Signal transduction in neuropathic pain, with special emphasis on the analgesic role of opioids – Part I: The basic science of phenotype expression in normal and regenerating nerves

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Neuronal injury evokes programmes of survival or apoptosis that have a natural history of at least 500 million years. In Part I of this trilogy, I present an in-depth synthesis of an hypothesis (which I call the ‘failsafe theory’) that emphasizes the absolute priority of these programmes. If survival/regeneration is initiated then it is essential that there is no distortion or interruption in signalling fidelity between the environment and cytoplasmic and nuclear processes of phenotype expression. It is now evident that many agents, notably the opioids, used to manage neuropathic pains, signal along pathways that converge with those of growth factors that regulate gene expression and promote survival. Through such convergence, some analgesics may compromise processes of regeneration. Consequently, such intervention may be opposed by systems of indescribable complexity that have evolved to minimize such threats and maximize survival. With a presentation of basic science in Part I, new options for pharmacotherapy are pursued in Part II and developed further in the final part of this trilogy; Part III is devoted to an appraisal of existing clinical entities and the opportunity for new therapies.

Pharmacological intervention is no match for an evolutilional need to ensure adequacy of neurotransmission

Introduction

The above heading can be less elegantly, but possibly more succinctly, restated as: ‘The need for survival outweighs the need for pain relief.’ Teleologically, even if survival is the ultimate goal, as part of this process we can assume that pain has evolved as an important and necessary signal. However, when pain ceases to provide any obvious useful function, why are some chronic pains, especially those associated with neuronal dysfunction (i.e. neuropathic/neurogenic pains), frequently so difficult to eradicate? The first part of this three-part article explores the notion that pharmacological intervention, aimed at eliminating abnormal sensations such as hyperalgesia or paraesthesia arising as a direct result of nerve
injury, activates adaptive responses that ensure adequacy of neurotransmission, regardless of whether such transmission ultimately evokes normal or abnormal sensations. Thus, by their nature, such adaptive responses will act to oppose and surmount any drug-induced intervention designed to diminish pain through attenuation of signal conduction. A corollary of this hypothesis is that even the most sophisticated novel pharmacological entities, when used to block the pain signal, represent substrates for evoking a repertoire of failsafe mechanisms that have evolved throughout a history of challenge and response. Moreover, as I argue later, activation of these responses may explain why treatment of neuropathic pains, particularly with opioids, can be so frustrating.

Modern medicine in perspective
Although the origin of life on earth probably took place about 10 000 million years ago, the earliest known fossils have been found in rocks from the Proterozoic era, which occurred over 7000 million years later. The vertebrates do not make their appearance in the fossil record until some 2500 million years later, in the Ordovician era. That is, our vertebrate lineage is approximately 400–500 million years old and our evolutionary heritage of cellular processes is probably older by about a further order of magnitude. The development of modern medicine dates back to Hippocrates (i.e. approximately 2500 years ago). If we use the life span of a human of 100 years as representing the time that has elapsed since the origin of life on earth, then modern medicine is only 13 minutes old! Using this perspective, we have only known of the existence of the cell (credited to Robert Hooke in 1665) for about one and a half minutes! In this way, if we assume that the earliest vertebrates (400–500 million years ago) experienced pain, then, relative to the life span of a modern human, we have been treating and studying pain for only about four and a half hours (early records indicate that Hippocrates used salicylate-containing extracts of the weeping willow tree to treat painful disorders, but of course Eastern medical records may predate such therapy), and we have known about the N-methyl-D-aspartate (NMDA) receptor for only two minutes. When viewed in this way, should we be so surprised that all of today’s commonly-used pain therapies came about by serendipity (e.g. opiates, valproic acid, paracetamol), or on the basis of false assumptions (e.g. tricyclic antidepressants) and mistaken beliefs (e.g. salicylates). With this relative time scale, it becomes easier to accept why so little progress has been made in attempts to diminish the pain associated with neuronal dysfunction (i.e. neuropathic/neurogenic pain). Modern medicine is faced with the formidable challenge of treating ‘malfunctions’ that occur in systems that have adapted successfully to environmental pressures encountered throughout 500 million years of evolution. Viewed in this way, in summary, neuronal injury represents a threat to signalling fidelity within a system of indescribable complexity such that, regardless of any intervention, the pain message will get through to the brain. On accepting such inevitability, the prospects seem bleak for research aimed simply at developing blockers of putative mediator synthesis, ion channel function, receptor activity, etc., which are involved in the transmission of neuropathic pain.

Opioids and neuropathic pain
The effectiveness of opioids in the management of clinical neuropathic pain is a controversial issue. Whereas it is widely assumed that neuropathic pain is only slightly responsive to opioid treatment, there is some evidence that opioids can produce a satisfactory outcome after dose escalation, to an endpoint determined by adequate relief of symptoms or the appearance of unacceptable side-effects (e.g. Portenoy et al.2). It is interesting that, in reviewing the role of opioids in the management of neuropathic pain, Benedetti et al.3 emphasize an important point concerning the use of opioids in managing acute postoperative pain. That is, it is the general view that pain arising from deep and superficial tissues, as a direct result of the surgical insult and subsequent inflammation, is highly sensitive to opioids. However, it should not be forgotten3 that 25% of postsurgical patients demonstrate relative insensitivity to the analgesic effect of opioids.4 Given that neuropathic pain frequently evolves into a distressing condition that erodes normal social and family interactions (see Genova-Strickland5 for a patient’s view), it seems strange
that, despite such clinical pressure, we have so little clarity on the role of opioids. Possible reasons for this situation include:

- There is a limited understanding of the relationship between neuronal injury and the subsequent reduction in opioid sensitivity of the ensuing pain, in comparison with that of inflammatory pain. For example, notwithstanding that a loss of presynaptic opioid receptors is commonly assumed, a recent investigation showed that, although expression of the mu-opioid receptor within the rat dorsal horn of the spine is significantly reduced at 14 days after segmented nerve section, expression returns to normal by 31 days after transection. Moreover, it is worth emphasizing that neuronal injury also results in long-lasting decreases in expression of other receptors such as the NMDA receptor; NMDA receptor antagonists, despite unacceptable toxicity, are generally remarkably effective in alleviating many neuropathic pains.

- Neuropathic pain is not a homogeneous condition. Sensory symptoms that are seen in neuropathic pain syndromes (spontaneous burning pain, cold-evoked allodynia, touch-evoked allodynia, lancinating pain, cramp-like pain, hyperalgesia, hyperpathia, etc.) may each be produced by a separate neuropathic mechanism. These separate mechanisms may coexist. The same symptom may be produced by different neuropathic mechanisms in different patients. Different neuropathic mechanisms may simultaneously produce the same symptom in a single patient.

- We have a limited understanding of how opioids effect analgesia in standard models of phasic (e.g. tail-flick) and tonic (e.g. formalin) pain, and we have virtually no understanding of their effects in models of nerve injury (e.g. chronic constriction injury). Additionally, we have practically no understanding of differences in mechanisms that exist between different opioid agonists of a similar subtype (e.g. mu-prefering agonists).

- In the current climate of novel entities (e.g. voltage-sensitive calcium channel (VSCC) antagonists such as SNX-111), financial incentives to pursue clinical studies with opioids are probably lacking.

What of the putative effectors for opioids? Does opioid analgesia mediated at such targets represent a threat to regeneration in the injured nerve? That is, in this case, do opioids evoke a response that is paradoxically ‘anti-opioid’? If yes, then why is this? Do opioids compromise successful regeneration?

In clinical practice, opioids are classified broadly as either full or partial agonists with principal activity at either µ-, δ- or κ-receptor subtypes, with experimental evidence for further division of the classification through effects at various isoforms (e.g. µ, µ), or at complexes of receptor subtypes (e.g. µ/δ). At clinical dosages, presynaptic actions of opioid agonists on transmitter release result from an opening of potassium channels and/or a closing of calcium channels, both of which lead to a reduction in \(\text{Ca}^{2+}\) influx into C-fibre terminals. In vivo, in addition to the direct effects of an opioid agonist on VSCC currents, it should not be forgotten that indirect mechanisms such as presynaptic hyperpolarization as a result of activation of \(\text{K}^{+}\) conductances, or direct mechanisms involving inhibition of exocytosis (e.g. by attenuation of phosphorylation of synapsin I), are also likely to play a role in the modulation of presynaptic release of neurotransmitter.

Presynaptically, a reduction of the voltage-sensitive calcium N current can be anticipated to result in diminished neurotransmitter release. However, after nerve injury, the use of a drug to block directly VSCCs may result in the activation of failsafe mechanisms that operate to oppose the blockade. Indeed, in contrast with normal neuronal function, it seems that nerve injury may be associated with, and characterized by, resistance to exogenous modulation of synaptic transmission. That is, in the treatment of neuropathic pain, to what extent does the rightward shift in the opioid analgesic dose–response curve represent ‘anti-opioid’ effects, which arise as a direct consequence of nerve injury? Such anti-opioid effects would be distinct from the phenomenon of tolerance attributable, for example, to an uncoupling of transducer proteins (i.e. G proteins), with receptor desensitization and internalization. In Part I, I review the nature of these
putative failsafe mechanisms, which, as I discuss next, are probably evoked after antagonism of calcium channel activity by an opioid.

Opioids work by blocking VSCCs and the nervous system works to unblock VSCCs blocked by opioids

**Opioid receptors and G proteins**

When an opioid, such as morphine, for example, binds to an opioid receptor, the conformational change induced in the receptor activates what is known collectively as a guanosine triphosphate (GTP)-binding protein (G protein) heterotrimer. It is the G protein that is responsible for carrying the analgesic signal (and probably other signals associated with opioids, which include adverse effects). G protein heterotrimers reside with the receptor in the vicinity of the nerve membrane, and consist of three units, α, β and γ (hence its description as a heterotrimer). The α subunit (Gα) can exist separately, but the β and γ units can only be separated chemically. Under physiological conditions, the β and γ units can only separate from the αβγ heterotrimer as the dimer, βγ (Gβγ). Traditionally, it has always been assumed that it is the α subunit that delivers the opioid-induced analgesia signal, and that the Gβγ functions as ‘ballast’ in order to keep Gα in an inactive state within the confines of the heterotrimer while awaiting activation by the opioid.

**Opioids, G proteins and VSCCs**

Endogenous modulation of the N-type VSCC is exceedingly complex and current understanding of these convergent processes is, at best, scant. During the 1990s there have been several published reports that direct interaction of Gβγ (i.e. the G protein βγ dimer) with the N-type VSCC is responsible for channel inhibition.9-12 Naturally, these observations have been proposed as a molecular mechanism by which presynaptic G protein-coupled receptors (GPCRs), such as those that bind morphine, for example, inhibit neurotransmission9,10 in addition to other mechanisms, which include activation of an inwardly rectifying K+ conductance.

Numerous reports indicate that the Go subclass of G proteins mediates inhibitory coupling of opioid receptors to neuronal VSCCs, especially the N type,13-34 and that such inhibition is probably mediated by direct interaction of the Gβγ subunit with the VSCC.9-12,35-40

**Signal integration by the VSCC**

The recent report of protein kinase C (PKC)-dependent upregulation of VSCC activity through antagonism of the Gβγ inhibitory action has been proposed as an additional mechanism for regulation and dynamic control of neurotransmitter release and synaptic efficacy.35 While G protein-dependent inhibition occurs through binding of Gβγ to the VSCC, ‘cross-talk’ resulting from the PKC-dependent phosphorylation of one of the Gβγ binding sites effectively antagonizes Gβγ-induced inhibition. In some part, this antagonistic effect of PKC might possibly explain the ability of the stimulated presynaptic cholecystokinin B-receptor subtype (CCKB) to exert anti-opioid effects; conversely, such regulation would also explain why, in paradigms of neuropathic pain, the administration of CCKB receptor antagonists restores opioid sensitivity (Figure 1). Stimulation of the CCKB receptor with subsequent activation of the G protein, Gq, results in an increase in activity of phospholipase C (PLC) isoforms, with resultant 1,2-diacylglycerol (DAG)-mediated activation of PKC (Figure 1).

The model in Figure 1 represents a gross simplification of the interaction by the VSCC of multiple (in this case two) presynaptic signals; however, in the normal intact neurone, this model is consistent with earlier observations by Wang et al.41 Using enzymatically-dissociated brain cells from newborn rats, these workers observed that potassium chloride produced a significant increase in intracellular calcium ion concentration ([Ca2+]i) and that this increase could be prevented by administering antagonists of VSCCs. While sulphated and desulphated forms of cholecystokinin octapeptide (CCK-8) did not affect the increase of [Ca2+]i after high potassium ion-induced membrane depolarization, they did reverse the suppression of a high potassium-induced increase in [Ca2+]i by the µ-agonist ohmefentanyl. Although the effects of CCK-8 on mobilization of intracellular Ca2+ or opioid

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binding affinity cannot be discounted, the recent demonstration\(^{42}\) that the anti-opioid effects of CCK\(_B\) receptor stimulation are abolished by the PKC-specific inhibitor, calphostin, accord with the model (Figure 1). Moreover, elegant investigations of CCK\(_B\) receptor signal transduction mechanisms emphasize the dominant role occupied by DAG formation (and subsequent PKC activation and translocation).\(^{43}\) In these studies it was demonstrated that, whereas CCK\(_B\) receptor stimulation results in rapid but transient elevations of inositol trisphosphates (InsP\(_3\)), and subsequent intracellular Ca\(^{2+}\) mobilization, DAG levels demonstrate a more pronounced biphasic response. Additionally, there is evidence to suggest that, after stimulation by CCK-8 for example, that PKC activation inhibits both InsP\(_3\) formation and the subsequent induction of cytosolic Ca\(^{2+}\) oscillations.\(^{44}\)

**Summary**

In summary, what inferences can be made? Endogenously, there exists an abundant source of G\(\beta\gamma\), and VSCCs represent tightly-coupled effectors for a variety of endogenous agonists of GPCRs. In physiological terms, Figure 1 represents a simple system of control and regulation. However, pharmacologically and teleologically we can also interpret Figure 1 to imply that blockade of a VSCC by G\(\beta\gamma\) can be surmounted. If this model is accepted, then under what circumstances would activity or expression of this ‘anti-G\(\beta\gamma\)/anti-opioid’ system be upregulated?

**Cross-talk between Gq (e.g. CCK\(_B\)) and Gi/o (e.g. opioid) is upregulated following neuronal injury**

Neuronal injury is associated with a very marked increase in the number of sensory neurones of all sizes that express CCK\(_B\) receptor mRNA,\(^{45}\) together with a dramatic increase in the number of sensory neurones in dorsal root ganglia that synthesize CCK.\(^{46}\) It is important that, in the normal dorsal root ganglia in the rat, mRNA for the CCK\(_B\) receptor is present at very low levels. Thus, the injury-associated increase described

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**Figure 1** At the VSCC, cross-talk results from the PKC-dependent phosphorylation of one of the G\(\beta\gamma\) binding sites. This phosphorylation antagonizes the G\(\beta\gamma\)-induced inhibition. The model is based on the recent observations\(^{35}\) that membrane-delimited inhibition of VSCCs (P/Q- and N-type) occurs through a direct binding of G\(\beta\gamma\) to the VSCC \(\alpha_1\) subunit domain I–II linker and that this interaction is directly affected by the PKC-dependent phosphorylation. The model is additionally based upon the assumption that PLC-activated PKC has access to VSCC-bound G\(\beta\gamma\).

PIP\(_2\): phosphatidylinositol-4,5-bisphosphonate.
above suggests an increased sensitivity to CCK for many primary sensory neurones of different modalities.45 A crushing injury to sensory nerves either has no effect47 or results in an increase48 in levels of mRNA for isoforms of PLC that are activated by Gq. Taken together, these findings indicate an upregulation of the pathway CCK_B → Gq → PLC → PKC (Figure 1), which suggests an increase in PKC-mediated antagonism of Gβγ-induced inhibition of VSCCs, in particular the N type.10,35

The following observations provide additional support for the above suggestion:

- In cultured dorsal root ganglion cells, activation of PKC partially reverses prior inhibition of VSCC current after G-protein activation with the nonhydrolysable GTP analogue, GTPγS49,50 (Gα•GTP is the active moiety after subunit dissociation).
- Inhibition of the Ca²⁺/calmodulin-regulated protein phosphatase, calcineurin, greatly attenuates the pertussis toxin (PTX)-sensitive G protein-coupled receptor-induced inhibition of the N-type VSCC current in rat sympathetic neurones51 (Gi is a PTX-sensitive G protein). There is now considerable speculation that, because PKC activation, in common with calcineurin inhibition, markedly attenuates GPCR-induced inhibition of the N-type channel activity, then PKC is likely to be the kinase that phosphorylates the calcineurin-regulated site, and that, consequently, PKC is indeed an important modulator of the N-type VSCC.51
- Immunophilins, protein receptors for immunosuppressant drugs such as cyclosporin A and FK506, are enriched far more in the brain than in the immune system. Indeed, levels within nervous tissue apparently exceed those in immune tissues manyfold. Drug–immunophilin complexes bind to calcineurin, inhibiting its phosphatase activity and leading to immunosuppressant effects. It is noteworthy that facial nerve crush markedly augments the expression of mRNA for the immunophilin, FKB-12, with a time course paralleling changes in mRNA for the growth-associated protein, GAP-43.52 After sciatic nerve lesions, similar increases in FKBP-12 mRNA occur in lumbar motor neurones and dorsal root ganglia neuronal cells.
- The expression of PKC isoforms is upregulated after neuronal injury.53–56 A functional link between PKC and VSCCs in the spinal cord has been proposed,57 and protein kinases and calcineurin are suggested to be functionally linked by association with common anchoring proteins.58 High densities of immunophilins co-localize with calcineurin in neural tissue.

These changes are consistent with the notion that injury-induced increases in levels of the calcineurin inhibitor group of compounds, the immunophilins, accord with the antagonism by PKC of Gβγ-mediated inhibition of VSCC activity. That is, it has been demonstrated that activating PKC, similar to inhibiting calcineurin activity, greatly attenuates Gβγ-induced inhibition of N-type Ca²⁺ channels.51

Taken together, all of these findings support the existence of the functional unit, VSCC/PKC/calcineurin/immunophilin. Finally, an increased expression of immunophilins augments depolarization-induced transmitter release from rat brain striatal synaptosomes. Synapsin I, a synaptic vesicle phosphoprotein, displays enhanced phosphorylation in the presence of immunophilin-stimulating drugs (e.g. FK506).59 It is worth emphasizing here that μ-, δ- and κ-opioid agonists attenuate the depolarization-induced phosphorylation of synapsin I60 and that this action has reasonably been interpreted as representing a further mechanism whereby opioid receptor agonists inhibit neurotransmitter release.61

Summary

There is ample evidence to suggest that, after nerve injury, cross-talk between Gq (e.g. CCK receptor-mediated) and Gi (e.g. opioid receptor-mediated) signalling is upregulated. This increase in activity effectively attenuates the GPCR-mediated modulation of at least the N-type VSCC and may in some part, explain the rightward shift in the analgesic dose–response curve for opioids in the management of neuropathic pain.
Depolarization-induced reversal of GPCR-mediated inhibition of Ca\(^{2+}\) currents

Voltage-dependent inhibition of high-voltage-activated Ca\(^{2+}\) currents by G\(\beta\gamma\) can be relieved transiently (an event synonymous with ‘facilitation’ of current) by a depolarizing ‘prepulse’ (i.e. a pulse of depolarization applied immediately before the test pulse). However, until recently it remained to be established whether this phenomenon was merely an experimental artefact, or if it could be induced under physiological conditions, by action potentials.

Using patch-clamp methods, Womack and McCleskey\(^26\) demonstrated that brief prepulses to very positive voltages increased (facilitated) the amplitude of current through Ca\(^{2+}\) channels during a subsequent test pulse in some, but not all, dorsal root ganglion sensory neurones. The amplitude of this facilitated current generally increased when the Ca\(^{2+}\) channels were inhibited by activation of the µ-opioid receptor. The facilitated current was blocked by the N-type VSCC blocker (within the picomolar range), \(\omega\)-conotoxin GVIA. It was activated in the range of high-threshold Ca\(^{2+}\) channels, and was inactivated at relatively negative holding voltages. Thus, facilitated current passes through N-type VSCCs in dorsal root ganglion cells for hundreds of milliseconds. It is interesting that they further demonstrated that, after inhibition of N-type VSCCs by activation of the µ-opioid receptor, facilitating depolarizations were generally more effective. Therefore, as they concluded, the facilitating prepulses diminish opioid inhibition of these channels. Such facilitation may be explained by the voltage-dependent release of G proteins (i.e. G\(\beta\gamma\)) bound to the channel, with the facilitation decaying as G\(\beta\gamma\) subunits rebind. In comparison with other neurones, facilitation (i.e. disinhibition of opioid inhibition) persists so much longer in dorsal root ganglion neurones; in some way this is due to a kind of ‘wind-up’ during trains of action potentials.\(^26\)

Can spontaneous ectopic discharges in injured nerves antagonize opioid-mediated analgesia?

As discussed above, Womack and McCleskey\(^26\) demonstrated that positive depolarizations can enhance current passing through N-type VSCCs in dorsal root ganglion cells for hundreds of milliseconds. It is interesting that they further demonstrated that, after inhibition of N-type VSCCs by activation of the µ-opioid receptor, facilitating depolarizations were generally more effective. Therefore, as they concluded, the facilitating prepulses diminish opioid inhibition of these channels. Such facilitation may be explained by the voltage-dependent release of G proteins (i.e. G\(\beta\gamma\)) bound to the channel, with the facilitation decaying as G\(\beta\gamma\) subunits rebind. In comparison with other neurones, facilitation (i.e. disinhibition of opioid inhibition) persists so much longer in dorsal root ganglion neurones; in some way this is due to a kind of ‘wind-up’ during trains of action potentials.\(^26\)

The ‘neuroma model’ of neuropathic pain\(^63\) has yielded critically important information about pathogenic mechanisms in damaged primary afferent neurones. For example, animal studies with this model suggested, and clinical studies in
humans with transected nerves confirmed, that spontaneous and evoked activity in primary afferent sprouts was a significant source of abnormal pain sensations. Animal studies have also shown that, not only the sprout of the damaged afferent neurone, but also its cell body in the dorsal root ganglion, was a source of ectopic discharge.

Studies in patients with painful peripheral neuropathies have led to the hypothesis that ongoing discharge in primary afferent nociceptors has two harmful effects: it is a source of ongoing ‘spontaneous’ pain, and it dynamically maintains a state of central hyperexcitability that underlies evoked-pain abnormalities like hyperalgesia and allodynia. Temporary suppression of nociceptor discharge by a local anaesthetic nerve block allows the central hyperexcitable state to normalize; hyperalgesia and allodynia are then absent or reduced, sometimes for many hours after the anaesthetic block has dissipated. However, according to the hypothesis, the pain, hyperalgesia and allodynia return because the resumption of nociceptor discharge rekindles the central hyperexcitable state. If the hypothesis is correct, then long-term suppression of spontaneous nociceptor discharge ought to be of therapeutic benefit. There is evidence that VSCCs, in particular the N type, play a role in the genesis of spontaneous ectopic discharges and the abnormal pains that occur after nerve injury. However, to my knowledge it has not previously been considered that continuous spontaneous ectopic discharges, while originating in some part from VSCCs within injured primary afferents, may also lessen the effectiveness of opioids to induce blockade of N-type VSCC. Although this additional formulation remains to be formally tested, it is worth reporting that clinicians have long noted that a local anaesthetic nerve block can give a patient days or weeks of relief. This result is very difficult to understand in terms of the known duration of the pharmacological effect and it is sometimes suspected to be a supernormal placebo effect, or presumptive evidence that the patient is neurotic or malingering. It is therefore of interest that rats with a chronic constriction injury likewise demonstrate prolonged relief (as measured by changes in the latencies of nocifensive withdrawal reflexes to painful stimulation) after application of a local anaesthetic block. I speculate that temporary abolition of the spontaneous ectopic discharges, in addition to resulting in a diminution of dynamically-maintained central hyperexcitability, may also produce an apparent increase in endogenous GPCR modulation of N-type VSCCs. Together these two mechanisms may nonlinearly augment (i.e. synergy) the effect of a local anaesthetic nerve block on hyperalgesia and allodynia of neuronal injury.

**An intracellular pool of VSCCs replenishes blocked VSCCs**

An intracellular pool of N-type VSCCs has been described in different neuronal cell lines (for review see Passafaro et al. 74). It is interesting that these intracellular channels can be recruited to the cell surface, over a time-scale of hours, if the cells are exposed to blockade by the snail toxin, ω-conotoxin GVIA. However, it remains to be determined whether blockade by SNX-111 or opioids also represents a similar stimulus for the translocation of intracellular VSCCs. It is also interesting to note that repetitive depolarization of cultured rat hippocampal neurones also results in an increase in VSCCs at the plasma membrane, presumably after recruitment of additional VSCCs from the intracellular pool. Whether this latter phenomenon is a paradigm of the neuroma-derived depolarizations (i.e. ectopic impulses) within the injured nerve and at subsequent stages of neuropathic pain is also thought-provoking, and likewise remains to be studied.

**More on injury, lipids and the VSCC**

Receptor-mediated stimulation of PLC isoforms results in the degradation of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to give two biologically active hydrolysis products, inositol 1,4,5-trisphosphate, which mobilizes Ca$^{2+}$ from sequestered intracellular stores, and DAG, which, as I have already discussed (Figure 1), activates PKC and promotes its translocation to the plasma membrane. However, in addition to the above generation of DAG by phosphoinositide-specific PLC isoforms, it is apparent that the PLC-catalysed degradation of other glycerophospholipids, especially phosphatidylcholine, can take place after activation of a variety of receptors. In recent years, evidence has accumulated that phos-
phatidylcholine can also undergo agonist-induced breakdown via the action of phospholipase D (PLD) in a wide variety of tissues and cells to yield phosphatidic acid (PA) and choline. PA can also serve as a precursor of DAG via the action of PA phosphatase.

**Phosphatidylcholine and nerve injury**

The presence of PLD in the nervous system has been documented for brain extracts, synaptosomal preparations, transformed and primary cultured cells of nervous system origin, and, more recently within intact peripheral nerve. In crushed sciatic nerve, incubated with labelled substrates, synthesis of phosphatidylcholine is significantly elevated by comparison with that in intact normal nerve. Such de novo synthesis of phosphatidylcholine occurs rapidly, with maximum synthesis occurring three days after crush injury, at which time the metabolism of other phospholipids remains unchanged. Later (within two weeks) there follows rapid axonal transport of labelled phosphatidylcholine and other glycerophospholipids, including phosphatidylethanolamine together with marked incorporation along the entire length of the proximal portion, including the ganglion and spinal terminations.

On the basis of the above data, I propose the straightforward speculation that, within the regenerating injured nerve, the considerably upregulated synthesis and transport of phosphatidylcholine, in providing increased amounts of substrate for the activity of PLD, results in an increase in levels of DAG and a subsequent increase in activity and translocation of PKC. Indeed, this notion is entirely consistent with the observation that full activation of PLD requires PKC (the activity of which is markedly upregulated following nerve injury) and a GTP-binding protein. With regard to the latter requirement, there is support for the coupling within nerves of opioid receptors to PLD. Overall, in the injured/regenerating nerve, antagonism by activated Gq (i.e. Figure 1: CCK$_B$ → Gqα → DAG → PKC↑) of the Gβγ-induced attenuation of VSCC activity may be enhanced by further increases in PKC activity and translocation mediated by coupling of the opioid-activated receptor to PLD activity (PLD → phosphatidylcholine → DAG → PKC↑). It is interesting to note that maximal activation of PLD occurs only under conditions that are permissive to PLC stimulation. Moreover, it should be noted that activation of PLD is Ca$^{2+}$-dependent. That is, PLD enhancement of PLC antagonism of Gβγ-mediated attenuation of VSCC currents will result in an increase in Ca$^{2+}$ current, which will further drive the process (Figure 2).

**Is the nicotinic acetylcholine receptor an ‘anti-opioid’ receptor?**

**Activation of nicotinic acetylcholine receptors augments VSCC-mediated exocytosis**

The Ca$^{2+}$ permeability of nicotinic acetylcholine receptors (nAChRs) has recently been investigated by several groups. Decker and Dani demonstrated that the nAChR has a high Ca$^{2+}$ permeability, and they predicted that, under physiological conditions, activation of the nAChR could provide a significant source of Ca$^{2+}$ influx. Vernino and coworkers observed that neuronal nAChRs have a sevenfold higher permeability to Ca$^{2+}$ than those in muscle, and that neuronal nAChR currents are enhanced in a dose-dependent manner by an increase in external Ca$^{2+}$ concentrations.

Mulle and coworkers, using rat medial habenular neurones, demonstrated that Ca$^{2+}$ entry through neuronal nAChRs was of the same order of magnitude as Ca$^{2+}$ entry through VSCCs. In accord with this observation, the results of more recent investigations suggest that an influx of Ca$^{2+}$ after activation of nAChRs may enhance synaptic transmission. Pursuing these observations, Harkins and Fox demonstrated that activation of nAChRs markedly (two to three times) augmented VSCC-mediated exocytosis in rat phaeochromocytoma (PC12) cells. Their results suggest that the ligand (nicotine)-activated nAChR exerted its effect primarily through Ca$^{2+}$ influx via the nAChR and that Ca$^{2+}$ release from cytoplasmic stores plays little or no role.

From this very recent study by Harkins and
Fox\textsuperscript{106} two points are worth emphasizing. First, even after removal of agonist, augmentation of VSCC-mediated exocytosis is likely to persist as a direct result of the slow deactivation of the nAChR (however, this may not be a general feature of all nAChRs). Secondly, at depolarized potentials (+20 mV) equivalent to the physiological activation of VSCCs, activation of nAChRs elicited little secretion, but significantly ($p < 0.005$) augmented the secretion elicited by the activation of Ca\textsuperscript{2+} channels. This latter observation, together with the presynaptic autoreceptor role of the nAChR, which is discussed below, indicates a likely physiological role of the nAChR in modulating (augmenting) VSCC currents.

**nAChR is a presynaptic facilitatory autoreceptor and heteroreceptor**

Recent evidence suggests that the majority of nAChRs within the central nervous system (CNS) are located presynaptically and subserve the function of enhancing the release of acetylcholine (ACh) (i.e. they act as facilitatory autoreceptors) and other neurotransmitters, which include noradrenaline, dopamine and glutamate (i.e. they act as facilitatory heteroreceptors) (for review see Langer\textsuperscript{107}). This demonstrated role of the nAChR as a facilitatory auto/heteroreceptor is entirely consistent with the facilitation of VSCC-mediated exocytosis by the activated nAChR, with a likely role in spinal nociceptive processing.
nAChR and spinal nociceptive processing

Participation of the nAChR in spinal nociceptive processing is strongly suggested by recent direct evidence that many neuronal nAChRs are associated with capsaicin-sensitive peptidergic neurones, including primary afferents, dorsal root ganglia and central nociceptive pathways. Moreover, capsaicin applied to the spinal dorsal horn of rats facilitates ACh release within the dorsal horn, which is inhibited by dorsal rhizotomy (a well-established method of depleting peptides within primary sensory afferents) and the administration of anti-substance P (sP). In addition, significant evidence exists to propose a physiological role for modulation of nAChR responsiveness by sP. In several systems it has been demonstrated that nAChR responses are attenuated by sP (for review see Min et al.). Recently, Min and coworkers confirmed that sP binds to the nAChR. Binding of sP to a high-affinity site is observed only in the presence of a cholinergic agonist, and the rate of association is much more rapid when agonist is added simultaneously with the peptide than when the receptor is pre-equilibrated with agonist before addition. These workers propose that it is likely that there is only one high-affinity site per receptor, and that this site may be within the ion channel pore, such that it is only accessible when the receptor is in the open state. Taken together, these results suggest feedback control by sP of the activity of the nAChR as a facilitatory autoreceptor.

nAChR and nerve injury

Cholinergic agonist binding sites in the spinal cord seem less sensitive to axonal damage than antagonist binding sites. Thus, a functional role of the nAChR in facilitating neurotransmitter release in a dysfunctional nervous system (i.e. after nerve injury) should be considered. It is interesting to speculate that, in some part, the preservation of the functional nAChR after neuronal injury may result in a rightward shift of the dose–response curve for opioid-induced analgesia/antinociception. This effect would be consonant with an apparent increase in facilitation of neurotransmitter release by the nAChR as a direct result of a decrease in the number of presynaptic opioid receptors subsequent to nerve injury; they may be reduced by up to 70%. On this basis, then, it may be anticipated that, at least at the level of the spinal dorsal horn, the use of a selective nAChR antagonist may nonlinearly augment the analgesic efficacy of an opioid in managing neuropathic pain.

Cross-talk between cholinergic (nAChR) and glutamatergic (NMDA) systems

Under physiological conditions, intracellular Mg$^{2+}$ may play a key role in the control of α-bungarotoxin (α-BGT)-sensitive nAChR activity in the CNS. Thus, normal physiological levels of intracellular Mg$^{2+}$ may be sufficient to block activation of α-BGT-sensitive nAChRs at depolarized potentials. Modulation by Mg$^{2+}$ may, however, not be a general feature of all nAChR CNS subtypes. For example, the native α4β2 CNS nAChR is not a likely candidate for control by Mg$^{2+}$, whereas the activity of α7-bearing CNS nAChRs would be regulated in this way.

In attempting to evaluate the physiological roles of nAChRs, Albuquerque and coworkers have proposed an elegant model of cross-talk between the glutamatergic and cholinergic neurotransmitter systems. It is important that new data strongly argue that therapies directed at the presynaptic NMDA receptor could ameliorate injury-evoked persistent pain states. In the light of these new data, the model of Albuquerque et al. provides an insight into those mechanisms that comprise a positive feedback network that facilitates and prolongs the transmission of nociceptive messages. Given that both the NMDA receptor and the nAChR are expressed on the surface of the terminals of small-diameter primary afferent fibres, it has been suggested that activation of these nAChRs modulates NMDA receptor function and activation of the NMDA receptors modulates nAChR function. At membrane potentials close to that of the resting membrane, the NMDA receptor will demonstrate little or no activity upon binding of glutamate, which is a result of the voltage-dependent blockade by extracellular Mg$^{2+}$. In contrast, the α-BGT-sensitive neuronal nAChR would be fully operational upon binding of ACh.

The activation of NMDA receptors in normally-functioning intact neurones can result in a
substantial increase in the intracellular concentrations of free Mg$^{2+}$ ([Mg$^{2+}$]i). On this basis, Albuquerque and coworkers reason that, when the NMDA receptor is fully operational after relief of the Mg$^{2+}$ blockade at depolarized membrane potentials, the α-BGT-sensitive nAChR would be inoperative because, at positive potentials, physiological levels of free Mg$^{2+}$ are likely to be adequate to cause inactivation of these nAChRs. However, activation of NMDA receptors and the concomitant rise in intracellular free Mg$^{2+}$ will ensure that these nAChRs remain inoperative at depolarized membrane potentials. Since both the NMDA receptor and the nAChR are highly permeable to Ca$^{2+}$, Albuquerque and coworkers propose that this cross-talk may represent a means by which rapid rises in intracellular Ca$^{2+}$ concentrations mediated by these receptors can be tightly controlled, thereby avoiding overloading of intracellular Ca$^{2+}$. However, regardless of the physiological significance of this proposed cross-talk, the model has significant implications when the nerve has been injured, with a subsequent increase in excitability of dorsal horn neurones of the spinal cord (i.e. central sensitization).

**Presynaptic nAChRs enhance glutamatergic synaptic transmission**

Using neurones of the medial habenular nucleus and the interpeduncular nucleus, McGehee and coworkers tested the notion that the localization of nAChRs to presynaptic terminals provides a mechanism whereby the activation of only a few nAChRs could alter excitability. They observed that nanomolar concentrations of nicotine enhanced glutamatergic synaptic transmission by activation of presynaptic nAChRs that increased presynaptic [Ca$^{2+}$]i. Pharmacological and subunit deletion experiments revealed that these presynaptic nAChRs include the α7 subunit. On the basis of their results, these workers concluded that presynaptic nAChRs can alter excitability through enhancing fast excitatory transmission, and that such modulation requires the activation of only a few nAChRs. These results were confirmed by Khan and coworkers in normally-behaving conscious rats. Using a dialysis tubing placed proximal to the intrathecal injection site, they observed that the nicotinic agonists, nicotine, cytisine and epibatidine, elicited dose-dependent increases in the spinal release of aspartate and glutamate.

More recently, Puttfarcken and coworkers investigated the role of several cholinergic channel activators (ChCAs) to effect nAChR-gated ion flux and modulate the release of sP in F11 cells, a dorsal root ganglia cell line with many characteristics of nociceptive neurones. They demonstrated that the prototypical agonists (−)nicotine and (−)cytisine, the novel ChCA, ABT-418 [(S)-3-methyl-5-(-1-methyl-2-pyrrolidinyl isoxazole] and (±)epibatidine evoked a concentration-dependent stimulation of $^{86}$Rb+ efflux. In addition, concentrations of (±)epibatidine, similar to those necessary to induce maximal $^{86}$Rb+ efflux, evoked spontaneous release of sP, which was blocked by the noncompetitive nAChR antagonist mecamylamine. Prolonged exposure to (±)epibatidine desensitized the functional response of the nAChR in this cell line. From these results, Puttfarcken and coworkers speculate that the release of sP after primary afferent stimulation correlates with the pain response elicited by the intrathecal administration of nicotinic agonists.

Finally, the recently reported antinociceptive actions of ChCAs may be explained in part by the ability of ChCAs to deplete stores of nociceptive transmitters, such as sP (although beyond the scope of this discussion, the antinociceptive actions of ChCAs may also derive from effects upon peripheral nAChRs and an activation of brainstem descending pain inhibitory systems).

**Do spinal presynaptic nAChRs occupy a special role in neuropathic pains?**

**nAChR – a wolf in sheep’s clothing?**

In the early 1990s, the role of the nAChR in somatosensory processing was little understood. With new studies, the emerging role of the nAChR within the dorsal horn as an auto/heteroreceptor and modulator of VSCC activity suggests that it occupies a pivotal position in establishing and maintaining the regenerating neuronal phenotype subsequent to injury. That
is, our new understanding of the role of the nAChR in regulating neurotransmitter release possibly positions this receptor as a candidate for therapeutic intervention in the management of neuropathic pain.

Studies with peripheral nerves demonstrate that injury by chemical neurotoxicant exposure, mechanical trauma, nerve transection, ischaemia or anoxia, is ubiquitously associated with a marked decrease in [Mg^{2+}]i.132 Similarly, both focal traumatic and diffuse axonal brain injury are also associated with a rapid decline in [Mg^{2+}]i.133–138 Moreover, in the injured nerve, facilitation of nociceptive messages by activated presynaptic NMDA receptors115 will be likely to amplify, enhance and sustain the injury-evoked decline in Mg^{2+}.139 Indeed, after experimental traumatic brain injury, the neuroprotective effects of NMDA antagonists are associated with the attenuation of [Mg^{2+}]i decline.139–140 In the light of the above injury-associated changes in neuronal [Mg^{2+}]i, and further to my earlier discussion within this article on cross-talk between neuronal glutamatergic (NMDA) receptors and cholinergic pathways (nAChR), a priori the low [Mg^{2+}]i at depolarized potentials in a spontaneously discharging injured primary afferent is consonant with both the NMDA receptor and the nAChR becoming fully active.113–114

Additional injury-associated changes will also further increase the probability for the open-channel configuration of both these receptors, notably the nAChR.141 The simultaneous activation of both cholinergic and glutamatergic pathways, resulting in a massive Ca^{2+} influx, will be associated with a subsequent increase in the release of arachidonic acid and lysophospholipids.142–143 It has recently been demonstrated that lysophospholipids produce long-lasting enhancement of nAChR currents.141,144 Although part of the mechanism for this effect involves a PTX-insensitive G protein-mediated activation of Ca^{2+}-dependent/-independent PKCs, with subsequent phosphorylation of the nAChR in general, phosphorylation of the nAChR by several routes is likely to represent an important means for modulating nAChR activity.113,145–150 Long-term changes of regeneration, neurite growth and synaptic remodelling are likely to involve tyrosine phosphorylation by protein tyrosine kinases (PTKs) of a subset of neuronal nAChRs.151 Whether phosphorylation of the nAChR renders the ion channel refractory to blockade by Mg^{2+} is a matter that requires investigation. If this was the case, then even if [Mg^{2+}]i were restored to normal in the regenerating neurone, maladaptive functions of the unregulated nAChR may, as I propose, represent some basis for the prolonged transmission of nociceptive messages in states of neuronal dysfunction. Indeed, on the basis of studies carried out in hippocampal cells in epileptic rats,152 it has been proposed that, at least for the NMDA receptor, the Mg^{2+} ion-dependent voltage-gating in rats with a chronic constriction injury of the sciatic nerve may be in a chronically deficient condition owing to decreased Mg^{2+} ‘binding affinity’ of the receptor-gated channel.153

Finally, the role of adenosine triphosphate (ATP) as a cotransmitter in activation of the nAChR113,154 may assume significance within the regenerating neurone; 32P-labelling of different nucleotides of the dorsal root ganglia of regenerating rat sciatic nerve after crush injury reflect an enhanced metabolism of nucleotides, including adenosine diphosphate (ADP) and ATP.155

**Opioids as noncompetitive nAChR agonists**

Paradoxically, the potency of an opioid to block the VSCC may be attenuated by concomitant activity of the opioid as an allosteric potentiating ligand of the nAChR.113,156–160 Primary structure–function investigations of the noncompetitive nAChR agonist (NCAA) physostigmine, identified the Amaryllidaceae alkaloid, galanthamine as a compound with similar activity.157 Pursuing these early studies with molecular modelling approaches with galanthamine as the template, based on structural co-ordinates stored in the Cambridge Structural Databank, the same workers157 identified opioid alkaloids of the phenanthrene type, which includes morphine and codeine, as possible candidates as NCAAs. However, since the structural requirements of compounds identified as NCAAs include protonation of a nitrogen atom at physiological pH, which is adequately separated within the opioid structural framework from a moiety with the characteristics of the phenoxide ion, then it is
possible that many opioids, other than phenanthrenes, can function as NCAAs.\textsuperscript{158}

When tested on phaeochromocytoma (PC12) cells, codeine, as predicted, evoked single-channel currents whose main conductance was similar to those obtained with either physostigmine or galanthamine, and indistinguishable from those induced by ACh or ACh-competitive nicotinic agonists. Whereas ACh antagonists such as BGT completely abolished the single-channel activity induced by ACh, these compounds exerted no significant effect on the frequency of channels activated by codeine. In contrast, the monoclonal antibody FK1\textsuperscript{161,162} virtually abolished the frequency of single-channel activity induced by codeine, whereas the activity induced by ACh or ACh-competitive agonists was unaffected. From these results it was concluded that, in common with physostigmine and galanthamine, codeine and ACh acted at different sites within the nAChR.

Whether allosteric augmentation of nAChR ion-channel activity by opioids has any pharmacological significance, particularly in the management of neuropathic pains, is an important issue for which currently there is no clear answer. NCAA activity of an opioid may possibly contribute to the rightward shift of the analgesic dose–response curve, which is considered characteristic of opioids when used to treat neuropathic pains. However, all the compounds that have been shown to bind to the ‘physostigmine/galanthamine/codeine’ site within the nAChR also have open-channel blocking properties or desensitizing effects upon nAChRs, and the concentrations at which these compounds can block or inactivate the nAChR overlap those at which they act as agonists.\textsuperscript{159,163} If these reports of allosteric modulation by opioids of the nAChR are of therapeutic significance, then it is clearly important to determine (1) if differences in potency exist between opioids as noncompetitive agonists of the nAChR, and (2) the concentrations of opioid at which the opioid augments (i.e. noncompetitive agonist) or antagonizes nAChR activity.

Can opioids modulate neuronal phenotype?

Classical view of opioid signal transduction

The opioid signal transduction pathway employs heterotrimeric GTP-binding proteins (G proteins), which consist of $\alpha$, $\beta$, and $\gamma$-subunits. Activation by an opioid agonist of a seven-transmembrane helix receptor results in a conformational change that allows the receptor to act as a nucleotide-exchange factor for the G$\alpha$ subunit. Once guanosine diphosphate (GDP) has been replaced with GTP, the G$\alpha$ monomer and the G$\beta\gamma$ dimer separate from each other and from the receptor. The signalling moiety G$\alpha$•GTP then binds to and modulates the activity of downstream effector(s). Termination of the signal is associated with hydrolysis of GTP to GDP and the dissociation of G$\alpha$•GDP from the effector. The G$\alpha\beta\gamma$ heterotrimer reforms and reassociates with the receptor.

Challenging traditional dogma

The subunit dissociation model has led investigators to test the effects of G$\alpha$ and G$\beta\gamma$ individually. Whereas early reports placed emphasis upon G$\alpha$•GTP as the signalling moiety and G$\beta\gamma$ as ‘ballast’ to capture and recycle G$\alpha$•GDP, the role of G$\beta\gamma$ in transducing the opioid signal is becoming more frequently demonstrated. These new observations have given rise to the emerging concept that the response of a cell (e.g. neuron) to the opioid agonist–receptor complex can lead to the activation of divergent pathways within the cell by producing not one, but two, species capable of mediating signal transduction. Is this new view an accurate portrayal of what happens in the cell, or does the picture of divergent signalling reflect extraordinary experimental conditions? For example, many investigations of subunit dissociation are conducted in solutions of detergents under conditions that are not physiological, but which include the use of non-hydrolysable GTP analogues or AlF$_4^-$ and high concentrations of Mg$^{2+}$ to activate G proteins. Thus, the use of such ‘stringent’ conditions in order to demonstrate apparent divergent signalling by G$\alpha$•GTP and G$\beta\gamma$ begs the question of whether effectors that might respond to
Gα•GTP and/or Gβγ could also respond to an activated G protein heterotrimer (i.e. Gαβγ•GTP).

Currently, there is no clear consensus about whether effectors bind Ga•GTP and/or Gβγ as separate ‘dissociated’ entities or as moieties contained within the activated heterotrimer.\(^{164-171}\) A key feature of the traditional model of opioid-induced analgesia is that receptors (i.e. μ, δ or κ) do not come into direct contact with effectors (e.g. adenyl cyclase, ATP-sensitive K\(^+\) channels, G protein-activated K\(^+\) channels, VSCCs), but communicate via shuttling of dissociated G proteins. Although various findings indicate that G protein association and subsequent dissociation from both receptors and effectors can occur, the notion of shuttling is purely speculative. A growing body of evidence exists that is difficult to reconcile with the idea that receptors activate effectors indirectly. Alternative schemes would necessarily imply the existence of heterooligomeric complexes containing receptor (R), G proteins (G), and effector (E), at least at some instant during transduction. The idea that signal transduction involves R–G–E complexes is attractive in that it provides inherent specificity within receptor–effector coupling pathways. The prevailing paradigm falls short in this regard, because experiments with purified components have shown that multiple receptors can activate the same G protein, one receptor can activate different G proteins, different G proteins can activate the same effector, and one G protein can activate different effectors. In addition, Gβγ subunits appear to be largely interchangeable among Gα subunits as well as among effectors and receptors. Effectors governed by Gβγ would thus be expected to show sensitivity to most receptors; evidence for such ubiquity however is not forthcoming.\(^{164,166,172-175}\) Cells typically have multiple types of receptors, G proteins and effectors, and it is difficult to understand how specific receptor–effector communication would result from a myriad of promiscuous protein interactions.

Attempts to reconcile these apparent differences between purified proteins and whole cells have led to the proposal that G proteins shuttle, as either the heterotrimer or dissociated subunits, between receptors and effectors within restricted microdomains. Although the suggestion of a ‘restricted collision-coupling model’ was first described by Gross and Lohse,\(^{176}\) these observations, together with those of Rodbell,\(^{177}\) have been incorporated into a concept that proposes explicit organization of effectors, G proteins and probably effectors into supramolecular complexes.\(^{174,178}\)

In 1998, Ford et al.\(^{169}\) identified effector-interacting residues on Gβ for several effectors, including β-adrenergic receptor kinase (β-ARK), PLC-β2, potassium channels and calcium channels. It is interesting that Gα•GDP, when bound to Gβγ, covered these distinct yet partially overlapping effector interaction regions on Gβγ and, as a result, blocked Gβγ regulation of all the effectors studied. Ford et al.\(^{169}\) conclude that this organization of interaction regions on Gβ for different effectors and Gα explains why subunit dissociation is crucial for signal transmission through Gβγ subunits. Finally, additional evidence for the existence of a pool of free Gβγ within intact cells was recently provided by the elegant studies of Lin and coworkers.\(^{179}\)

Together, these new findings revitalize the case in support of a signalling role for Gβγ as a dissociated subunit.

**Opioids have access to neuronal phenotype via Gβγ**

The ubiquitous mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine kinases that are involved in the transduction of externally derived signals regulating cell growth, regeneration, division and differentiation. Upon activation, MAPKs translocate to the nucleus, where they phosphorylate and activate transcription factors for transcriptional regulation of several particular genes that are essential for the induction of survival (or apoptosis) and the subsequent regeneration process.\(^{180-182}\) A number of stimuli have been demonstrated to activate the MAPKs through seven-membrane receptors that are coupled to heterotrimeric G proteins, such as Gq and Gi (i.e. GPCRs).

The mechanisms by which GPCRs activate the MAPK signalling cascade are poorly understood. Early investigations using transiently transfected COS-7 cells demonstrated that the activation of receptors coupled to either Gs, Gq or Gi resulted in stimulation of the MAPK pathway.\(^{183}\) They
further demonstrated that, whereas $\beta_1$, $\beta_2$, $\gamma_1$ or $\gamma_2$ G protein subunits alone did not increase p44MAPK (44 kDa isoform of extracellular signal-regulated kinase (ERK)) activity, the $\beta_1\gamma_2$ combination and, to a lesser extent, $\beta_2\gamma_1$ and $\beta_2\gamma_2$ did increase the activity of pp44MAPK. Subsequently, using Chinese hamster ovary (CHO)-K1 cells, it was demonstrated that coexpression of G$\beta\gamma$ with the Ras guanine nucleotide exchange factor, son of sevenless (Sos)1, resulted in a synergistic increase in MAPK activation. Expression of either G$\beta\gamma$ or Sos alone resulted in a two to threefold or a fivefold increase in MAPK activation respectively, whereas coexpression of both G$\beta\gamma$ and Sos resulted in a 15–20-fold increase. These workers further demonstrated that, as in COS-7 cells, the ability of G$\beta\gamma$ to synergize with Sos1 in CHO-K1 cells was dependent upon the activity of the PH-domain-containing enzyme, phosphatidylinositol 3-kinase-gamma (PI-3K$\gamma$). Ito and coworkers, in the same year, also reported that in human embryonic kidney (HEK) 293 cells, overexpression of G$\beta\gamma$ induced the activation of Ras, c-Raf and MAPK. More recently, van Biesen and coworkers also reported the activation of MAPK by a novel PKC-dependent mechanism, which was PTX-sensitive, insensitive to the G$\beta\gamma$-sequestering carboxy-terminal fragment of the $\beta$-ARK1ct peptide, and Ras-independent. Since the inhibition of MAPK activity by PTX was specifically rescued by a PTX-insensitive mutant of G$\alpha$, in this study a role for G$\alpha$ was suggested.

G$\beta\gamma$ subunits derived from either PTX-sensitive or -insensitive heterotrimeric G proteins have been demonstrated to mediate Ras-dependent MAPK activation. In order to activate a MAPK cascade, G$\beta\gamma$ subunits appear to require the activity of a PTK because the PTK inhibitors genisten and herbimycin A attenuate G$\beta\gamma$-stimulated MAPK activation in a dose-dependent manner. It seems that G$\beta\gamma$ uses the same pathway to stimulate MAPK as several tyrosine kinase-dependent cell-surface receptors that lack intrinsic tyrosine kinase activity. These cell-surface receptors appear to recruit Src (also known as pp$^{\text{ src}}$ or c-Src) family kinases (for a review of Src kinases see Brown and Cooper and Rudd et al.) such as Lck, Fyn and c-Src. It has been proposed that G$\beta\gamma$ initiates tyrosine phosphorylation of the Src homology 2/alpha-collagen-related adapter protein, Shc, which is then able to assemble a multiprotein complex for regulating activity of the low molecular weight G protein, p21-ras. The tyrosine phosphorylation of Shc is accompanied by a simultaneous increase in complex formation between Shc and another adapter protein, Grb2, which is constitutively associated with Sos1. These early events serve to recruit and translocate the p21-ras guanine nucleotide exchange factor Sos1 to the cytoplasmic surface of the membrane. That is, translocated Sos1 activates p21-ras by catalysing GDP for GTP exchange. GTP-bound p21-ras initiates activation of the MAPK cascade by activating the serine/threonine kinase, Raf-1, followed by the sequential phosphorylation and activation of MAPK kinase (MAPKK), which in turn mediates the activation of MAPK (Figure 3).

In addition to the observation that G$\beta\gamma$-mediated Shc phosphorylation is sensitive to tyrosine kinase inhibitors, Touhara and coworkers observed sensitivity to the PI-3K inhibitor, wortmannin. These earlier results have been confirmed by the demonstration that overexpression of PI-3K$\gamma$ in COS-7 cells results in G$\beta\gamma$-dependent activation of MAPK and that expression of a catalytically inactive mutant of PI-3K$\gamma$ abolishes the response to stimulation by G$\beta\gamma$ or a GPCR. These workers concluded that PI-3K$\gamma$ mediates G$\beta\gamma$-dependent regulation of the MAPK pathway. It is interesting that, in contrast with PI-3K$\gamma$, some other members of the PI-3 kinase family, demonstrated not to be activated by G$\beta\gamma$ alone, could be synergistically activated by G$\beta\gamma$ and phosphotyrosyl platelet-derived growth factor (PDGF) receptor peptides. Collectively, these observations, together with those of other workers, allow the conclusion that PI-3K$\gamma$ activity promotes tyrosine kinase (Src) activation resulting in Shc phosphorylation, and that in some way PI-3K$\gamma$ is coupled to G$\beta\gamma$ facilitation of the tyrosine phosphorylation of Shc. On reviewing the results of more recent studies, Lopez-Ilasaca et al. conclude that ‘free G$\beta\gamma$ recruits PI-3K$\gamma$ to the plasma membrane, enhancing the activity of an Src-like kinase, which in turn leads to the activation of the Shc-Grb2-Sos-Ras pathway, resulting in increased MAPK activity (Figure 3).
Signal transduction in neuropathic pain: part I

**Summary**

These data suggest that alterations in the levels of expression of G\(b\gamma\) will influence the MAPK-mediated phosphorylation and activation of nuclear transcription factors that regulate nerve cell growth and regeneration. Moreover, the above description of the G\(b\gamma\)-mediated Ras-dependent activation of the MAPK pathway is consistent with emerging reports that implicate agents that activate opioid, \(\alpha_2\)-adrenergic and M\(_2\)-muscarinicACh receptors as activators of the Ras-MAPK pathway via GPCRs. Depending upon the cellular context, activated MAPKs may mediate pleiotropic responses in the membrane, cytoplasm, nucleus or cytoskeleton. Upon activation, MAPKs translocate to the nucleus, where they phosphorylate and activate nuclear transcription factors involved in DNA synthesis and cell division.

**After neuronal injury, could exogenous opioids compromise the exquisite intimacy between VSCCs and changing phenotype expression?**

**Introduction: The traditional model of growth factors and neuronal phenotype**

Activation of the ERK cascade (a member of the MAPK cascade family) by peptide growth factors
such as epidermal growth factor (EGF), PDGF, and fibroblast growth factor (FGF) has been studied extensively.\textsuperscript{231-234} The EGF, PDGF, and FGF receptors are single transmembrane domain proteins that possess intrinsic ligand-stimulated receptor tyrosine kinase (RTK) activity. Upon ligand binding, these RTKs dimerize and transphosphorylate on their cytoplasmic domains. The resulting phosphotyrosine residues serve as docking sites to recruit components of the mitogenic signalling complex to the receptor. The recruitment of adaptor proteins, such as Shc and Grb2, to the phosphorylated RTK serves to assemble a multiprotein complex for regulating the low molecular weight G protein p21-ras. Recruitment of Grb2 brings the constitutively-associated Sos1 to the cytoplasmic surface of the membrane, where it activates p21-ras, which in turn initiates the ERK cascade by activating the Raf-1 serine/threonine kinase, followed by the sequential phosphorylation and activation of the MAPK kinase (MEK) and ERK kinases (Figure 4).

\textbf{Figure 4}  Activation of the p21-ras/ERK-signalling pathway by RTKs. The binding of ligand to the extracellular domain of RTKs, such as the PDGF and EGF receptors (PDGFR, EGFR), leads to receptor dimerization and autophosphorylation. The resulting phosphotyrosine residues on the intracellular domain of the RTK serve as high-affinity binding sites for the Shc and Grb2 adapter proteins. The nucleotide exchange factor, Sos1, catalyses the exchange of GDP for GTP on p21-ras, leading to its activation. GTP-bound p21-ras binds to and activates Raf kinase, thereby initiating a kinase cascade resulting ultimately in the stimulation of ERK activity.
**Opioids modulate growth factor activation of MAPK**

Because of their pleiotropic potential, MAPK (e.g. ERK) activities are tightly controlled by both positive and negative mechanisms. Belcheva and coworkers reported in 1998 that, in kidney COS-7 cells cotransfected with µ-, δ-, and κ-opioid receptors and ERK1- or ERK2-containing plasmids, with corresponding agonists, chronic opioid agonist treatment (>2 h) resulted in attenuation of the stimulation by EGF of ERK1 activity. Moreover, this opioid-mediated inhibition of EGF-induced ERK1 activity was transduced by Gβγ, probably along the same routes whereby acute opioid treatment stimulates ERK1 activity. At the present time, the significance of this modulatory role of opioids upon growth factor (i.e. RTK)-induced stimulation of ERK activity remains unknown. Polakiewicz et al. recently reported that inhibition of the MAPK pathway blocks desensitization of µ-opioid receptor signalling as well as the loss of receptor density due to internalization. From these results, these workers suggest that a feedback signal emanating from the MAPK cascade is required for µ-opioid receptor desensitization. It is difficult to imagine how neuronal injury would impinge upon this system of regulation, and only further studies can provide the answers.

Despite our considerable lack of understanding in this area, it is clear that within the injured/regenerating/dysfunctional neurone there is a need for dynamic control of intracellular Ca²⁺ levels within tight limits (see earlier sections on VSCCs). Accordingly, some form of cross-talk and/or feedback control must exist between Ca²⁺ channels, notably the VSCC, and MAPK-driven changes of phenotype expression. That such an intimate relationship exists between VSCC and p21-ras-dependent activation of MAPK is becoming evident as our level of understanding slowly increases.

**p21-ras modulates VSCCs**

An increasing number of reports suggest that many of the signalling proteins involved in cell growth, including p21-ras, growth factors and receptor- and nonreceptor-tyrosine kinases, are additionally involved in regulating the activity of ion channels, notably VSCCs. The acute application of oncogenic p21-ras has been found to enhance N-, L- and T-type Ca²⁺ channel currents in a variety of neuronal cells, which include embryonic chick dorsal root ganglion neurones, NG108-15 cells, and snail neurones. VSCCs in rat dorsal root ganglion cells appear to be tonically upregulated by endogenous p21-ras. Although this effect appears largely to involve RTK activation of p21-ras, in addition, Src tyrosine kinase can also upregulate VSCCs, possibly via p21-ras (see below). Although it is not clear whether p21-ras exerts a direct effect upon VSCCs or uses downstream signalling, recent evidence suggests that modulation of VSCCs by p21-ras is dependent on the activation of a MAPK.

**Growth factors modulate VSCCs**

There is considerable evidence to suggest that several growth factors, including neurotrophic growth factor, insulin-like growth factor, PDGF, and the nonreceptor tyrosine kinase, Src, can also enhance N- and L-type VSCC currents in PC12 cells, snail neurones, embryonic basal forebrain neurones, SK-N-SH neuroblastoma cells, cerebellar granule neurones and vascular smooth muscle.

**Convergence of signalling by Ca²⁺ and growth factors**

Important in vivo studies have implicated Ca²⁺ in the control of a wide array of distinct effects in neurones which overlap with those elicited by neuronal growth factors. Such effects include neurite growth, neuronal apoptosis, neuronal differentiation and synaptic plasticity. In neuronal cell culture, depolarization-induced Ca²⁺ entry through VSCCs has been shown to mimic and complement neuronal growth factor actions, including neuronal survival and the maintenance of neurites after growth factor deprivation. It is highly likely that a signalling cassette, which includes Src and p21-ras, underlies the broad range of Ca²⁺ actions in the nervous system.

**VSCCs activate MAPK via p21-ras**

Ca²⁺ influx through VSCCs leads to stimulation of tyrosine phosphorylation of Shc and its association with Grb2, most likely mediated via...
nonreceptor tyrosine kinases (which include PYK2) and the subsequent activation of p21-ras. Evidence has also been presented for the involvement of a Ca$^{2+}$-binding nucleotide exchange protein for p21-ras, p140 Ras-GRF, in mediating p21-ras activation in response to the elevation of intracellular Ca$^{2+}$ levels (Figure 5). This alternative pathway to p21-ras activation would appear to be involved in some CNS neurons in which Ca$^{2+}$-stimulated Shc tyrosine phosphorylation is not observed. Ca$^{2+}$ influx results in translocation of p140 Ras-GRF from the cytosol to the plasma membrane. Recent reports confirm earlier speculation that Ca$^{2+}$-mediated activation and translocation of p140 Ras-GRF requires the co-operative binding to p140 Ras-GRF of calmodulin (binding to IQ domains) and Gβγ subunits (binding to PH domains). In summary, these observations define two distinct pathways by which VSCCs can activate p21-ras and the MAPK cascade, leading to transcriptional induction of immediate early genes; the ability of dominant negative forms of Src and p21-ras to block Ca$^{2+}$-mediated induction of immediate early genes further attests to the stimulation of at least the Src-ras pathway by Ca$^{2+}$.

**Summary**

In attempting to answer the question posed in the title at the commencement of this section, it is interesting to report that PI-3K (i.e. p110$^\gamma$) is also one of the effectors of p21-ras. The ability of PI-3Kγ (i.e. p110γ) to also activate p21-ras suggests that PI-3Kγ is involved in the stimulation of at least the Src-ras pathway by Ca$^{2+}$.

![Figure 5](image-url) Enhanced consumption of opioid-derived Gβγ subunits after neuronal injury may compromise Gβγ-mediated inhibition of VSCC current. After neuronal injury, there is a marked increase in expression and translocation of calmodulin. This increase may drive calmodulin-dependent processes towards regeneration and away from analgesia. This diversion results in an enhanced sequestration of Gβγ, with a possible ‘winding-down’ of opioid-coupled receptor activity. In some part this may explain the need for an increase in dose of an opioid in order to restore analgesic efficacy (relative to that needed to treat nociceptive pain) in the management of neuropathic pain. CaM: Calmodulin; ††: Activation; ††: Inhibition; ††: Upregulation of activity/expression following nerve injury; PI-3Kγ: Phosphatidylinositol 3-kinase γ; PYK2: Protein tyrosine kinase; PYK2/Src: PYK2-mediated activation of Src-like kinase; p140-Ras-GRF: Ras-specific guanine nucleotide exchange factor (i.e. activator of p21-ras).
ing PI-3Kγ activity in intact cells, at least when it is overexpressed. Because PI-3Kγ is the ‘point of entry’ of opioids into the cycle of p21-ras activation (Figure 5), then, a priori, opioids have access to the interrelationships between p21-ras, VSCCs and growth factors (i.e. RTKs) described above (Figure 5). As we have no understanding of the effects of neuronal injury upon the dynamic equilibrium between these factors, then, in the absence of new research, this important question must remain largely unanswered.

However, after nerve injury there is an intense increase in expression and translocation to the plasma membrane of calmodulin which, in the normal nerve, is necessary for activation of p21-ras at several steps (Figure 5). Calmodulin binds to known Gβγ units and these interactions are Ca2+-dependent. Inspection of Figure 5 allows the notion that upregulation of p21-ras activation following, for example, the increase in activation and translocation of p140 Ras-GRF by binding of calmodulin would result in increased activation of PI-3Kγ, an effector of p21ras. In this way, enhanced sequestration (or ‘consumption’) of opioid-derived Gβγ units, by binding of Gβγ, not only to calmodulin and PI-3Kγ, but also to p140 Ras-GRF and Raf (Figure 5), may result in a ‘winding down’ of opioid-coupled receptor activity. That is, diversion of Gβγ from the normal cycling of receptor-activated Gα (i.e. Gα•GTP) and Gβγ subunits may possibly diminish the availability of these signalling units for actions upon other effectors, such as VSCCs, adenyl cyclase etc., which are putative mediators of opioid analgesia. If this scenario is accepted, then adaptation to injury apparently results in an upregulation of survival (or apoptotic) mechanisms at the expense of opioid signalling within nociceptive pathways.

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