effects of the enkephalin mutation on specific nociceptive signaling pathways.

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We are also using a forward genetic approach in order to identify novel genes involved in nociceptive signaling. For this purpose, we are participating in a large scale ethylnitrosourea (ENU)-mutagenesis program which is sponsored by the German Human Genome Project and carried out at the GSF – National Research Center in Neuherberg, Germany. ENU, an alkylating agent, is one of the most powerful mutagens for the production of mutations in mice. It creates mainly point mutations, as well as small intragenic lesions[6]. Systematic ENU-mutagenesis screens have been successfully performed in *Drosophila melanogaster*, *Caenorhabditis elegans*, and in zebrafish. Male mice are injected with ENU and mated to females to produce mutant F1 offspring. These F1 animals are then analyzed for dominant traits, or bred further to screen for recessive phenotypes. We have screened over 1600 F1 animals for nociceptive behaviors using the hotplate test. Mice that deviate in three subsequent tests (performed at weekly intervals) by more than two standard deviations from the strain means, are considered variants. All variants are crossed back to wild type mice and the F2 offspring of this backcross are analyzed in the hotplate test. If this analysis indicates Mendelian (autosomal dominant) transmission of the trait, then the variant is called a mutant. To this date, we have identified over 30 variants and 5 mutants.

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Studies in mice in which the preprotachykinin-A gene is deleted reveal a novel relationship between tachykinins and the delta opioid receptor

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Recent immunocytochemical studies have demonstrated that the delta opioid receptor (DOR) differs considerably in its subcellular distribution from that of the mu opioid receptor (MOR). The MOR is located on the plasma membrane of cell bodies, dendrites and axon terminals; DOR is present in the membrane of dense core vesicles that contain neuropeptides. We recently reported that mice with a deletion of the gene that encodes the preprotachykinin-A (PPT-A) gene do not respond normally to a range of intense noxious stimuli. Because the PPT-A peptide products, substance P (SP) and neurokinin A (NKA), are found in large dense core vesicles, we assessed the distribution of DOR immunoreactivity in these animals. We found that the density of DOR immunoreactivity parallels that of the tachykinins; it is reduced in heterozygotes and absent from the dorsal horn of the PPT-A knockout mice. However, immunocytochemical localization of a variety of other molecules, including calcitonin-gene related peptide (which is co-stored in dense core vesicles with the tachykinins) as well as the MOR and various opioid peptides, is not altered in the PPT-A mutant mice. Importantly, in regions of the brain where DOR is not co-localized with tachykinins, there is no loss of DOR immunoreactivity. On the other hand, although the immunocytochemical demonstration of DOR was also lost in the trigeminal ganglia and in dorsal root ganglia (DRG), RT-PCR established that DOR message persists in the ganglia. Furthermore, Western blotting revealed that the protein is present in both trigeminal ganglia and dorsal horn. These results raise the possibility that DOR and tachykinins are co-trafficked to the synaptic terminal, via the dense core vesicle. By ligating the sciatic nerve and demonstrating build up of DOR immunoreactivity at the ligature, we established that distal (and presumably proximal) transport is not disrupted in the mutant mice. Taken together these results indicate that when tachykinins are not produced there is an alteration in the distribution of the delta opioid receptor. One possibility is that the DOR, rather then ending up in the dense core vesicle, is inserted into the plasma membrane. Conceivably the density of the DOR protein on the plasma membrane is not sufficient to be detected by immunocytochemistry. Alternatively, the confirmation of the DOR molecule in the plasma membrane may be sufficiently altered so that it is no longer recognized by available DOR antisera. Regardless of the explanation, these results provide evidence for a novel relationship between tachykinins and a major class of presynaptic opioid receptor.

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Targeting spinal cord neurons involved in the conduction of persistent inflammatory and neuropathic pain

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Within the past decade there has been a revolution in our ability to visualize the interaction between neurotransmitters and their receptors.

Using confocal microscopy and digital imaging techniques, investigators have shown that following a painful stimulus a specific set of neurotransmitters is released in the spinal cord to signal pain. The released neurotransmitters in turn bind to receptors expressed on the surface of the post-synaptic neuron which induces a dynamic translocation of both the receptor and the neurotransmitter from the outside (plasma membrane) to the inside (cytoplasm) of the post-synaptic neuron. Aside from providing a pharmacologically specific view of the population of neurons activated by the release of a neurotransmitter in response to a painful stimulus this data suggests that receptor internalization could provide a portal of entry into specific populations of receptor bearing cells. Building on these observations we have recently used intrathecal infusion of a neurotransmitter-toxin conjugate substance P-saporin (SP-SAP) to target and destroy spinothalamic and spinoparabrachial neurons, as these neurons preferentially express the substance P receptor (SPR) and that are involved in the ascending conduction of chronic pain. Thus, following intrathecal infusion of the SP-SAP, which undergoes ligand-induced internalization by the SPR expressing spinothalamic and spinoparabrachial neurons, responses to mildly painful stimuli and morphine analgesia remained unchanged. In contrast, hyperalgesia and allodynia associated with chronic inflammatory or neuropathic pain was reduced even at long time points following treatment.

As only a small subset of dorsal horn neurons express the SPR, this identifies a target for the treatment of persistent inflammatory or neuropathic pain.

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IS SUBSTANCE P THE NEUROTRANSMITTER FOR MECHANICALLY INSENSITIVE ("SILENT") NOCICEPTORS? STUDIES IN NK1 RECEPTOR KNOCKOUT MICE

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Substance P acting via the NK1 receptor is known to be essential for neurogenic inflammation, and recent studies have revealed that mechanically-insensitive nociceptors are responsible for neurogenic inflammation in various species (see 1). Furthermore, the proportion of primary afferents expressing substance P is similar to the proportion that are mechanically insensitive in both cutaneous (~25%) and visceral (~50-80%) nerves (see 2). Here, we tested the hypothesis that SP may be expressed by mechanically-insensitive nociceptors and mediate pain responses due to their activation, by comparing the phenotype of NK1 -/- mice with the known response properties of mechanically insensitive nociceptors (1,2). We examined the behavioural and reflex responses of NK1 -/- (3) and wild-type mice to either neurogenic or non-neurogenic stimulation of the skin or viscera.

NK1-/- mice showed abbreviated pain behaviour and no hyperalgesia to intraplantar capsaicin and no pain or referred hyperalgesia after intracolonic capsaicin. The NK1 -/- mice also showed reduced pain to a prolonged visceral neurogenic inflammation (cyclophosphamide cystitis). In anaesthetised -/- mice, reflex responses to intracolonic acetic acid were absent, and primary hyperalgesia (enhanced responses to distension of the inflamed colon) did not develop. However, NK1 -/- mice accurately detected brief mechanical stimuli, both somatic (von Frey hairs) and visceral (writhing test with i.p. acetylcholine or hypertonic saline), and showed normal pain and referred hyperalgesia after a visceral inflammatory stimulus that evokes tissue damage (intracolonic mustard oil).

We conclude that NK1 receptors are essential in mediating pain and hyperalgesia evoked by neurogenic noxious stimuli, and that the phenotype of NK1-/- mice is consistent with a loss of function of the mechanically-insensitive nociceptors. We propose that substance P is preferentially expressed in this group of primary sensory neurones, rather than in conventional, mechanically-responsive nociceptors.

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Supported by: CICYT, DGICYT, Europharma S.A., Comunidad Autónoma de Madrid and Generalitat Valenciana, Spain, and by the MRC (ROPA) and the BBSRC, U.K.

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Session 4: Opioid peptides

Chair: Carlos Belmonte

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Involvement of different receptors and intracellular messengers in opioid-induced antinociception, tolerance and dependence

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The neurobiological mechanisms involved in opioid-induced antinociception, tolerance and dependence have been recently investigated by using mice with a genetic disruption of genes related to opioid responses. In a first study, the pharmacological effects induced by acute and chronic opiate administration was investigated in mice with a genetic disruption of genes encoding mu, delta and kappa opioid receptors. Antinociceptive responses induced by morphine were completely abolished in mice deficient in mu opioid receptors, as well as the rewarding effects induced by repeated morphine administration in the place conditioning paradigm. The activation of the endogenous opioid system by stress and/or administration of the inhibitor of the enkephalin catabolism RB 38A produced an antinociceptive response in mu-deficent mice, but less intense than in wild-type controls. Besides, chronic morphine did not develop any behavioral manifestation of dependence in these mu-deficient mice (Matthes et al., 1996). Antinociceptive and rewarding effects of morphine were preserved in mice deficient in kappa opioid receptors. These mice presented an increase in the rewarding properties induced by morphine when this opioid was administered at a high dose. In contrast, the manifestation of the behavioral signs of morphine dependence was slightly attenuated in kappa-deficient mice (Simonin et al., 1998). Antinociceptive and rewarding properties of morphine were also preserved in mice lacking delta opioid receptors. However, these mice showed a slight increase in the expression of some of the behavioral signs of morphine withdrawal. These results clearly indicate a cruciai role of the mu opioid receptors in morphine-induced antinociception as well as in the different components of morphine dependence, whereas the role of kappa and delta opioid receptors does not seem to be important.

Behavioral and biochemical consequences of opioid tolerance and dependence were also evaluated in mice with a genetic disruption of the cAMP-responsive element-binding protein (CREB). Previous molecular analysis revealed that these mice lack the alpha and delta isoforms of CREB, but contain an up-regulated beta isoform of this transcription factor. The expression of the behavioral and vegetative symptoms of morphine withdrawal was strongly attenuated in CREB mutant mice. The development of tolerance to the antinociceptive responses induced by morphine was also significantly decreased in these mutant mice. However, the increase in immunoreactivity for c-FOS and c-JUN in locus coeruleus and amygdala, and the enhancement of adenylyl cyclase activity in cortex produced by morphine withdrawal were similar in wild type and CREB knockout mice. The rewarding effects induced by morphine, cocaine or food in the place conditioning paradigm were not modified in these mutant mice. These data indicate that CREB-dependent gene transcription is crucially involved in the adaptive changes responsible for the behavioral espression of morphine withdrawal but not in the rewarding effects induced by this drug.

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Analgesia in mice lacking opioid receptors

B. L. Kieffer

Opioid receptors mediate the strong analgesic and addictive properties of opiate drugs. These receptors and their endogenous ligands modulate numerous physiological functions, including the regulation of nociception, mood control and responses to stress. Three receptors classes, mu, delta and kappa, were described by pharmacological approaches and their genes have been cloned recently (see ref 1). We have used gene targeting in mice to produce null mutants (Ref 2, 5, unpublished results). The observation of mice lacking either mu, delta or kappa receptors does not reveal any obvious developmental deficit. Careful evaluation of their responses to acute painful stimuli reveals subtle phenotypes for mu- (thermal pain, see Ref 4) and kappa-receptor knock-out mice (chemical visceral pain, see Ref 5). We have measured analgesic responses of mutant mice to standard mu-, delta - and kappa-preferring opioids in order to reevaluate drug selectivity under in vivo experimental conditions. The data show that morphine and U-50,488H analgesia is abolished in mice lacking the mu- and the kappa-receptor gene, respectively. They also show that DPDPE and deltorphin analgesia is attenuated in both mu- and delta-receptor knock-out mice, suggesting a mixed activity of the compounds. In addition to nociceptive responses and drug-induced analgesia, we have investigated other spontaneous (unpublished) or pharmacological (Ref 3) responses. Together, the data will illustrate the usefulness of these genetic animal models (i) to reevaluate the molecular mode of action of classical, as well as newly developed opiates of clinical interest and (ii) to clarify the specific implication of each opioid receptor in adult physiology. Our studies will be discussed in the general context of opioid receptor- and opioid peptide-deficient mice which have been developed in several laboratories (see Ref 6). In the future, the comparative study of single and combinatorial mutants should elucidate the contribution of each component of the opioid system in the development of chronic pain, stress-induced behavior and drug addiction.

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Molecular approaches to pain and pain control

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New molecular approaches to pain control are evolving which emerge directly from the fields of gene therapy, molecular biology and receptor biochemistry. These treatments when used in combination with image-guided local administration can provide a new potential for long-term pain control that was previously unattainable.

Our laboratory is exploring three new approaches to chronic pain control. One involves gene therapy for pain, an area also addressed by two other laboratories. We have called our approach the "paracrine paradigm" for in vivo viral-mediated gene transfer. This paradigm uses either an adenovirus and/or adeno-associated virus (AAV), but it is likely that other therapeutic virsus could also be used. In our system an expression cassette composed of a growth factor leader sequence is fused upstream of the coding sequence for β-endorphin, in between is a proteolytic cut site. The leader sequence directs the fusion protein to the non-vesicular secretory pathway which allows □-endorphin to be secreted from non-neuronal cells. Thus an intrathecal injection of adenovirus transduces the cells of the pia mater, however it does not cross the pia into the spinal cord. The pial cells are closely adherent to the surface of the spinal cord and these cells provide an in vivo secretory source for β-endorphin release. This treatment produced an antihyperalgesic effect in the carrageenan inflammation model. The anti hyperalgesic action is naloxone reversible. More recent work has focused on adapting the expression cassette to adeno-associated virus. A new AAV-β-endorphin virus has been produced that also yields high level secretion from transduced cells. Repication defective Herpes virus has been engineered to express preproenkephalin by two other groups. At least one group has carried out behavioral studies after administration of virus to the skin and subsequent expression in the dorsal root ganglion. This approach is aimed at altering the presynaptic mixture of peptide released from the central and peripheral endings of primary afferents in the DRG.

The second and third approaches involve selective cell deletion. Both treatments attenuate pain sensation by preventing information transfer to the brain through permanent removal of either pain-sensing primary afferent neurons in the dorsal root ganglion, or the second order relay neurons in the spinal cord. Selective loss of primary afferent nociceptive neurons is achieved by intraganglionic or epidural application of the highly potent plant compound resiniferatoxin (RTX). The receptor for RTX, referred to as the vanilloid receptor 1 (VR1), transduces thermal pain and is exclusively expressed by pain-sensing primary afferent neurons. Exposure of the dorsal root ganglion to RTX kills VR1 expressing cells through a Ca^{++} influx mechanism, and produces a long lasting analgesia that is proportional to the dose used. The other treatment deletes the very next cell in the pain transmission circuit, the second-order nociceptive neuron in the spinal cord. We have developed a ligand-cytotoxin (substanceP-Pseudomonas exotoxin conjugate, SP-PE), that when administered into the subarachnoid CSF space selectively targets pain transmission neurons in the superficial layers of the dorsal horn. Loss of these cells blocks pain transmission from the spinal cord to the brain and produces a profound analgesia to thermal and mechanical nociception. Administration via the intrathecal route circumvents the toxicity to periperal organs that can accompany therapeutic use of these types of agents. We see RTX and SP-PE being incorporated into pain management strategies for patients with chronic, intractable pain. Assuming that toxicity to other neurons in the cord can be avoided, clinical implementation will fundamentally transform palliative care in patients for whom conventional pain therapy has failed or is inadequate.

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Exogenous agmatine, a novel neuromodulator, modulates the spinal plasticity of chronic pain

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Antagonists of glutamate receptors of the N-methyl-D-aspartate subclass (NMDAR) or inhibitors of nitric oxide synthase (NOS) prevent nervous system plasticity[1-8]. Inflammatory and neuropathic pain rely on plasticity, presenting a clinical opportunity for NMDAR antagonists and NOS inhibitors in chronic pain. Agmatine, an endogenous neuromodulator expressed in brain[9], has *both* NMDAR antagonist[10] and NOS inhibitor[11, 12] activities. We report here that exogenously administered agmatine selectively relieves allodynic, hyperalgesic and autotomy-like states accompanying spinal nerve injury, peripheral muscle inflammation and excitotoxic spinal cord injury (SCI), respectively. Moreover, as in brain neurons [13, 14], we have detected agmatine expression in spinal cord, indicating that agmatine may be an endogenous modulator of pain pathways.

Agmatine is an amine and organic cation formed by the decarboxylation of L-arginine by the enzyme arginine decarboxylase in bacteria, plants, invertebrates [15] and mammals [9, 16] notably in the central nervous system. In brain, agmatine meets most of the criteria of a central neurotransmitter/neuromodulator [17]: it is synthesized, stored, and released from specific networks of neurons [13, 14], is inactivated by energy-dependent reuptake mechanisms [18], is enzymatically degraded [19], and binds with high affinity to alpha-2-adrenergic and imidazoline receptors [9, 20]. In addition, agmatine has *both* NMDAR antagonist [10] and NOS inhibitor [11, 12] activities. Either NMDAR antagonists *or* NOS inhibitors prevent adaptive changes in neuronal function, including opioid tolerance [3, 21], persistent pain [7, 8] and spinal cord injury [1, 2, 4, 5]. Rodents tolerate large doses of agmatine (100 nmol intrathecally or 100 mg/kg systemically), giving it an advantage over novel therapeutic NMDAR antagonists or NOS inhibitors with low therapeutic indices [21, 25]. An agent, like agmatine, with *both* activities should be effective at alleviating chronic pain accompanying inflammatory, neuropathic or spinal cord injury conditions.

We first examined the effects of agmatine in a model of inflammatory pain and two models of neuropathic pain. Carrageenan, injected into the triceps of rats, produces muscle hyperalgesia, reflected by a reduction in the animal's ability of a rat to grip a transducer, an effect reversed by opioids, steroids, and non-steroidal antiinflammatory drugs (NSAID) [26]. Agmatine, administered intrathecally (60 nmol), enhanced recovery from carrageenan-evoked muscle hyperalgesia. We also tested agmatine's ability to modulate neuropathic pain elicited by a central chemical insult or direct injury to a peripheral nerve. Hypersensitivity to innocuous cutaneous pressure delivered to the hindpaw (mechanical allodynia) can be induced in normal mice by intrathecal injection of dynorphin 1-17 [27]. A single injection of agmatine dose-dependently reversed this allodynia for at least five days. This effect was also replicated in another model of neuropathic pain in mice (Chung Model[28],

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— tight ligation of the L5 spinal nerve (SNL)) distal to the dorsal root ganglion induces mechanical allodynia and hyperalgesia, evidenced by cutaneous hypersensitivity of the ipsilateral hindpaw to innocous and noxious mechanical force. A single intrathecal post-treatment with agmatine dose-dependently reversed SNL-induced allodynia (2 wk) and hyperalgesia (3 wk). Thus agmatine administered intrathecally decreases established allodynia and hyperalgesia induced by chemical, mechanical, and inflammatory insults in rodents.

Spinal cord injury (SCI) also is a cause of chronic pain syndromes and shares neurochemical and pathophysiological mechanisms with it. Like chronic pain, NMDAR [29-31] and AMPA/kainate receptors [32-35], as well as NOS [36, 37], participate in the negative consequences of traumatic spinal cord injury, suggesting a comparable cascade of secondary pathological changes in both states. We therefore investigated whether agmatine could alleviate the painful behaviors associated with excitotoxic lesions of spinal cord and whether any beneficial effects would be accompanied by reductions in neuronal damage like that which agmatine produced in cerebral ischemia [24]. SCI was produced by intraspinal injection of the AMPA/metabotropic agonist quisqualic acid (QUIS, 125 nmol), which produces an excitotoxic injury with pathological characteristics similar to those associated with ischemic and traumatic spinal cord injury (SCI) [38, 39]. Agmatine (1 nmol, 5 nmol, 10 nmol) injected with QUIS reduced the injury produced by QUIS alone. Pathological effects of spinally administered agmatine alone were not evident at doses below 15 nmol. Spinally or systemically administered agmatine produced dose-dependent neuroprotection from quisqualate-induced injury. Furthermore, co-treatment and post-treatment (2 weeks) with agmatine significantly improved locomotor function and reduced pain behaviors (excessive grooming) following traumatic SCI. The effect of agmatine to reduce persistent pain and SCI supports a view that both models share common mechanisms. Furthermore, agmatine clearly demonstrates a *post*-injury therapeutic potential in multiple preclinical models of persistent pain.

To determine whether agmatine produced antinociception or only affected the *sensitized* allodynic and hyperalgesic responses, we examined the effects of agmatine in normal mice measuring tail flick latency [40] or aversive behavior elicited by substance P (SP, i.t.) [41]. Agmatine (i.t.) neither prolonged tail flick latencies nor inhibited SP-induced behavior (Fig. 3a). These results concur with previous observations that NMDAR antagonists and NOS inhibitors do not inhibit responses to acute nociceptive stimuli [42] and distinguish agmatine from conventional analgesics.

Agmatine inhibits NMDA-evoked currents in cultured hippocampal neurons from both mouse [43] and rat [10] by channel blockade. Agmatine could therefore exert its anti-allodynic/anti-hyperalgesic effects through an antagonist action at this receptor. To directly test this possibility we administered agmatine (0.3, 1, 10, 40, 70, 100 nmol, i.t.) with a dose of NMDA (0.3 nmol, i.t.) that produced a characteristic scratching and biting behavior directed to the hindlimbs in mice and hyperlocomotion (circling behavior) and "spontaneous tail flicks" in rats [44]. Agmatine effectively and dose-dependently antagonized this NMDA-elicited behavior in both species (Fig. 3b). Consistent with these results, we observed that iontophoretically applied agmatine inhibits NMDA-evoked firing in 7 of 20 rat spinal neurons studied (Fig. 3c[45]). Interestingly, the ED_{50} value of agmatine (30 nmol) to antagonize NMDA-evoked behavior is 30 to 500,000 times higher than that of clinically (ketamine, memantine, dextromethorphan) and scientifically (MK801, LY235959, aminoguanidine, ifenprodil) used NMDA receptor antagonists in this test (Table 1). This low potency may indicate an improved therapeutic potential for agmatine, relative to previously used agents [46]. Taken together, these data underscore the spinal relevance of this mechanism of action and provide an estimate for intrathecal doses of agmatine (30 nmol) sufficient to block NMDA receptors; lower intrathecal doses of agmatine (1 nmol) likely act at other effectors (e.g. NOS [12]). Therefore, exogenous agmatine

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appears to produce its therapeutic effects by a combination of low dose (NOS inhibition) and high dose (NMDAR antagonist) actions. This result suggests that endogenous agmatine may play a similar role.

Endogenous control of spinal plasticity by agmatine would require localization in spinal tissue. We therefore investigated the presence and localization of *endogenous* agmatine in the spinal cord using high performance liquid chromatography (HPLC) and immunocytochemistry. HPLC analysis of quick-frozen spinal tissue from uninjured animals showed that the mean agmatine level in naive mouse lumbar spinal cord is 0.96 ± 0.14 μg/g (n = 5), a value comparable to previous measurements in mammalian brain [9, 47]. Intrathecal administration of agmatine (60 but not 0.3 nmol) increased spinal agmatine levels more than two-fold (2.6 μg/g ± 0.65 S.E.M. wet weight, n = 6). The localization of agmatine within the cord was determined immunocytochemically using laser confocal microscopy (LCM) and a specific immunofluorescent labeled antibody to agmatine [13]. Agmatine-like immunoreactivity (agmatine-LI) was sparse, but was consistently observed in all areas of the spinal cord gray matter. Immunoreactivity was not observed in tissue treated with pre-immune serum and was decreased by pre-incubation of antiserum with agmatine sulfate (10 mM, data not shown). Agmatine-LI revealed a pattern suggestive of neuronal processes and/or puncta in distinct clusters throughout the spinal cord. A neuronal source is consistent with the previous demonstration of agmatine-LI associated with small synaptic vesicles in axons and axon terminals [14]. Agmatine-LI was also observed in fiber-like structures in the surrounding white matter in both rat and mouse. The immunoreactivity pattern showed distinct localization relative to other commonly used neuronal (NeuN[48]; TuJ1[49], neurochemical (DβH[50]) or glial (GFAP[51]) immunomarkers. Although we did not observe co-localization of agmatine-LI with neuronal or glial markers using immunofluorescence, we cannot exclude neuronal or glial localization of agmatine without ultrastructural studies. Regardless, the distribution of agmatine-LI indicates that cells or processes highly enriched in agmatine reside in spinal cord. Taken together, quantification (HPLC) and localization (LCM) of agmatine in lumbar spinal cord supports the proposal that endogenous agmatine may play a distinct role in the spinal processing of pain and plastic changes associated with abnormal, chronic pain syndromes.

Our observations that exogenous, spinal agmatine *post*-treatment significantly reduces inflammatory, neuropathic, and spinal cord injury-induced pain suggests a new therapeutic direction for treatment of plasticity-mediated neurodysfunction. The doses necessary for enhanced recovery from inflammation-induced hyperalgesia (60 nmol i.t.) and for protection from excitotoxic injury (5 nmol) are comparable to the dose (30 nmol i.t.) required to inhibit NMDA-evoked behavior; this correspondence suggests that these effects of agmatine require NMDA-R antagonism. That the optimal dose (0.3 nmol i.t.) for rescue from neuropathic pain is 100-fold lower than that required to antagonize NMDA-R-mediated action suggests that the mechanism for agmatine-mediated recovery from neuropathic pain requires activity other than NMDA-R blockade (e.g., NOS inhibition). The present study illuminates an endogenous source and possible mechanisms for its antiplasticity action in spinal cord. As might be expected of an endogenous substance, agmatine does not present the behavioral side effects of hyperlocomotion (e.g. MK801 [14]) or sedation (e.g., LY274614[21]) observed with the most commonly used NMDA receptor antagonists and NOS inhibitors [21]. The apparently low toxicity and selective anti-allodynic (non-analgesic, non-sedating) profile of agmatine make the compound a novel and potentially advantageous therapeutic agent for treatment of chronic pain and acute spinal cord injury.

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REDUCED NICOTINE-ELICITED ANTINOCICEPTION IN MICE LACKING NEURONAL NICOTINIC RECEPTOR SUBUNITS L.M. Marubio*, M.M. Arroyo-Jimenez, M. Cordero-Erausquin, C. Léna, N. Le Novère, A. de Kerchove d'Exaerde, M. Huchet, M.J. Damaj§, and J.P. Changeux. Institut Pasteur, Biotechnologies, 25 rue du Dr. Roux, 75724 Paris Cedex 15, FRANCE. §Dept. of Pharmacology, Medical College of Virginia, Richmond 23298-0613, USA.

Nicotine exerts antinociceptive effects by interacting with one or more of the subtypes of nicotinic acetylcholine receptors (nAChR) present throughout the pain pathways, however, the involvement of a particular nAChR subunit in nicotine analgesia has been difficult to assess. Pharmacological approaches have suggested a possible contribution of the widely-expressed neuronal α4 subunit which co-assembles *in vivo* with the β2 nAChR subunit (and possibly other nAChR subunits) to form functional receptors. To identify particular subunits involved in the antinociceptive effect of nicotine, mice lacking the α4 and the β2 nicotinic acetylcholine receptor (nAChR) subunits were investigated. Both types of mutant mice display a reduced antinociceptive response on the hot-plate test and diminished sensitivity to nicotine in the tail-flick test. Systemic injection of the antagonist, hexamethonium, revealed a role for peripheral, non-α4, non-ß2-subunit containing nAChRs in the tail-flick test. Furthermore, in the thalamus and the raphe magnus there is a loss of ^{3}H-nicotine and ^{3}H-epibatidine binding sites in mutant mice, indicating a contribution of the α4 and β2 nAChR subunits to functional receptors in areas implicated in supraspinal nicotine-elicited antinociception. Thus, the α4 nAChR subunit, possibly associated with the β2 nAChR subunit, plays a critical role in nicotine-elicited antinociception.

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Session 5: Neurotrophins and other central mechanisms

Chair: Fernando Cervero

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Genomic strategies for identification of molecular targets of pain

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Peripheral pain can be classified into three broad areas, nociceptive pain, inflammatory pain and neuropathic pain. Nociceptive pain is also refereed to as physiological pain and serves as a defense mechanism throughout the animal kingdom. Inflammatory pain, arising from trauma and/or associated with inflammatory infiltrates, can be well controlled by NSAID-like drugs, steroids and opiates. Neuropathic pain is thought to arise from inherent defects in sensory or sympathetic neurons and can be secondary to trauma. However, the etiology and management of neuropathic pain is not well understood.

Several interesting and novel genes have been discovered in the past 5 years that suggest new approaches to the modulation of persistent neuropathic pain. We are utilizing a comprehensive, high-throughput genomic strategy to search and mine genes relevant to pain in mammals. The thrust of our strategy is to use both animal models and human specimens to generate cDNA's for the high-throughput analyses. Using cDNA sequencing, data base mining and micro arrays of cDNAs, and more rapid genetic analyses it is now possible to profile the transcriptional events, both short and long term, as they pertain to the onset and persistence of pain. With a new technology platform developed at Millennium we are mining gene targets of particular classes (ion channels, cell surface receptors, enzymes, transporters, etc.) homologous to those known in the literature, as well as distinct genes that are specific to particular pain syndromes. We rationalize that targets derived from the peripheral nervous system are of strategic benefit in that candidate compounds do not need to cross the blood-brain barrier, that they act on the initiation site of pain, and are devoid of CNS toxicities. Using these techniques in a variety of well-established experimental animal paradigms, we are trying to identify novel molecular targets involved in pain generation and propagation, in order to design new therapeutic strategies for the management of neuropathic pain.

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Insights into nociception from $GABA_A$ receptor knock-down mice

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The γ-aminobutyric acid$(GABA)_A$ receptor is formed by the heteromeric arrangement of α_{1-6}, β_{1-4}, γ_{1-4}, δ, π, ε and ρ_{1-3} subunits (Barnard et al., 1998). The β subunit is of particular interest because it is present in virtually all $GABA_A$ receptors and forms one interface of the binding domain for GABA (Stephenson, 1995). Recently, a line of knockout mice was created in which the *gabrb3* gene, which encodes the β_3 subunit, was inactivated resulting in a 50% decrease in the number of $GABA_A$ receptors (Homanics et al., 1997). Substantial neuroanatomical, pharmacological and electrophysiological evidence implicates GABA and $GABA_A$ receptors in the spinal cord, medulla and pons in the modulation of nociception (Hammond, 1997). The existence of a line of mice in which the number of $GABA_A$ receptors is substantially reduced provides another, complementary approach to studies of the role of GABA and $GABA_A$ receptors in nociception. Mice deficient in the β_3 subunit are particularly relevant to studies of the role of GABA in nociception because $GABA_A$ receptors in the dorsal horn of the spinal cord and the dorsal root ganglia contain the β_3 subunit almost exclusively (Zhang et al., 1991; Ma et al., 1993). The mRNA for the β_3 subunit is also expressed by neurons in the medullary and pontine nuclei that modulate nociceptive transmission at the spinal level (Zhang et al., 1991; Luque et al., 1994). This study characterized the responsiveness of $\beta_3^{-/-}$ mice to different intensities of noxious thermal and innocuous mechanical stimuli, and also examined the ability of $GABA_A$ and $GABA_B$ receptor agonists to produce antinociception after s.c. or i.t. administration. Homozygous null ($\beta_3^{-/-}$) mice displayed enhanced responsiveness to low-intensity thermal stimuli in the tail-flick and hot-plate test compared to C57BL/6J and 129/SvJ progenitor strain mice, and their wild-type ($\beta_3^{+/+}$) and heterozygous ($\beta_3^{+/-}$) littermates. The $\beta_3^{-/-}$ mice also exhibited enhanced responsiveness to innocuous tactile stimuli compared to C57BL/6J, 129/SvJ and to their $\beta_3^{+/+}$ littermates as assessed by von Frey filaments. The presence of thermal hyperalgesia and tactile allodynia in $\beta_3^{-/-}$ mice is consistent with a loss of inhibition mediated by presynaptic and postsynaptic $GABA_A$ receptors in the spinal cord. As expected, s.c. administration of the $GABA_A$ receptor agonist THIP did not produce antinociception in $\beta_3^{-/-}$ mice, whereas it produced a dose-dependent increase in hot-plate latency in C57BL/6J, 129/SvJ, $\beta_3^{+/+}$ and $\beta_3^{+/-}$ mice. However, the antinociceptive effect of the $GABA_B$ receptor agonist baclofen in the tail-flick and hot-plate tests was also reduced in $\beta_3^{-/-}$ mice compared to the progenitor strains, $\beta_3^{+/+}$ or $\beta_3^{+/-}$ mice after either s.c. or i.t. administration. This unexpected finding suggests that GABA and $GABA_A$ receptors play an important role in the production of antinociception by other drug classes as well.

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Nociceptors and ionotropic receptor expression

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Dorsal root ganglion (DRG) neurons are a diverse collection of sensory afferents that include the nociceptors as a major subpopulation. The nociceptors themselves are a heterogeneous group of sensory afferents whose subpopulations have been defined by a variety of parameters. These include sensory modality, peptide expression and expression of the newly cloned vanilloid heat receptors, VR-1 and VRL-1 (Caterina et al., 1997; 1999). All of these approaches to identifying nociceptor subpopulations are supported by the observation that each has specific laminar terminations in the superficial dorsal horn, a major site of nociceptive input. Interestingly, however, there is no clear pattern that has yet emerged for how each of these subsets of nociceptors defined by one set of criteria is related to those defined by another. Yet, defining the relationships among these groupings may help to establish good and practical criteria for nociceptor identification.

Another set of potentially important properties expressed by nociceptors is ligand-gated ion channels. A variety of ligand-gated channels are expressed on different subpopulations of nociceptors suggesting specific receptor functions could be associated with the specific sensory modalities of those subpopulations. For example, the ATP-gated ion channels, called the P2X receptors, are expressed by sensory neurons in the DRG. However, only one subset of nociceptors, those projecting to inner lamina II and expressing the binding site for the lectin IB4, has so far been identified as expressing the P2X3 subunit that mediates a rapidly desensitizing response to ATP (Guo et al., 1999). These IB4-positive neurons are also defined by expression of the Ret receptor and their associated sensitivity to GDNF (Molliver et al., 1997). However, only 60-80% (Tominaga et al., 1998; Guo et al., 1999) of these neurons express VR1 suggesting either that the IB4-labeled neurons are not a uniform subpopulation of nociceptors or that VR1 expression has a dynamic component.

Nociceptors express all three of the glutamate-gated ion channel families found in neurons of the central nervous system including NMDA, kainate and AMPA receptors. NMDA receptors are expressed at the central terminals of nociceptors in the superficial dorsal horn and their activation is tightly coupled to release of substance P following painful stimuli to the periphery (Liu e al, 1997). We have observed functional AMPA receptor expression in nociceptors from postnatal rat pups. When nociceptors are defined by the expression of the intermediate filament protein, peripherin, we observe co-expressed AMPA receptor subunits as indicated by immuno-staining with antibody to GluR1 and GluR2/3. The staining patterns are consistent with at least 2 different types of AMPA receptors in different subpopulations of nociceptors: an AMPA receptor with high Ca^{2+} permeability and one with low Ca^{2+} permeability.

We have recently found that kainate receptors are expressed by different subpopulations of nociceptors when the nociceptors are defined by carbohydrate surface markers. These markers, named LD2, SSEA4, and LA4, were originally studied by Dodd and Jessell (1985) and they identify subpopulations of nociceptors that have different patterns of termination in the superficial dorsal horn. Using DRG from newborn rats, our preliminary data suggest that 90% of the LD2 positive neurons are responsive to kainate receptor activation

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observed by Ca^{2+} imaging. In contrast, our preliminary results show that none of the SSEA4 positive neurons express functional kainate receptors.

There may be multiple roles of kainate receptors in nociceptor function, including a developmental role. Functional kainate receptors are consistently expressed by nociceptors throughout postnatal development. However, the receptor undergoes a change from a Ca^{2+} permeable to a Ca^{2+} impermeable form. This change occurs over the first few days after birth. Consistent with the developmental timing of this switch in Ca^{2+} permeability, we have observed that Ca^{2+} permeable kainate receptors are functional on growth cones of nociceptors from newborn rats and thus may contribute to the fine tuning of synaptic connections between nociceptors and their dorsal horn target neurons.

There are indications that at least some of these ionotropic receptors, including the P2X and glutamate receptors, are expressed on or near the central terminals of nociceptors after synaptogenesis is complete (Liu et al., 1997; Gu and MacDermott, 1997). There is also evidence of ionotropic receptor expression on the peripheral terminals of nociceptors (Carlton and Coggeshall, 1995; Jackson et al., 1995; Cook et al., 1997). In addition, glutamate and P2X receptors on the peripheral terminals are up-regulated following injury or inflammation, with larger numbers of nociceptors showing evidence of receptors (Novakovic et al., 1999; Carlton and Coggeshall, 1999). Understanding the role of these ionotropic receptors in nociceptor function may be aided by pre-identification of nociceptor subpopulations. This will allow us to study the functional properties of receptors in characterized subpopulations of nociceptors under the divergent conditions of activation, injury and development.

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FUNDACION JUAN MARCH EX-LIBRIS

DESENSITIZATION OF THE AVERSIVE RESPONSE EVOKED BY REPETITIVE OCULAR APPLICATION OF CAPSAICIN IN NK1 RECEPTOR KNOCKOUT MICE.

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Application of a capsaicin solution onto the ocular surface, produces an immediate sensation of pain, evidenced in experimental animals by an aversive response that includes increasing of blinking frequency and scratching movements directed to the eye.

Painful sensation evoked by topical administration of capsaicin was evaluated quantitatively in adult OF-1 mice eyes, by counting during 1 minute the number of scratching movements directed to the eye, evoked by a 2.5 µL drop of a capsaicin solution onto the ocular surface. The eye was extensively washed with saline afterwards. EC50 was calculated from the corresponding dose-response curve (increasing concentrations from 1 nM to 3.3 mM). In a separated set of experiments, capsaicin at 333 µM (EC50 concentration) was applied five times with 20 min intervals.

Experiments were carried out in intact mice (WT) and in animals with a targeted deletion of the gene codifying for the NK1 receptor (KO) (de Felipe, C. et al., 1998). Response to repetitive capsaicin application was also evaluated in WT animals pre-treated with the NK1 antagonist RP67580.

Dose-response curves for capsaicin were sigmoidal, being the EC50s 85 µM (WT) and 54 µM (KO), and the maximum number of scratching movements 48 ± 2 (WT) and 19 ± 3 (KO). As in OF-1 mice, repetitive application of capsacin at EC50 concentration for five times did not induce a decrease of the number of scratching movements in WT and KO mice. Interestingly, when capsaicin was applied at a two-fold EC50, the response was progressively reduced in KO mice (19 ± 1 vs. 6 ± 1 scratching movements after the first and the last application of capsacin, respectively) but not in WT animals (31 ± 1 vs. 26 ± 1). This desensitizing effect was reproduced in WT animals pre-treated with topical RP67580 (27 ± 2 vs. 15 ± 3).

The present data suggest that substance P, acting through NK1 receptors, is necessary for the perception of moderate-severe noxious stimuli. SP could also prevent the desensitization of nociceptive nerve terminals, mainly polymodal nociceptors, after stimulation with capsaicin.

Supported by grants CICYT SAF99-0066-C02-01 and 02. A.A. is a Fellow of the Generalitat Valenciana, Spain.

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Integrity of pain control and ascending spinal pathways in the NK1 receptor gene knockout mouse

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Homologous recombination has been used to disrupt the NK1 receptor gene in mice. Experimental analysis suggested that activity within spinal pathways had been disrupted resulting in impaired descending inhibitory pain controls. 1) This project was therefore designed to investigate descending pain controls using the anatomo-functional technique of noxiously-evoked c-Fos expression. 2) In parallel, the integrity of the main ascending pathways originating in the lamina I neurones, and terminating in the thalamus and the parabrachial area have been investigated.

1) Mice of both genotypes were anaesthetised with halothane and stimulated using a 50°C waterbath, either on the hindpaw only (10"), or concurrently to a forepaw (40") stimulus starting 15" before and ending 15" after the hindpaw stimulation. Animals recovered from anaesthesia, and were perfused 2 hours later under terminal anaesthesia. Similar procedures were used with wild type mice injected i.v. either with the NK1 antagonist (RP67580), or its inactive enantiomer (RP68651). Immunohistochemical detection the c-Fos protein was performed on 50 µm thick sections of the lumbar cord, and analysed with light microscopy.

2) Iontophoretic injections of tetramethylrhodamine and biotinylated dextran amine (Molecular Probes) were made in the right parabrachial area and Po/VPM thalamus respectively. Cell counts of retrograde labelling were performed in the lumbar and cervical cord.

C-Fos immunoreactive neurones (Fos-ir) were observed mainly in the superficial laminae following hindpaw stimuli in mice of both genotypes. Conditioning stimulation of the fore paw led, in wild type mice, to a decrease of 44% in Fos-ir neurones in the laminae I-II of the lumbar cord. No such effect was observed in knockout mice. Similarly, in wild type mice, treatment with the NK1 antagonist blocked the descending inhibitory effects, while treatment with the inactive enantiomer to the antagonist produced the normal reduction in c-Fos expression.

We conclude that the NK1 receptor is essential for descending control of nociceptive processing within the spinal cord.

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Repeated treatment with NGF and RP 67580 in experimental diabetic rats:Reversal of reduced sciatic substance P levels without exacerbation of mechanical hyperalgesia

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There are decreased levels of substance P (SP) in sensory neurons in the experimental diabetic rat, which are reversed by nerve growth factor (NGF) treatment. NGF treatment may be a therapeutic strategy for diabetic neuropathy, but it may induce hyperalgesia, reflected as increased sensitivity to both thermal and mechanical noxious stimuli. The aim of this study was to evaluate the effect of repeated treatment with NGF on diabetic rat mechanical hyperalgesia. We also included treatment with a selective NK1 antagonist, RP 67580 to block NGF hyperalgesic effects mediated by SP systems. Responses to noxious mechanical stimulation and SP levels in sciatic nerves has been assessed following repeated administration of NGF and /or RP 67580, a Neurokinin 1 antagonist, to control and diabetic rats. Repeated administration of RP 67580 (1 mg/kg, i.p.; 3 times per week for 4 weeks) induced antinociception in control and a transient antinociceptive effect in diabetic rats. Repeated NGF treatment (0.2 mg/kg, s.c.) induced transient mechanical hyperalgesia, not reversed by RP 67580 cotreatment, in control rats, but did not alter mechanical hyperalgesia in diabetic rats. Acute administration of either NGF or RP 67580 did not alter mechanical thresholds in control or diabetic rats. SP levels in sciatic nerves were reduced by repeated administration of RP 67580 in controls, but unaltered by NGF treatment. In contrast, decreased SP levels in diabetic rats were reversed by repeated administration of NGF. Alterations in SP systems do not explain mechanical hyperalgesia in experimental diabetes. Interestingly, this study shows that repeated treatment with a low dose of NGF is effective in restoring reduced SP levels in diabetes, but did not exacerbate hyperalgesia of the rats, endorsing its possible use as a therapeutic agent in diabetic neuropathy.

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Nicotine's analgesic properties: analysis of its target through knockout mice

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Morphine remains the painkiller of choice, however, undesirable side effects (such as respiratory depression and its addictive properties) have given the impetus to consider alternative antinociceptive drugs, such as nicotinic agonists. Nicotine itself exhibits an antinociceptive effect[1] as the agonists, epibatidine (isolated from the skin of an Ecuadorian frog) [2] and ABT-594 (generated by Abbott Laboratories)[3] which have both been shown to be 100 times more potent than morphine in models of acute pain.

Nicotine exherts pharmacological effects by interacting with one or more of the subtypes of nicotinic acetylcholine receptors (nAChRs) present in the nervous system. At least ten neuronal nAChR subunits (α2-α9, β2-β4) have been identified in the vertebrate brain which associate into a variety of pentameric oligomers with discreet, yet overlapping, distribution patterns and different physiological and pharmacological properties. In this study, the mechanism of action of nicotine-elicited antinociception was investigated using α4 and β2 knockout mice[4]. Behavioural tests, as well as binding and electrophysiological experiments have been performed on these mice.

In conclusion, deletion of the α4 and of the β2 nAChR subunits, which together form a high affinity receptor for nicotine, reveals that this particular receptor is an important, though not exclusive, component of the nicotinic pain pathway. Pharmacological agents targeted to this receptor may prove to be therapeutically useful for analgesia.

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Rewarding Effects of Opiates are Absent in Mice Lacking the Receptor for Substance P_1

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Modulation of Substance P activity has been reported to offer a radical new approach to the management of depression, anxiety and stress. The substance P receptor is highly expressed in areas of the brain that have been implicated in these behaviours, but also in other areas such as the nucleus accumbens which mediate the motivational properties of both natural rewards such as food and of drugs of abuse such as opiates . We have examined the rewarding properties of morphine in mice with a genetic disruption of the substance P receptor. Using the place preference test we found a loss of the rewarding properties of morphine in substance P receptor knockout mice. The loss was specific to morphine as both groups of mice responded when cocaine or food were used as rewards. The physical response to opiate withdrawal was also reduced in substance P receptor knockout mice as was the locomotor response to morphine, a putative characteristic of addictive drugs. We conclude that substance P plays an important and specific role in mediating the motivational aspects of opiates and may represent a major new pharmacological route for the control of drug abuse.

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EXPRESSION OF SODIUM CHANNEL SNS/PN3 AND ANKYRIN$_G$ mRNAS IN THE TRIGEMINAL GANGLION AFTER INFERIOR ALVEOLAR NERVE INJURY IN THE RAT.

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The inferior alveolar nerve is a sensory branch of the trigeminal nerve that is frequently damaged, and such nerve injuries can give rise to persistent paraesthesia and dysaesthesia. The mechanisms behind neuropathic pain following nerve injury is poorly understod. However, remodeling of voltage-gated sodium channels in the neuronal membrane has been proposed as one possible mechanism behind injury-induced ectopic hyperexcitability. The TTX-resistant sodium channel SNS/PN3 has been implicated in the development of neuropathic pain after spinal nerve injury. We here study the effect of chronic axotomy of the inferior alveolar nerve on the expression of SNS/PN3 mRNA in trigeminal sensory neurons. The organization of sodium channels in the neuronal membrane is maintained by binding to ankyrin, which help link the sodium channel to the membrane skeleton. Ankyrin$_G$, which co-localizes with sodium channels in the initial segments and nodes of Ranvier, and is necessary for normal neuronal sodium channel function, could be essential in the reorganization of the axonal membrane after nerve injury. For this reason, we here study the expression of ankyrin$_G$ in the trigeminal ganglion and the localization of ankyrin$_G$ protein in the inferior alveolar nerve after injury. We show that SNS/PN3 mRNA is down-regulated in small-sized trigeminal ganglion neurons following inferior alveolar nerve injury but that, in contrast to the persistent loss of SNS/PN3 mRNA seen in dorsal root ganglion neurons following sciatic nerve injury, the levels of SNS/PN3 mRNA appear to normalize within a few weeks. We further show that the expression of ankyrin$_G$ mRNA also is downregulated after nerve lesion, and that these changes persist for at least 13 weeks. This decrease in the ankyrin$_G$ mRNA expression could play a role in the reorganization of sodium channels within the damaged nerve. The changes in the levels of SNS/PN3 mRNA in the trigeminal ganglion, which follow the time course for hyperexcitability of trigeminal ganglion neurons after inferior alveolar nerve injury, may contribute to the inappropriate firing associated with sensory dysfunction in the orofacial region.

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The inhibitory effect of 5-HT on C-fibre mediated reflexes is mediated by a 5-HT receptor positively coupled to adenylate cyclase: an *in vitro* study.

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Serotonin (5-HT) plays an important role in the descending control of sensory transmission through the spinal cord and has been shown to depress the early (monosynaptic) and late (polysynaptic) components of the dorsal root -ventral root reflex in the spinal cord of young rats *in vitro* (Hedo & Lopez-Garcia, 1999 a). The aim of this study was to elucidate whether the 5-HT receptor that mediates the depression of the C fibre mediated reflexes was positively or negatively coupled to adenylate cyclase (AC).

Spinal cords were dissected from urethane anaesthetised (2g kg^{-1} i.p.) rats pups, hemisected and superfused with standard oxygenated ACSF. Responses to electrical stimulation of the L5-L6 dorsal root were recorded from the ventral root via suction electrodes and a DC amplifier. 5-HT and AC modulators were superfused at known concentrations and full recovery was allowed after drug applications. The stimulation consisted of a train of twenty high intensity stimuli (200 μs x 300 μA) at 1Hz before, during and after drug application. The responses were quantified in terms of integrated area and rise rate to trains of stimuli. Data are presented as mean percentages of control response (± S.E.M).

5-HT (1-50 μM), superfused for 7 min, had a concentration dependent inhibitory effect on the area of the response (EC_{50} ≈34 μM) whereas the rise rate was only marginally reduced at concentrations up to 10 μM and a significant reduction of this parameter was only achieved with 50 μM 5-HT (28.2 ± 10.6 %).

Forskolin (1-50 μM), an AC activating agent, had a concentration dependent inhibitory effect on the area and the rise-rate of the response to trains of stimuli. The time course of this effect was slow (30-40 min). Forskolin 5 μM reduced the integrated area and the rise-rate to 48 ± 3.2 % and to 41 ± 1.5 % of control values. Forskolin 5 μM superfused for 30 min occluded the depressant effect of 5-HT (10 μM). 2'3'-dideoxyadenosine (100 μM, 30-40 min), an irreversible AC inhibitor, had no significant effect on the integrated area and on the rise rate of the response but by itself but attenuated forskolin- and 5-HT-induced depressions.

These data suggest an important contribution of 5-HT receptors positively coupled to AC to the inhibition of spinal C-fibre mediated reflexes and together with previous pharmacological data (Hedo & Lopez-Garcia, 1999b) suggest that the receptor largely responsible for the effects of serotonin is the 5-HT_6 receptor.

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Supported by the Ministry of Education, Spain (Grant CICYT SAF 97-0104 to Dr. Cervero)

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GDNF CAN PREVENT THE UPREGULATION OF GALANIN IN A MODEL OF PERIPHERAL NEUROPATHY.

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Damage to a peripheral nerve is known to induce an increase in the expression of the neuropeptide galanin. The functional significance of galanin's altered expression as a result of nerve injury remains unknown. Several factors have been implicated as positive signals which can induce galanin expression in primary sensory neurons including the cytokines interleukin-6 (IL-6) and leukemia inhibitory factor (LIF). Conversely, it has also been shown that molecules belonging to the neurotrophin family such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) can inhibit galanin upregulation resulting from nerve injury, both *in vivo* and *in vitro*.

In this study we have examined the effects on the expression of galanin following unilateral injury of the L5 spinal nerve in rats treated intrathecally with another neurotrophic factor, glial cell derived neurotrophic factor (GDNF). GDNF was able to prevent the full expression of galanin following transection of the L5 spinal nerve. There was nearly a 50% reduction in the percentage of cells expressing galanin following L5 transection in GDNF treated rats compared to saline controls. Galanin expression was also limited to small cell bodies in GDNF treated animals compared to saline treatment where galanin immunoreactivity was also found in medium and large primary sensory neurons.

These results indicate that GDNF along with other neurotrophins such as NGF can regulate the expression of neuropeptides following nerve injury and thus may prevent some of the injury induced alterations in primary sensory neuron phenotype and excitability.

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Spinal amplification of low threshold inputs following inflammation: an in vitro study.

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We have shown that the induction of peripheral inflammation in rat pups causes a decrease in thermal and mechanical thresholds and that the spinal reflexes studied in the isolated cord from these animals are significantly larger than those seen in control animals but only when sufficiently long periods of inflammation are allowed (Hedo et al 1999). For this study we have focused on changes of threshold of the dorsal root-ventral root reflex (DR-VRR) which appear *in vitro* when using animals pretreated with carrageenan.

Electrophysiological experiments were performed on the isolated spinal cord of rat pups which had received an intraplantar injection of carrageenan 3 h (n = 21) or 20 h (n = 21) prior to the extraction or no pretreatment (n = 50). The spinal cords were dissected under urethane anaesthesia, hemisected and kept *in vitro* following standard procedures (see REF). The L5 dorsal root and the corresponding ventral root were placed in tight-fitting suction electrodes. Responses to electrical stimulation of the dorsal root were recorded from the ventral root via a DC coupled amplifier. The electrical threshold (T) for the DR-VRR was determined using a 20 μs pulse. Then trains of 20 stimuli at 1 Hz were delivered using two different intensities within the low threshold range (2T and 50 μs per 50 μA). The preparations were then assigned to other pharmacological experiments. In addition, the sciatic nerve trunk attached to dorsal root ganglia and the corresponding dorsal roots was extracted from control (n = 3) and 3 h pre-treated animals (n = 3) and kept *in vitro* under the same experimental conditions. The electrical threshold for the A-fibre volley was determined using stimulating and recording suction electrodes placed at each end of the nerve.

The electrical thresholds found in cords from carrageenan-injected animals were significantly smaller than those found in control animals (control:60 ± 4.2 μA; pre-treated 3 h: 36 ± 3.7 μA; pre-treated 20 h: 42.2 ± 4.7 μA). The integrated area of the response to trains of stimuli at 2T intensity was very similar for control and 3 h pre-injected animals and significantly greater for 20 h pre-injected animals (control:2.6 ± 0.3 mV.s; pre-treated 3 h: 2.7 ± 0.3 mV.s; pre-treated 20 h: 5.5 ± 0.4 mV.s). The integrated area of the response to trains of 50 μs per 50 μA increased gradually (control:3.2 ± 0.3 mV.s; pre-treated 3 h: 5.7 ± 0.5 mV.s; pre-treated 20 h: 7.2 ± 1.2 mV.s). The electrical threshold for the A-fibre nerve volley was very similar for both control and carrageenan groups.

We conclude that after peripheral inflammation two different amplification systems for low threshold inputs appear in the spinal cord which are not due to changes in the properties of primary afferent fibres. The first is a linear system which reflects a lowering of the threshold and manifests as an increased response to an fixed stimulus intensity. This amplification can be detected in vitro as soon as 3 h after induction of inflammation. The second system is non-linear and appears unrelated to changes in threshold. This manifests as an increased response to 2T stimulus intensity and is detected only 20 h after inflammation.

Hedo, G., Laird, J.M.A., y Lopez-Garcia J.A. (1999) Neuroscience 92, 309-318

Supported by the Ministry of Education, Spain (Grant CICYT SAF 97-0104 to Dr. Cervero)

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Intrathecal NGF and GDNF and the release of substance P from the spinal cord following sciatic nerve transection

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The neuropeptide substance P (SP) is synthetized in the cell bodies of a sub-population of small diameter sensory neurones (Aδ and C) in the dorsal root ganglia (DRG). The central axons of these neurones terminate in the superficial laminae of the spinal cord where SP is released and contributes to the transmission of nociceptive signals.
Following lesions of sensory neurones, SP is down-regulated in the small fibres (Hökfelt et al., 1994) and *de novo* expressed by the large diameter myelinated fibres (Aβ) (Noguchi et al., 1995) which can contribute to the establishment of abnormal neuropathic pain sensation by releasing SP (Malcangio et al., in press).

The aim of this study was to evaluate whether 2-week-treatment with intrathecal neurotrophins induced any change in the release of SP from the spinal cord 2 weeks following sciatic nerve transection. SP release was assayed with an *in vitro* dorsal roots-attached spinal cord preparation, in which the roots were stimulated at A or C fibre strength, and SP levels were measured by radioimmunoassay (Malcangio et al., 1997).

Spinal cord slices obtained from sciatic nerve transecetd-rats intrathecally-injected with saline (controls, n=5) did not show any *de novo* release of SP following A fibre stimulation. However, they did release significantly less SP then sham-rat cords (n=3) following stimulation of the dorsal roots at C-fibre strength. Intrathecal delivery of nerve growth factor (NGF, 12 μg/day for 2 weeks, n=10), whilst not changing the lack of SP release after A-fibre stimulation, did induce a significant recovery of C-fibre-evoked release of SP. In contrast, two-week intrathecal delivery of glial cell-derived neurotrophic factor (GDNF, 12 μg/day, n=10), induced *de novo* release of SP from A fibres but did not modify the reduced SP release from C fibres.

These data show that distal lesions to peripheral nerve did not induce *de novo* release of SP from A fibres but did cause a significant reduction in SP release from C fibres.
NGF treatment enhanced the reduced release of SP from C fibres suggesting that this neurotrophin does not only up-regulate SP in normal rats (Malcangio et al., 1997) but brings toward normal values reduced SP levels in fibres expressing the trkA receptor. Interestingly, GDNF induced *de novo* release of SP from A fibres without changing the reduced release from C fibres. Accordingly, cells expressing GDNF receptor components do not normally contain SP but large cells start expressing GDNF receptor after axotomy (Bennett et al., in press).
Central delivery of neurotrophic factors selectively targets different populations of sensory neurones that can release SP.

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MODELS OF VISCERAL PAIN AND HYPERALGESIA IN MICE

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The generation of transgenic mice which lack or overexpress genes relevant to pain is becoming increasing common. However, only one visceral pain model, the writhing test, is widely used in mice. Here we describe a range of models of visceral pain and hyperalgesia that we have established in our laboratory, including models described in rats which we have adapted to mice (cyclophosphamide cystitis, colon distension) and a novel model which we have developed for use in mice in our laboratory (chemical stimulation of colon).

Cyclophosphamide (CP) cystitis (1,2). The toxic metabolites of systemically administered CP are excreted in the urine, and induce bladder inflammation, spontaneous pain-related behaviour and referred hyperalgesia. CP (300 mg/kg i.p) during 4 hrs video observation evoked a 53% reduction in locomotor activity, 22 ± 6 min of "crises" of visceral pain-related behaviour and significant plasma extravasation in the bladder, but not other abdominal tissues (Evan's Blue method). The pain behaviour was correlated with bladder inflammation.

Distension of normal or inflamed colon (3,4): Mice were anaesthetized with sodium pentobarbital and vital parameters monitored and maintained. A 3.5 cm long balloon connected to a pressure transducer and a reservoir was inserted in the colon via the anus. Colon distensions (30s, 5-75 mmHg) evoked cardiovascular reflex responses proportional to stimulus intensity. Instillation of 0.6% acetic acid into the colon resulted in significantly greater responses to distension (primary hyperalgesia), and significant colon plasma extravasation.

Chemical stimulation of colon: Mice were placed on a raised grid and 50 µl of saline, mustard oil (0.25–2.5 %) or capsaicin (0.03-1%) was administered using a fine cannula inserted via the anus, and visceral pain-related behaviors counted. Before intracolonic administration, and 20 min after, the frequency of withdrawal responses to the application of von Frey probes (1, 4, 8, 16, 32 mN) to the abdomen was tested. Mustard oil and capsaicin but not saline evoked dose-dependent visceral pain behaviors, referred hyperalgesia (significant increases in responses to von Frey hairs) and colon plasma extravasation.

We conclude that these models represent a useful battery of tests for phenotyping mutant mice with regard to visceral primary and secondary hyperalgesia and visceral pain.

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Supported by: CICYT (to Prof. F. Cervero), Comunidad Autónoma de Madrid, DGICYT, FIS(99/9335), Instituto UPSA del Dolor and Europharma S.A., Spain.

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ACUTE ANTINOCICEPTION IS OBSERVED AFTER NON-SELECTIVE BLOCKADE OF CICLOOXIGENASE ACTIVITY BUT NOT AFTER SELECTIVE BLOCKADE OF COX-2 ACTIVITY.

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Aim of investigation: To investigate and compare the analgesic potency of a selective COX-2 inhibitor, the Rofecoxib, and two non-selective COX inhibitors: the S(+)-ketoprofen trometamol (Dexketoprofen) and the S(+)-flurbiprofen trometamol in rat hind limb withdrawal reflexes.

Methods: Single Motor Units (SMU) were recorded in male Wistar rats under α-Chloralose anaesthesia. The responses elicited by noxious mechanical stimulation of the hind toes were pooled an used for analysis. All drugs were injected i.v. in cumulative doses in normal rats and in rats with carrageenan induced monoarthritis.

Results: Rofecoxib did not have any significant effect on the SMU responses in normal animals. Flurbiprofen induced a depression of SMU activity only significant with the highest dose used (2620 nmol/kg). Dexketoprofen produced a dose-dependent inhibition of the responses which was significant from doses of 100 nmol/kg. A complete inhibition was observed with 1600 nmol/kg, the ID50 being 100 nmol/kg. In monoarthritic animals both Dexketoprofen and Flurbiprofen induced a dose-dependent inhibition of the responses with ID50s of 415 and 706 nmol/kg respectively. Rofecoxib did not have any effect on the depression of SMU activity.

Conclussions: Dexketoprofen trometamol was the only NSAID effective in the reduction of withdrawal reflexes in normal animals. In animals with monoarthritis both Dexketoprofen and Flurbiprofen, but not Rofecoxib, were potent antinociceptive agents. We conclude that inhibition of COX-1 but not COX-2 alone is needed to inhibit nociceptive responses in acute experiments.

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ARGININE-RICH PEPTIDES ARE BLOCKERS OF VR-1 CHANNELS THAT SHOW ANALGESIC ACTIVITY

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Vanilloid receptors (VR1) play a fundamental role in the transduction of painful responses resulting from peripheral tissue injury and /or inflammation. Thus molecules that modulate VR1 channel activity may act as selective and potent analgesics. We have addressed this issue and used the cloned VR1 channel as a molecular target to identify and characterize novel receptor antagonists. We report that arginine-rich hexapeptides block homomeric VR1 channel expressed in *Xenopus* oocytes with submicromolar efficacy. VR1 blockade was voltage-dependent inhibiting capsaicin-activated currents exclusively at negative membrane potentials. Naturally occurring arginine rich peptides such as dynorphins blocked VR1 channel activity with micromolar affinity. Notably, arginine-rich peptides attenuated the ocular irritation produced by topical application of capsaicin to mice. This analgesic activity correlated well with the efficacy blocking VR1 channels expressed in *Xenopus* oocytes. Taken together, our results show that VR1 channel blockers such as arginine-rich peptides display significant analgesic activity. These findings may expand the development of novel analgesics by targeting receptor sites distinct from the capsaicin binding site.

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AN 'IN VITRO' STUDY ON THE RESPONSIVENESS OF REGENERATING NERVE ENDINGS IN EXPERIMENTAL NEUROMAS: MECHANICAL SENSITIZATION AFTER CHEMICAL STIMULATION.

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The purpose of the present investigation was to explore the characteristics of the response to mechanical and chemical stimuli of regenerating nerve endings in an 'in vitro' saphenous nerve neuroma model.

Neuromas of rat saphenous nerve were developed in 5mm long silicone tubes The nerve was cut just distal to the ligature and the cut end placed inside a 5mm-silicone tube; 1-40 weeks old neuromas were obtained, placed in a perfusion chamber with oxygenated physiological salt solution at 35°C (pH 7.4). The proximal stump of the nerve was located in a separated compartment filled with mineral oil. Monopolar recording of electrical activity was performed from thin nerve strands. Conduction velocity, spontaneous activity and responsiveness to mechanical (von Frey hairs), or chemical stimuli (acidic solutions, pH 6.5, 6.0 and 5.5 and inflammatory soup, IS) were studied.

Forty-three units conducting in the A-δ or C range were studied. Twenty-five of them (58%) responded to mechanical and chemical stimuli and were classified as polymodal units and 18 (42%) were classified as pure chemosensory units. Conduction velocity measurements performed in 16 units showed that about two thirds were C-fibres (mean= 0.8±0.3m/s, range: 0.4-1.1m/s, n=11) and the rest were A-δ fibres (mean= 3.5±0.9m/s, range: 2.6-4.4m/s, n=5). In addition, 16 of the polymodal (61%) and 4 of the pure chemosensory units (23%) developed spontaneous activity at the beginning of the experiment. Mechanical threshold varied from 5 to 40 mN (n= 25). Either polymodal or pure chemical units responded similarly to different acidic concentrations (pH 6.5, 6.0,5.5) and to IS. The firing rate during the stimulus increased and the latency of the first evoked impulse discharge decreased with the lower pH values of the test solutions. The impulse response evoked by IS did not diminished by repetition of stimulus. A significant reduction of mechanical threshold was observed in polymodal units following their exposure of the neuroma to an acidic solution (pH 6.0, n= 10, $p \leq 0.05$) and to IS (n=10, $p \leq 0.01$). Furthermore, nine of the pure chemosensory units developed sensitivity to mechanical stimulation after chemical activation (pH and IS). The possibility that neuroma endings became sensitised by endogenous chemicals could explain the generation of pain sensations by innocuous mechanical stimulation of neuromas.

The model of the isolated superfused neuroma *'in vitro'* may be useful to study the physiological and pathophysiological responses of regenerating nociceptive nerve endings and their modulation by drugs under controlled experimental conditions.

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The cell adhesion molecules axonin-1 and F11 affect the axonal pathfinding of different subpopulations of sensory afferents in the spinal cord in vivo

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In order to study the molecular mechanisms of axon pathfinding, we have established in vivo approaches to test the role of individual candidate guidance cues in the complex environment of the developing vertebrate nervous system. Due to its easy accessibility we have chosen the embryonic chicken as a model system. Based on our expertise and knowledge gained in in vivo studies of commissural axon pathfinding, we have established techniques to study the pathfinding of sensory afferents of the spinal cord.

The sensory neurons are divided into different subpopulations and establish connections within specific laminae of the gray matter depending on their sensory modality. We have injected function-blocking antibodies against cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily into the central canal of the spinal cord in ovo. In order to visualize axonal trajectories after perturbation of CAM/CAM interactions, we have used FastDil injected into the DRGs in transverse sections of the lumbosacral level of the spinal cord. Using this approach, we could show that perturbations of axonin-1 and F11 interactions result in subpopulation-specific pathfinding errors. Whereas Ia afferents depend on F11 interactions, the perturbation of axonin-1 interactions affects pathfinding of nociceptive fibers.

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TRANSPLANTATION OF OLFACTORY BULB ENSHEATHING GLIA PROMOTES INGROWTH OF NOCICEPTIVE AFFERENTS AND REDUCES AUTOTOMY AFTER BRACHIAL PLEXUS DORSAL RHIZOTOMY. **Taylor, J.S., Muñetón, V.C., and Nieto-Sampedro, M.** Dpto. Plasticidad Neural, Instituto Cajal, CSIC, Madrid.

Self-mutilation following dorsal rhizotomy in rats has been proposed as a model of deafferentation pain associated with brachial plexus lesions in humans. However, the possibility also exists that autotomy is an attempt to remove an appendage that the animal sees as foreign. Recent evidence for functional neuroregeneration of the central branch of sectioned dorsal roots (Navarro et al., 1999; Taylor et al., 1999) indicates that transplantation of olfactory bulb ensheathing cells (OBECs) into either the dorsal root entry zone (DREZ) or the dorsal horn, promotes the ingrowth of nociceptive afferent fibres. Here we report that restoration of nociceptive afferent input to second order neurons after OBEC transplantation correlates with the termination of autotomy.

Dorsal roots C4 to T2 were rhizotomized unilterally. Roots C7/C8 were reapposed to the DREZ using fibrin glue and OBECs were injected into the ipsilateral dorsal horn at the C7/C8 level. The remaining brachial plexus dorsal roots (C4, C5, C6, T1 and T2) were resected. Behavioural and electrophysiological correlates of afferent ingrowth and reinnervation by C7/C8 dorsal roots was monitored using Hargreave's plantar test and biceps EMG recordings in response to median nerve stimulation. Immunohistochemical analysis of regenerated C7/C8 afferents was detected using antibodies against CGRP and cholera toxin. Activity in second order dorsal horn neurons after thermal activation of regenerated afferent was identified by *c-fos* expression in the superficial laminae.

These results suggest that afferent fibres regenerated into the spinal cord using OBEC transplants limit the onset of autotomy in rats, in parallel with the restitution of specific nociceptive sensory input after brachial plexus dorsal rhizotomy. The mechanism by which OBECs modulates autotomy will need to be addressed.

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Work financed by European Community Contract BMH4-97-2586 (Biomed II).

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*14 **Workshop on Frontiers of Alzheimer Disease.**
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*15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
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16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organizers: E. Donnall Thomas and A. Grañena.

*17 **Workshop on Cell Recognition During Neuronal Development.**
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18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
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*22 **Workshop on Molecular Bases of Ion Channel Function.**
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*24 **Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.**
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*34 **Workshop on Computational Approaches in the Analysis and Engineering of Proteins.**
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35 **Workshop on Signal Transduction Pathways Essential for Yeast Morphogenesis and Cell Integrity.**
Organizers: M. Snyder and C. Nombela.

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39 **Workshop on Molecular Basis for Biodegradation of Pollutants.**
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*40 **Workshop on Nuclear Oncogenes and Transcription Factors in Hematopoietic Cells.**
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*41 **Workshop on Three-Dimensional Structure of Biological Macromolecules.**
Organizers: T. L Blundell, M. Martínez-Ripoll, M. Rico and J. M. Mato.

42 **Workshop on Structure, Function and Controls in Microbial Division.**
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*43 **Workshop on Molecular Biology and Pathophysiology of Nitric Oxide.**
Organizers: S. Lamas and T. Michel.

*44 **Workshop on Selective Gene Activation by Cell Type Specific Transcription Factors.**
Organizers: M. Karin, R. Di Lauro, P. Santisteban and J. L. Castrillo.

45 **Workshop on NK Cell Receptors and Recognition of the Major Histocompatibility Complex Antigens.**
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46 **Workshop on Molecular Mechanisms Involved in Epithelial Cell Differentiation.**
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64 **Workshop on Novel Biocatalysts.**
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76 **Workshop on Initiation of Replication in Prokaryotic Extrachromosomal Elements.**
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87 **Workshop on Novel Approaches to Study Plant Growth Factors.**
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88 **Workshop on Structure and Mechanisms of Ion Channels.**
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89 **Workshop on Protein Folding.**
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91 **Workshop on Eukaryotic Antibiotic Peptides.**
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98 **Workshop on Telomeres and Telomerase: Cancer, Aging and Genetic Instability.**
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99 **Workshop on Specificity in Ras and Rho-Mediated Signalling Events.**
Organizers: J. L. Bos, J. C. Lacal and A. Hall.

100 **Workshop on the Interface Between Transcription and DNA Repair, Recombination and Chromatin Remodelling.**
Organizers: A. Aguilera and J. H. J. Hoeijmakers.

101 **Workshop on Dynamics of the Plant Extracellular Matrix.**
Organizers: K. Roberts and P. Vera.

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103 **Workshop on the Neural Mechanisms of Addiction.**
Organizers: R. C. Malenka, E. J. Nestler and F. Rodríguez de Fonseca.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 28th of February through the 1st of March, 2000, at the Instituto Juan March.

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