MECHANISMS OF THE DOWNREGULATION OF PROSTAGLANDIN E_2-ACTIVATED PROTEIN KINASE A AFTER CHRONIC EXPOSURE TO NERVE GROWTH FACTOR OR PROSTAGLANDIN E_2

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DEDICATION

To my sons, Raphael and Gabriel, my wife Virginia, my mother Loulou and my father Refaat, and my sister Lillian, the people who made the cloudiest of days, sunny.

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> “And God shall wipe away all tears from their eyes; and there shall be no more … pain: for the former things are passed away.”
>
> (KJV, Revelation 21:4)

ABSTRACT

Ramy Refaat Habashy Malty

Mechanisms of the downregulation of prostaglandin E_2-activated protein kinase A after chronic exposure to nerve growth factor or prostaglandin E_2

Chronic inflammatory disorders are characterized by an increase in excitability of small diameter sensory neurons located in dorsal root ganglia (DRGs). This sensitization of neurons is a mechanism for chronic inflammatory pain and available therapies have poor efficacy and severe adverse effects when used chronically. Prostaglandin E_2 (PGE_2) is an inflammatory mediator that plays an important role in sensitization by activating G-protein coupled receptors (GPCRs) known as E-series prostaglandin receptors (EPs) coupled to the protein kinase A (PKA) pathway. EPs are known to downregulate upon prolonged exposure to PGE_2 or in chronic inflammation, however, sensitization persists and the mechanism for this is unknown. I hypothesized that persistence of PGE_2-induced hypersensitivity is associated with a switch in signaling caused by prolonged exposure to PGE_2 or the neurotrophin nerve growth factor (NGF), also a crucial inflammatory mediator. DRG cultures grown in the presence or absence of either PGE_2 or NGF were used to study whether re-exposure to the eicosanoid is able to cause sensitization and activate PKA. When cultures were grown in the presence of NGF, PGE_2-induced sensitization was not attenuated by inhibitors of PKA. Activation of PKA by PGE_2 was similar in DRG cultures grown in the presence or absence of NGF when phosphatase inhibitors were added to the lysis and assay buffers, but significantly less in cultures grown in the presence of NGF when phosphatase inhibitors were not added. In DRG cultures exposed to PGE_2 for 12 hours-5 days, sensitization after re-exposure to PGE_2 is maintained and resistant to PKA inhibition. Prolonged exposure to the eicosanoid caused complete loss of PKA activation after PGE_2 re-exposure. This desensitization was homologous, time dependent, reversible, and insurmountable by a higher concentration of PGE_2. Desensitization was attenuated by reduction of expression of G-protein receptor kinase 2 and was not mediated by PKA or protein kinase C.

The presented work provides evidence for persistence of sensitization by PGE_2 as well as switch from the signaling pathway mediating this sensitization after long-term exposure to NFG or PGE_2.

Michael R. Vasko, Ph.D., Chair

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AC	Adenylyl cyclase
AKAP	A-kinase anchor protein
AP	Action potential
ASIC3	Acid sensing ion channel
ATP	Adenosine-5'-triphosphate
BDNF	Brain derived neurotrophic factor
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	Complimentary DNA
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX1 and 2	Cyclooxygenases 1 and 2
$cPGI_2$	Carbaprostacyclin
$cPLA_2$	Cytosolic phospholipase A_2
CTX	Cholera toxin
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Erk	Extracellular signal-regulated kinase
FSH	Follicle stimulating hormone
GDNF	Glial derived neurotrophic factor
GEF	G-protein exchange factors
GIRK	G-protein coupled inward rectifying potassium channels
GPCRs	G-protein coupled receptors
Grk2	G-protein receptor kinase 2
HCN	Hyperpolarization-activated cyclic nucleotide gated channels
HETEs	Hydroxyeicosatetraenoic acids
HIV-gp 120	Human immunodeficiency virus glycoprotein 120
I-2	Inhibitor-2
iCGRP	Immunoreactive calcitonin gene related peptide
IGF1	Insulin-like growth factor 1

IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IP3	Inositol-1,4,5-trisphosphate
iSP	Immunoreactive substance P
IκB	Inhibitor of κB
LPA	Lysophosphatidic acids
LysoPLD	Lysophospholipase D
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MCS-LR	Microcystin-LR
MKP1	MAP kinase phosphatase1
mPGES	Microsomal PGE synthase
NGF	Nerve growth factor
NO	Nitric oxide
NTRK1	Neurotrophic tyrosine kinase receptor 1
p75NTR	p75 neurotrophin receptor
PAR2	Protease-activated receptor 2
PDE	Phosphodiesterase
PGE_2	Prostaglandin E_2
PGHS1 and 2	Prostaglandin H synthases 1 and 2
PGI_2	Prostacyclin
PIP2	Inositol-4,5-bisphosphate
PKA	Cyclic AMP-activated protein kinase
PKC	Protein kinase C
PKCε	Protein kinase C epsilon
PLCβ	Phospholipase Cβ
PP2A	Protein phosphatase 2A
PSPs	Serine/threonine protein phosphatases
PTEN	Phosphatase and tensin homologue deleted on chromosome10
PTPs	Protein tyrosine phosphatases
RGS	Regulator of G-protein signaling
sAC	Soluble adenylyl cyclase
SDF-1	Stromal-derived factor-1
SP	Substance P

TNF-α	Tumor necrosis factor-α
tNSAID	Traditional non-steroidal anti-inflammatory drug
TrkA	Tropomyosin receptor kinase A
TRPA1	Transient receptor potential ankyrin repeat 1
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TTX-R	Tetrodotoxin-resistant
TXA_2	Thromboxane A_2
βAR	β-adrenergic receptor
βARK	β-adrenergic receptor kinase

INTRODUCTION

Biology of Nociception

Pain as an important physiological function and as a disease

Pain is the most common condition for which patients seek medical care. According to a study in 2003, 105 million people (35.5 percent of all US population) suffer from chronic pain. The cost of pain, whether it is the primary reason for seeking medical care, or secondary to another ailment, amounts to $100 billion annually (Melnikova, 2010). In a study from Europe, 46,000 subjects were interviewed and 19 percent were found to suffer from chronic moderate to severe pain. Sixteen percent of these chronic pain sufferers reported that their pain is sometimes severe enough that they want to die[1] (Tsuda et al., 2005). In addition to high prevalence of chronic pain, there are multiple drawbacks with available therapies including severe side effects, liability for abuse and most importantly, failure in alleviating patients' suffering (Woolf, 2010a). Moreover, treatment of chronic pain is currently conducted in the clinic on an empiric basis. This means that the physician has to prescribe different drugs at different dosing regimens till an effective agent and dose are found. All these factors significantly increase the cost of therapy and thus the burden on the health care system (Finnerup et al., 2007).

It is important to differentiate pain as a symptom of an underlying disease versus pain as a disease. The former serves as a protective mechanism, while the latter serves no known function (Tsuda et al., 2005). Pain as a symptom serves a very important biological and evolutionary function; it helps the organism to identify noxious stimuli to avoid further harm and to accelerate healing and resolution of the injury. Reduced or lost ability to perceive pain results in severe and often life threatening conditions. Various studies on individuals who have mutations in genes encoding proteins that are essential for pain perception provide strong evidence for the importance of pain as a protective mechanism. These patients have total loss or severe reduction of the ability to perceive pain. Over time the afflicted individuals suffer severe undetected injuries due to the lack of the protective behavior initiated by pain sensation that can lead to death (Bejaoui et

[1] http://www.paineurope.com/healthcare-professional/pain-surveys/pain-in-europe-survey/key-findings.html (accessed November 7, 2012)

al., 2001; Cox et al., 2006; Indo et al., 1996; Slaugenhaupt et al., 2001). As a disease, pain is highly prevalent. Chronic pain is a significant problem from the health care point of view. Not only do chronic pathological pain disorders cause physiological and psychological distress, but they can also be life threatening (Fertleman et al., 2006)[2].

Definition of pain, nociception, hypersensitivity, hyperalgesia, hypernociception, allodynia and nociceptive neuron

Pain is defined as an experience involving both the sensation and the reaction that are initiated by a harmful stimulus whether that stimulus caused tissue damage or not (Merskey and Bogduk, 1994). According to this definition, pain involves affective and psychological aspects (Basbaum et al., 2009; Julius and Basbaum, 2001). Since there is no way for an experimental animal to communicate its affective state to the experimenter, the term pain is appropriate for use in clinical studies performed on humans only (Le Bars et al., 2001) whereas the term nociception is most accurate for experiments involving animal models (Vierck Jr, 2006). Nociception (literally means "to perceive harm"; derived from the Latin *nocere*, which means "to harm" and *percipere* which means "to seize") is a general term that can be used when describing experimental results using animal models (Loring and Meador, 1999; Sherrington, 1906). Nociception can be defined as the neural process by which a noxious stimulus is encoded and processed (Loeser and Treede, 2008). A noxious stimulus is a stimulus that is capable of producing tissue damage in an organism (Loeser and Treede, 2008). A nociceptive neuron is a peripheral or central neuron that mediates nociception (Loeser and Treede, 2008). Increased responsiveness of these neurons is collectively known as sensitization (Basbaum et al., 2009). Since nociceptive neurons can be divided into peripheral or central neurons, their sensitization can also be classified into peripheral or central sensitization respectively (Loeser and Treede, 2008). Hypersensitivity is a broad term that can be applied to shift to the left of the stimulus-response curve of neuronal cultures, animal models of pain or human studies. On the other hand hyperalgesia, which is defined as increased sensitivity to painful stimulus (Loeser and Treede, 2008), is better reserved within the clinical context. Hypernociception, unlike hyperalgesia, can be used to describe increased sensitivity of any organism to a noxious stimulus,

[2] (2001) Practice guideline for the treatment of patients with borderline personality disorder. American Psychiatric Association. *Am J Psychiatry* **158**(10 Suppl): 1-52.

including experimental animals (Parada et al., 2003a). Allodynia is a type of sensitization, which is defined as pain in response to non-noxious stimulus (Loeser and Treede, 2008). As mentioned above, because pain properly describes the human experience, the term allodynia should best be reserved for the clinical setting. However, allodynia continues to be used in the context of experiments involving animal models.

Dorsal root ganglia, classification of nociceptive neurons

As mentioned above, nociception is mediated by peripheral and central nociceptive neurons. The cell bodies (soma) of the peripheral nociceptive neurons are located in dorsal root ganglia (DRG) which are groups of specialized neurons that are situated on both sides of the vertebral column, outside the spinal cord. The existence of these excitable cells, which were named neurons by Heinrich Wilhelm von Waldeyer in 1891 (Lopez-Munoz et al., 2006), that connect the peripheral tissues to the spinal cord was first proposed by Sherrington CS (Sherrington, 1906) and subsequently substantiated by Gasser and Erlanger and others using the bull frog sciatic nerve (Adrian, 1926a; Adrian, 1926b; Adrian and Zotterman, 1926a; Adrian and Zotterman, 1926b; Gasser and Erlanger, 1922). DRGs contain two major classes of sensory neurons, low threshold sensory neurons (activated by touch, vibration, innocuous heat, innocuous cold, etc.) and high-threshold neurons. Peripheral sensory neurons (as well as other classes of sensory neurons located in the DRG) are pseudounipolar, i.e. they have a very short axon that bifurcates and branches into peripheral and central branches. The peripheral branch can be long and innervates peripheral tissues (skin, muscles, viscera, connective tissue, etc.). The central branch is relatively shorter and it projects to the dorsal horn of the spinal cord and there it relays the nerve impulses, through synapses, to spinal neurons.

Peripheral nociceptive neurons communicate exogenous or endogenous noxious stimuli to the CNS. These stimuli can be thermal, mechanical or chemical (Julius and Basbaum, 2001). The type of noxious stimuli that the neuron is able to detect can be used as a means to classify nociceptive neurons into thermal and mechanical (McMahon and Koltzenburg, 2006). Other bases of classification of peripheral nociceptive neurons include axonal diameter and its status of myelination (Schmalbruch, 1986), soma size (Swett et al., 1991), conduction velocity (Harper and Lawson, 1985; Yoshida and Matsuda, 1979) and the assortment of proteins they express (Snider and McMahon,

1998). Evidence of such diversity in function existed as early as 1926 (Adrian, 1926a; Adrian, 1926b; Adrian and Zotterman, 1926a; Adrian and Zotterman, 1926b).

As mentioned above, one way to classify peripheral nociceptive neurons is based on their status of myelination. Using this criterion, there are two classes of peripheral nociceptive neurons; the thinly myelinated Aδ fibers and the unmyelinated C fibers, which have medium and small diameter cell bodies, respectively. Both classes are activated at higher thresholds of stimulation than large-diameter DRG neurons that communicate touch and proprioception (McMahon and Koltzenburg, 2006). The small diameter neurons can be further classified into peptidergic and non-peptidergic. Peptidergic neurons express calcitonin gene related peptide (CGRP), substance P (SP), transient receptor potential cation channel, subfamily V, member 1 (TRPV1) and tropomyosin receptor kinase A (TrkA) receptor. The non-peptidergic neurons also associate with an extracellular matrix (ECM) proteoglycan named versican which is able to bind isolectin B4 (Bogen et al., 2005) and hence these neurons often named IB4+ neurons (Silverman and Kruger, 1990; Streit et al., 1985). In rats, there is significant overlap between TRPV1-expressing and IB4+ neurons (Woodbury et al., 2004). This is a widely accepted classification of nociceptive neurons in an uninjured (normal) organism. The criteria of classifying nociceptive neurons become less clearly defined under pathological conditions. For example low-threshold sensory neurons that associate with the myelinated Aβ fibers express SP under inflammatory conditions (Neumann et al., 1996). Also, IB4+ neurons were shown to express TRPV1 after induction of peripheral inflammation (Breese et al., 2005).

Pain is generally classified into four basic categories; direct or acute pain (sometimes referred to as nociceptive pain because it involves activation of nociceptive neurons without detectable tissue damage), inflammatory pain (resulting from tissue damage and inflammation that spares the nerves), neuropathic pain (which results from nerve damage) and idiopathic pain (pain that is perceived without detectable noxious stimulus, tissue or nerve damage) (McMahon and Koltzenburg, 2006; Mogil, 2009; Woolf, 2010b). In the rest of this dissertation, I will focus on inflammatory pain.

Acute nociception

As mentioned previously, a noxious stimulus can activate the nociceptive neuron leading to the occurrence of what is known as acute or nociceptive pain. An example of

such noxious stimulus is brief exposure of glabrous skin of the hand to temperatures between 43-50°C (LaMotte and Campbell, 1978). Interestingly, heat applied in this manner does not lead to significant tissue damage or inflammation yet it is perceived as noxious (Caterina et al., 1997; Leffler et al., 2007). The main receptor responsible for this heat sensitivity is the well-known TRPV1. TRPV1 is a ligand-gated non-selective cation channel. It can be selectively activated by the compound capsaicin (the pungent ingredient in peppers), noxious heat that is at or above 43°C (Caterina et al., 1997), and by protons. There is a debate whether a number of lipids act as endogenous ligands of the TRPV1 channel. Metabolites belonging to the endocannabinoids (such as anadamide) are argued to be endogenous ligands for the activation of TRPV1 channel (Jordt and Julius, 2002; Zygmunt et al., 1999). Another group of oxidized heat-generated catabolites of linoleic acid was recently found to activate TRPV1 as well (Patwardhan et al., 2010; Patwardhan et al., 2009). When TRPV1 channel is activated, it allows an influx of cations, most notably calcium (Dray et al., 1990; Oh et al., 1996; Wood et al., 1988). The influx of cations leads to two major consequences: the first is depolarization of the neuronal membrane that is enough to fire action potentials and thus initiate a nerve impulse (Williams and Zieglgansberger, 1982), and the second is the release of neurotransmitters and hence communicating the stimulus to the CNS (Saria et al., 1988). It is noteworthy that peripheral termini of sensory neurons can also release neurotransmitters due to propagation of retrograde action potentials from the cell body. This phenomenon is implication in neurogenic inflammation, the discussion of which is beyond the scope of this dissertation (Chiu et al., 2012). TRPV1-knockout mice (Caterina et al., 2000) have reduced sensitivity to noxious heat between 43°C and 49°C. However, these animals are not totally devoid of thermal sensation at lower or higher temperatures. This is due to the presence of many other channels that become activated at different temperatures providing a whole spectrum of thermal sensitivity (Dhaka et al., 2006; Hardie, 2007; Nakagawa and Hiura, 2006). In addition to TRPV1, nociceptive neurons express many other TRP channels and receptors that enable them to detect and transduce a wide range of thermal, chemical and mechanical noxious (Basbaum et al., 2009; Gold and Gebhart, 2010; Julius and Basbaum, 2001; Ren and Dubner, 2010; Woolf and Ma, 2007).

Acute and chronic inflammatory hyperalgesia

Tissue damage leads to production of a vast array of inflammatory mediators, which in turn sensitize nociceptive neurons. This sensitized state of nociceptive neurons can be relatively short lived, referred to as acute sensitization, or it can last for a long time, referred to as chronic sensitization (Reichling and Levine, 2009). Distinction between acute and chronic inflammatory sensitization is usually based on the time scale in which they occur. These time scales have been set without much scientific evidence or rationale other than the feasibility of classification. Due to the lack of clear demarcation between acute versus chronic sensitization, the accurate definition of these situations varies in different clinical settings as well as historically over time (Reichling and Levine, 2009). Acute inflammatory sensitization can be viewed as part of the adaptive function that nociception serves, but chronic inflammatory sensitization is considered to be maladaptive and serves no beneficial role. On the contrary, it is detrimental to the organism (Basbaum et al., 2009; Tsuda et al., 2005).

Peripheral sensitization and mechanisms of maintenance of pain (signaling switch, plasticity, priming)

Hyperalgesia and inflammatory mediators

Sensitization or hypersensitivity of nociceptive neurons ensues when the responsiveness of the neuron to a given stimulus is increased or when the threshold of response to the stimulus is lowered. Sensitization is caused by inflammatory mediators that are released upon the occurrence of tissue damage, infection, disorder of the immune system or exposure to certain pain-producing xenobiotics known as algogens (Basbaum et al., 2009; Gold and Gebhart, 2010; Julius and Basbaum, 2001; Ren and Dubner, 2010; Woolf and Ma, 2007). Inflammatory mediators belong to a wide variety of chemical groups and include but are not limited to; amines (such as histamine and the catecholamine epinephrine), nucleotides (such as adenosine-5'-triphosphate [ATP] and adenosine), peptides (such as bradykinin, endothelins, CGRP and SP), lipid mediators (such as prostaglandins [including the most studied member, PGE_2], leukotrienes, thromboxanes and hydroxyeicosatetraenoic acids [HETEs]), neurotrophins (such as nerve growth factor [NGF], glial derived neurotrophic factors [GDNF] and brain derived

neurotrophic factor [BDNF]), chemokines (such as monocyte chemotactic protein-1 [MCP-1], stromal-derived factor-1 [SDF-1] and fractalkine), cytokines (such as tumor necrosis factor-α [TNF-α] and interleukin-1β [IL-1β]), complement components, enzymes (such as matrix metalloproteinases), free radicals such as nitric oxide (NO) and even protons (Klippel et al., 2001; Pezet and McMahon, 2006). These inflammatory mediators are produced by different kinds of cells; such as neutrophils, macrophages, mast cells, fibroblasts, epithelial and endothelial cells, keratinocytes, microglia, Schwann cells and activated sensory neurons themselves (Basbaum et al., 2009; Klippel et al., 2001). Different subpopulations of nociceptive neurons express one or more receptors for each of these inflammatory mediators (Gold and Gebhart, 2010; Ren and Dubner, 2010).

One of the most important inflammatory mediators is NGF which is released during inflammation and can sensitize nociceptive neurons (Nicol and Vasko, 2007). One of the effectors of NGF-induced sensitization is the channel TRPV1. This channel has a unique role in development of sensitization because when it is knocked out in genetically modified mice, hypersensitivity caused by noxious high temperatures up to 43°C is significantly reduced (Caterina et al., 2000). As detailed later in this thesis, NGF acutely sensitizes TRPV1 channels through phosphorylation by multiple kinases including protein kinase C (PKC) and extracellular signal related kinase (Erk) (Nicol and Vasko, 2007). NGF also increases sensitivity of sensory neurons to capsaicin through increasing the translocation of TRPV1 channel to the cell membrane. So, despite that the overall mRNA and protein levels of TRPV1 do not change rapidly, the expression of TRPV1 in the cell membrane is increased leading to enhanced sensitivity of the nociceptive neuron (Zhang et al., 2005b).

NGF can also cause long-term (chronic) sensitization through increasing both the mRNA and protein expression of TRPV1 (Winston et al., 2001; Zhuang et al., 2004), TRPA1 (Diogenes et al., 2007), sodium channels (Fjell et al., 1999a; Fjell et al., 1999c; Gould et al., 2000), purinergic 2X receptors (Ramer et al., 2001), acid sensing ion channels (Mamet et al., 2003) and other ion channels. NGF also increases the expression of mRNA and peptide levels of CGRP and SP (Lindsay and Harmar, 1989; Lindsay et al., 1989). These and much more evidence indicate that NGF is an inflammatory mediator that can cause both acute and chronic sensitization via multiple mechanisms.

NGF is only one of many inflammatory mediators that are released upon the occurrence of tissue damage. Different inflammatory mediators can be involved in

different phases of sensitization and through variety of mechanisms that lead to increased responsiveness of nociceptive neurons.

The conundrum with chronic inflammatory pain

One of the most important effects of chronic inflammatory diseases is the perpetuation of sensitization of nociceptive neurons leading to chronic inflammatory pain. In different models of chronic inflammation, relatively high amounts of various inflammatory mediators are produced (Feghali and Wright, 1997; Kidd and Urban, 2001). These inflammatory mediators acutely sensitize nociceptive neurons for a long period of time. Several lines of evidence support the notion that PGE_2 is an important inflammatory mediator in sensitization of nociceptive neurons (Zeilhofer, 2007). Sequestration of PGE_2 using a selective monoclonal antibody attenuates inflammation induced by carrageenan as well as in experimental adjuvant arthritis (Mnich et al., 1995; Portanova et al., 1996). Antagonists of the PGE_2 receptor EP4 also diminish both carrageenan and adjuvant-induced hypersensitivity (Clark et al., 2008; Murase et al., 2008; Nakao et al., 2007). Inhibition of synthesis of COX, the enzyme synthesizing PGE_2, using non-steroidal anti-inflammatory drugs (NSAIDs) reduces sensitization in various models of inflammation (Anderson et al., 1996; Malmberg and Yaksh, 1992a; Malmberg and Yaksh, 1992b). In humans, NSAIDs are also able to alleviate chronic pain conditions (Chou et al., 2007; Lanas, 2002; Morlion, 2011; O'Dell, 2004; Sarzi- Puttini et al., 2010; Shah and Mehta, 2012b), which implies that prostaglandins maintain their ability to enhance the sensitivity of nociceptive neurons.

Prostaglandins sensitize nociceptive neurons by activating a group of G-protein coupled receptors (GPCRs). However, homeostatic mechanisms that terminate activity of these receptors are known to exist. These mechanisms evolved to prevent excessive stimulation of the receptor and thus avoid deleterious effects on living cells (Shenoy and Lefkowitz, 2011b; Sibley et al., 1987; Sibley et al., 1988). The conundrum is how PGE_2 maintains its ability to cause sensitization, despite the built-in biological mechanisms that function to terminate receptor activation and hence the subsequent sensitization.

One attempt to explain the persistence of PGE_2-induced sensitization despite the receptor-downregulating homeostatic mechanisms is that prostaglandin receptors of the E-series (EP receptors) are atypical and that they are not subject to canonical desensitization. In order to study this possibility, Vasko and his co-workers showed that

induction of inflammation in the rat paw using CFA (complete Freund's adjuvant) leads to downregulation of [H3]-PGE_2 binding in membrane fractions prepared from the dorsal horn of the spinal cord where the central termini of nociceptive neurons end (Southall et al., 2002). This reduction in receptor binding was reversed by the intrathecal injection of ketorolac, a traditional NSAID (tNSAID). In a previous study, Vasko's group showed that peripherally-induced inflammation using CFA significantly increased of Immunoreactive SP (iSP) and immunoreactive CGRP (iCGRP) release from the dorsal horn of the spinal cord, and that this augmentation of release was attenuated by intrathecal ketorolac injection (Southall et al., 1998). The conclusion from both studies is that peripherally induced inflammation causes increased synthesis of eicosanoids including PGE_2 in the dorsal horn of the spinal cord. This increased synthesis is accompanied by increased binding to and activation of the receptor, and thus initiates hyperalgesia. The increased activation of the receptors also engaged the mechanisms that causes internalization of these receptors (Pierce et al., 2002). The same phenomenon was also observed in cultures of rat sensory neurons, after prolonged exposure to PGE_2. This supports the notion that long-term exposure to PGE_2 causes downregulation of the receptor in the membranes of sensory neurons. According to the canonical models of downregulation of receptor activity, the effects initiated by these receptors should also cease (Gainetdinov et al., 2004; Lefkowitz, 2004). However, from the work by Vasko's group and others it is evident in experimental animals that behaviors associated with CFA-induced hypernociception persist for several days after induction of inflammation. Also, *in vitro*, PGE_2-induced sensitization of bradykinin-evoked iSP release persists even after chronic exposure to PGE_2 for 24-hour (Bolyard et al., 2000; Southall et al., 2002). Thus the conundrum is that expression of EP receptors in the cell membrane fraction downregulates after prolonged exposure to PGE_2, and thus EP receptors behave as typical GPCRs, yet sensitization is maintained.

Another potential explanation for the persistence of sensitization is that the differential expression profile of EP receptors in sensory neurons changes with inflammation, i.e., receptors involved with acute sensitization are downregulated while others are upregulated. Grubb and co-workers showed that the mRNA level of various EP receptors is decreased in CFA-injected rats DRGs 1 or 3 days post injection (Donaldson et al., 2001). On the other hand, Vasko and his group showed that mRNA level of all four subtypes of EP receptors do not change in adult rat DRG cultures after 24 hours exposure to PGE_2, TNF-α or IL-1β (Fehrenbacher et al., 2005). It is clear from

these studies that the argument that an increase of the level of expression of PGE_2 receptors may offset the receptor downregulation after long-term exposure is unlikely.

A third potential mechanism for maintained PGE_2-induced sensitization despite receptor downregulation is alteration in the level of expression of molecules in the PGE_2 signaling pathway. As will be explained later, PGE_2 induces sensitization through activation of the $G_{\alpha s}$/adenylyl cyclase/cAMP pathway (Ferreira and Nakamura, 1979; Hingtgen et al., 1995; Taiwo et al., 1989). It is possible that an increase in the level of expression of heterotrimeric G-protein or adenylyl cyclase may enhance signal amplification. Therefore, even after downregulation of cell surface receptors, signaling by the remaining fraction of receptors is amplified to a greater extent and thus sensitization persists. However this mechanism seems unlikely since there is no change in the expression and/or activity of $G_{\alpha s}$ and adenylyl cyclase (Southall et al., 2002).

The phenomenon of spare receptors may explain resistance of EP receptors to desensitization after long-term exposure to PGE_2 (Brodde, 1993; Pollet and Levey, 1980). A cell possesses spare receptors to a particular ligand if occupancy of a submaximal fraction of these receptors produces a maximal effect. Spare receptors can constitute up to 99 percent of a specific population of a receptor in some cell types (Levitzki, 1984). Since maximal responses do not require occupancy of all receptors, it is possible that downregulation of receptors expressed on the cell surface does not reduce the biological activity associated with activation of these receptors. An example of this phenomenon was found with prostacyclin (PGI_2) receptors (IP receptors) in the neuronal hybrid cell line NCB-20 (Leigh and MacDermot, 1985). In NCB-20 cells, 14 hours exposure to $cPGI_2$ (a stable analogue of PGI_2) caused an increase in the concentration of the drug needed to achieve half-maximum enzyme activity, while no change in affinity of the drug to IP receptors was observed. The observed effects appear to be secondary to loss of spare receptors. Similarly $PGF_{2\alpha}$ is thought to have spare receptors in the cat iris (Sharif et al., 2008). However there was no evidence of spare receptors to PGE_1 in rat liver membranes (Rice et al., 1981). Lack of spare receptors may explain why a particular ligand/receptor desensitizes when another does not. For example, in a hamster cell line, SK-N-MC, the pattern of desensitization of β-adrenergic receptor 1(βAR1) differs from that of dopamine receptor 1 (D1R). The authors attributed that difference to the presence of spare βAR1 but the lack of spare D1R (Zhou et al., 1993). Also in in CHO cells, μ-opioid receptors desensitize relatively rapidly because of the absence of spare receptors (Pak et al., 1996). Overexpression of histamine H2R

receptor rendered U937 cells resistant to downregulation of cAMP signaling (Monczor et al., 2006). Monczor and his colleagues attributed this induced resistance to desensitization to the fact that overexpressed H2Rs act as spare receptors and occupancy of a small fraction of the receptors is sufficient to activate the signaling pathway. Therefore, even after downregulation, the remaining fraction is sufficient to fully activate signaling. From these studies, it is clear that spare receptors represent an important mechanism to account for persistence of sensitization, however, as I will demonstrate in the results section, a signaling pathway that mediates PGE_2-induced sensitization is independent of PKA after long-term exposure to NGF or to PGE_2. This also indicates that spare receptors cannot fully account for persistence of PGE_2-induced sensitization in DRG neurons.

Persistent hyperalgesia and hyperalgesic priming

There is an emerging concept that chronic sensitization results from a phenomenon called persistent hyperalgesia or “hyperalgesic priming” (Hucho and Levine, 2007; Reichling and Levine, 2009). Priming of sensory neurons, such as by pre-exposure to CFA, allows sensitization of sensory neurons by a lower concentration of a sensitizing agent and for more prolonged period of time. In the absence of a sensitizing agent, the threshold for activating nociceptive sensory neurons by noxious stimuli remains unaltered (Hucho and Levine, 2007). In other words, a primed nociceptive neuron will respond more vigorously and for a much more prolonged period of time to an inflammatory mediator, than a naïve (an unprimed) one (Aley et al., 2001; Khasar et al., 1999a; Parada et al., 2003b; Parada et al., 2003c).

Originally Ferreira and coworkers showed that daily intraplantar PGE_2 injection for 14 days caused hyperalgesia that lasted for a month after cessation of injections, which they named persistent hyperalgesia. Dipyrone, but not indomethacin (both are tNSAIDs, dipyrone is not used clinically anymore), was able to attenuate the persistent hyperalgesia, but subsequent administration of a small dose of IL-1β or PGE_2 reversed the effect of dipryone and restored it (Ferreira et al., 1990). The effect of dipryone is mediated by activation of the nitric oxide/PKG pathway that leads to opening of ATP-sensitive potassium channels causing an increase of potassium currents and hence hyperpolarization of the cell membrane and restoration of its resting membrane potential (Sachs et al., 2004).

In work published by Levine and co-workers, hyperalgesic priming is induced by exposure to carrageenan, which induces acute hyperalgesia that resolves within a few hours to days. Subsequent injection of PGE_2, 5-HT or epinephrine leads to stronger and much more prolonged hypersensitivity in the primed animals than the unprimed ones (Aley et al., 2000; Parada et al., 2005). This primed state persisted for weeks after the original carrageenan challenge. Carrageenan causes the increased production of a multitude of inflammatory mediators, including TNF-α, IL-1β and interleukin-6 (IL-6) (Loram et al., 2007). Levine's group showed that injection of TNF-α or IL-1β were also able to induce hyperalgesic priming (Dina et al., 2008; Parada et al., 2003b). Thus the carrageenan-induced hyperalgesic priming is likely secondary to these cytokines.

Potential mechanisms underlying persistent sensitization

There are several potential mechanisms that may account for hyperalgesic priming. One such mechanism is the notion of a switch of the intracellular signaling that occurs upon prolonged stimulation of a given receptor by its cognate ligand. This prolonged stimulation leads to turning off the classical signaling pathway, while an alternative signaling pathway is activated. Presumably, this alternative signaling pathway remains activated for a longer duration and thus its activation leads to lasting changes in the nociceptive neuron function. This could result in a feed-forward mechanism that orevents the loss of hypersensitivity. There is extensive evidence to support the presence of a switch in signaling activated by GPCRs after prolonged exposure to their ligands. Lefkowitz and his group showed in their seminal work that the prolonged exposure to agonists leading to GPCRs desensitization also leads to recruitment and activation of a multitude of alternative signaling pathways (Shukla et al., 2011). These alternative signaling pathways remain active for longer duration than the pathway acutely activated by the receptor thus effectively switching and maintaining signaling by the GPCR.

Of primary importance to receptor desensitization and signaling switch are two classes of molecules, G-protein coupled receptor kinases (Grks) and β-arrestins (Daaka et al., 1997; Lefkowitz, 1998; Lefkowitz et al., 1983). Recently, it was shown that Grks can mediate both desensitization and switch in signaling independent of β-arrestins (Penela et al., 2010; Penela et al., 2006; Penela et al., 2003; Ribas et al., 2007). Kavelaars and colleagues demonstrated that models of chronic inflammation show

downregulation of G-protein receptor kinase 2 (Grk2) (Eijkelkamp et al., 2010b; Lombardi et al., 1999). This reduction in expression of Grk2 was mimicked by developing and using heterozygous knockout mice. These genetically modified mice demonstrated a phenotype that is strikingly similar to that described under hyperalgesic priming phenomenon. Kavelaars' work demonstrated that cell specific reduction of Grk2 in nociceptive neurons or glial cells caused the hypersensitivity produced by either PGE_2 or epinephrine to significantly increase both in intensity and in duration. It was also demonstrated that the signaling pathway mediating PGE_2-induced hypersensitivity is altered in these genetically modified mice (Eijkelkamp et al., 2010a; Eijkelkamp et al., 2010b; Wang et al., 2011; Willemen et al., 2010). Collectively, this work provides the potential of Grk2 as a key mediator of the shift and perpetuation of PGE_2-activated signaling. However the exact mechanism by which Grk2 mediates this switch is still not well understood.

Overall, the literature supports the notion that PGE_2-induced sensitization persists despite receptor downregulation and that a switch of the signaling pathway mediating this PGE_2-induced sensitization is a possible mechanism for persistence of sensitization. Therefore the two questions that I addressed in the current dissertation are:

1- Does long-term exposure to the inflammatory mediators PGE_2 or NGF alter PGE_2-activated PKA in adult rat sensory neuronal cultures?
2- If such alteration exists, what are the mechanisms mediating it?

In order to study the long-term effects of exposure to PGE_2 and NGF, I will briefly review the current knowledge about the role of both autacoids as inflammatory mediators capable of sensitizing sensory neurons in the following sections of the introduction. I will also discuss in some detail the signaling pathway that mediates acute PGE_2-induced sensitization including EP receptors and the rest of the components of the signaling pathway that were the basis for experiments performed in this work.

Eicosanoids

Historic background

Prostaglandins were first discovered in human seminal fluid as agents that cause contraction of human uterine muscles (Kurzrok and Lieb, 1930). The finding was independently confirmed and the compound dubbed prostaglandin since it was erroneously thought to be synthesized in the prostate gland (Goldblatt, 1933; von Euler, 1934; von Euler, 1936). It was thought at the time that two different compounds existed, prostaglandin and vesiglandin, the latter being derived from seminal vesicles. Later it was discovered that seminal vesicles actually produce far larger concentrations of prostaglandins and thus the compound should more properly be named vesiglandin, but the misnomer persisted (Eliasson, 1959). Prostaglandins are derived from polyunsaturated fatty acids containing 20 carbon atoms. The fatty acid that contributes the most as a precursor for prostaglandins is arachidonic acid, chemically known as 5, 8, 11, 14-eicosatetraenoic acid. Since both arachidonic acid and prostaglandins contain 20 carbons, the family of compounds was collectively named eicosanoids (*eicosa*-, Greek for 20) (Bergström et al., 1962; Smith et al., 2000b).

The second major discovery regarding prostaglandins was the finding that aspirin, one of the first chemically-synthesized drugs (Botting, 2010; Mahdi et al., 2006), exerts its analgesic, anti-inflammatory and antipyretic actions by inhibiting the synthesis of prostaglandins (Ferreira et al., 1971; Smith and Willis, 1971; Vane, 1971).

Role of prostaglandins in hyperalgesia

Another milestone in the history of prostaglandins is the finding that they are able to induce nociception by themselves at high doses in experimental animals (Collier et al., 1968; Collier and Schneider, 1972). Of paramount importance prostaglandins of the E-type (such as PGE_2 and PGE_1) were found to augment pain (cause sensitization) in humans at concentrations too low to produce pain by themselves, but high enough to induce inflammation (Ferreira, 1972; Ferreira et al., 1978). It was shown in independent studies that PGE_1 did not cause pain by itself at doses up to 100 µg/ml, but sensitized bradykinin-induced pain sensation in human at 0.1 µg/ml (Horton, 1963). This fit remarkably well with the definition of a sensitizing agent; a substance that by itself does

not cause overt pain, but it sensitizes to the action of another pain producing substance. A causal relationship between PGE_2 and hyperalgesia was further confirmed by the use of PGE_2 selective sequestering antibodies. These anti-PGE_2 antibodies were able to ameliorate CFA-induced hyperalgesia, inflammation and even IL-6 production in rats (Mnich et al., 1995; Portanova et al., 1996). Alternatively, it was found that IL-1β induces the expression of cyclooxygenase 2 (COX2) in the dorsal horn of the spinal cord (Samad et al., 2001) and both TNF-α and IL-1β induces COX2 expression in the dorsal root ganglion (Fehrenbacher et al., 2005). Prostaglandins were also found to directly sensitize second order neurons in the dorsal horn of the spinal cord, and thus also act as central sensitizing agents (Baba et al., 2001; Ferreira and Lorenzetti, 1996; Taiwo and Levine, 1988). Inhibition of COX enzymes in the dorsal spinal cord by intrathecal injection of NSAIDs attenuated second-phase hyperalgesia produced by formalin injection in the paw as well as thermal hyperalgesia caused by intrathecal SP and glutamate receptor agonists injections (Malmberg and Yaksh, 1992a; Malmberg and Yaksh, 1992b). Intrathecal injection of PGE_2, but not PGI_2, PGD_3, $PGF_{2\alpha}$, was found to cause mechanical and thermal sensitization (Reinold et al., 2005). Also carrageenan-induced hyperalgesia in the rats resulted in increased production of PGE_2 for higher levels and longer durations than other eicosanoids (Guay et al., 2004). The studies described here are but a small representation of much research that provides overwhelming evidence for the important role of PGE_2 as a sensitizing agent.

Effectors mediating PGE_2-induced sensitization

One of the more commonly studied effectors that were shown to mediate the hyperalgesic effect of PGE_2 is the TRPV1 channel (Lopshire and Nicol, 1997; Moriyama et al., 2005). Under normal conditions the TRPV1 channels are activated at approximately at 43°C. In the presence of PGE_2, the channel can be activated at 35°C, lower than core body temperature. PGE_2 also sensitizes DRG neurons via phosphorylation of and inhibition of potassium currents via the cAMP/PKA pathway (Evans et al., 1999). PGE_2 also increases calcium conductance through its channels in avian DRG neurons (Nicol et al., 1992). Other effectors for PGE_2-mediated sensitization are the tetrodotoxin-resistant sodium channels Nav1.8 and Nav1.9 (Akopian et al., 1999; England et al., 1996a; Gold et al., 1996; Rush and Waxman, 2004). PGE_2 was also found to increase trafficking of the TTX-resistant Nav1.8 in DRG neurons (Liu et al.,

2010a). In the spinal neurons of the superficial dorsal horn (second order neurons), PGE_2 inhibits glycine receptors (neuronal inhibitory receptors) through activation of the EP2/$G_{\alpha s}$/cAMP/PKA pathway (Ahmadi et al., 2002). This pathway inhibits glycine receptors by phosphorylating GlyRα3 subunits in the aforementioned spinal neurons (Harvey et al., 2004). It is also well known that PKA-mediated phosphorylation of synaptic vesicle proteins modulate neurotransmitter release (Dubois et al., 2002; Hansel et al., 2001; Sudhof, 2004). Some of these proteins are shown to be directly functionally involved with sensory neurons in simpler model organisms such as the *Aplysia californica* (Leenders and Sheng, 2005) as well as in rodent models of hyperalgesia (Schmidtko et al., 2005). PGE_2 increases synthesis of BDNF, a well-known inflammatory mediator in DRG explants (Cruz Duarte et al., 2012). PGE_2 also increases the synthesis and release of IL-6 in DRG neurons (Ma and Quirion, 2005; St-Jacques and Ma, 2011). This brief review of the different mechanisms by which PGE_2 causes sensitization of peripheral sensory neurons demonstrates the importance of the eicosanoid as an inflammatory mediator and hence, as an attractive target for therapy. However, for over 100 years, inhibition of prostaglandins synthesis has been the only mechanism used for therapies targeting eicosanoids, highlighting the need for discovery of novel therapeutic targets in the eicosanoid pathway causing sensitization.

Synthesis

The major precursor for synthesis of all eicosanoids is the essential fatty acid arachidonic acid, which contains 20-carbon atoms and four unsaturated (double) bonds and thus is named eicosatetr(5,8,11,14)aenoic acid (Samad et al., 2002). Arachidonic acid is liberated in a biphasic manner. An early acute burst is liberated by cytosolic phospholipase A_2 ($cPLA_2$) through hydrolysis of phospholipids on the inner leaflet of the cell membrane. A second delayed wave is mainly mediated by secreted PLAs ($sPLA_2$) (Clark et al., 1991; Irvine, 1982; Kudo and Murakami, 2002; Ma and Quirion, 2005). Following the liberation of arachidonic acid, several enzymes catalyze the first committed (irreversible) step by converting arachidonic acid to an intermediate, PGH_2 (Smith et al., 2000b). The enzymes catalyzing this step are known as prostaglandin endoperoxide H synthases 1 and 2 (PGHS1 and 2) or cyclooxygenase 1 and 2 (Smith and Dewitt, 1996). It is thought that COX enzymes and PLA_2 are coupled in a fashion that allows for the arachidonic acid released by PLA_2 to be directly delivered to COX

(Funk, 2001). In general, COX1 is constitutively active while COX2 is inducible (Smith et al., 2000b). There are crucial exceptions to this simplification. One such exception is that in endothelial cells, PGI_2 is synthesized mainly by COX2 the expression of which is increased after exposure of platelets to shear stress (Gimbrone et al., 2000). However, since blood platelets are constantly exposed to shear stress as they flow through blood vessels, COX-2 expression in them is viewed as being "static" (FitzGerald, 2003).

PGHSs catalyze the synthesis of the intermediate product PGG_2 which is then converted to another intermediate product known as PGH_2 (Smith et al., 2000b). In the process of this catalysis, the enzyme undergoes suicide inactivation; i.e. undergoes covalent modification of the protein followed by degradation (Callan et al., 1996; Smith et al., 1996; Wu et al., 1999). However, the amount of PGHSs is in vast excess of the substrate enabling rapid accumulation of product when surges in production are needed for homeostatic processes (Gimbrone et al., 2000).

PGH_2 is then converted by a variety of enzymes to the different prostaglandins (Funk, 2001). The two prostanoids that are most important for sensitization of sensory neurons are PGE_2 and PGI_2, which are synthesized by microsomal PGE synthase (mPGES) and prostacyclin synthase respectively (DeWitt and Smith, 1983; Jakobsson et al., 1999).

Transport, metabolism and bioactive metabolites

It is noteworthy that PGE_2 and other prostanoids are produced intracellularly, but they are transported out of the cell to act as first messengers binding to the extracellular portions of their receptors and thus A transporter belonging to the superfamily of organic anion transporters dubbed prostaglandin transporter (PGT) mediates this function of pumping PGE_2 outside the cell membrane (Kanai et al., 1995; Schuster, 1998).

Once released from cells producing them, prostanoids have short half-lives. PGE_2 is almost totally eliminated by the kidney and liver (Gerkens et al., 1978). PGE_2 is rapidly metabolized by an enzyme called prostaglandin dehydrogenase which is located intracellularly. Therefore in order for PGE_2 to be metabolized it has to be transported back through the cell membrane via prostaglandin transporter (PGT, see below) (Nomura et al., 2004). On the other hand, prostacyclin (PGI_2) has a shorter half-life compared to PGE_2. It was reported that at pH of 7.4 and temperature of 25°C, 50 percent of PGI_2 degrades within 3-4 minutes (Stehle, 1982). This lead to the speculation

that the site of synthesis/secretion of prostanoids has to be closely associated with their site of action, i.e. the receptors (Funk, 2001). In fact, recent insights from literature about lysophosphatidic acids (LPA), a different group of lipid mediators, provide evidence on the existence of such tightly coupled production/action. It was found that the enzyme lysophospholipase D (LysoPLD), also known as autotaxin, which produced LPA extracellularly, is bound directly or indirectly to the GPCRs on which they act (Moolenaar and Perrakis, 2011; Nishimasu et al., 2011; Tabchy et al., 2011). It was found that the nascent LPA is delivered directly to the receptor binding site. This is of particular importance considering the rapidity with which LPA is broken down in the extracellular space (Albers et al., 2010). Despite the fact that $cPLA_2$ and mPGES are intracellular molecules, one can imagine that some mechanism might exist to extrude the produced prostanoid in a site that is close or even coupled to the prostaglandin receptor.

Several pathways mediate the breakdown of prostanoids, including spontaneous hydrolysis and breakdown of PGI_2 and thromboxane A_2 (TXA_2) (Anggard et al., 1971), or enzymatic oxidation (Lands, 1979). As discussed above, prostaglandins are short-lived compounds that are rapidly eliminated, a property that evolved to allow for tight regulation of their function either on the very cells that synthesize them (autocrine action), or very closely associated ones (paracrine action) (Grunnet and Bojesen, 1976; Lin and Rao, 1977). In fact, abnormalities in prostanoid catabolism is believed to lead to diseases such as patent ductus arteriosus (Coggins et al., 2002) and colorectal cancer (Backlund et al., 2005; Myung et al., 2006).

What complicates the matter, is the fact that prostanoid metabolites show biological activity (Nishigaki et al., 1996). It is thought that the increased production of PGE_2 metabolites in certain tissues and organs cause desensitization of prostaglandin receptors. This leads to tonic desensitization of these receptors and thus low biological activity of the ligand (Anggard et al., 1971; Crutchley and Piper, 1975). Genetic ablation of EP4 receptors in experimental animals inhibits hyperalgesia in different experimental models (Lin et al., 2006; McCoy et al., 2002). Even if PGE_2 metabolites contribute to sensitization, it is most likely that they would do so through the canonical EP receptors, thus the downstream signaling should be identical. However, it is known that different ligands to the same receptor might not activate the same signaling pathways due to the phenomenon known as biased agonism (for detailed discussion see below) (Rajagopal et al., 2010b). Indeed it was shown that different ligands of EP4 receptor show various degrees of biased agonism (Leduc et al., 2009).

An additional layer of complexity in prostanoid biology is whether prostaglandins or their metabolites can act as ligands for peroxisome proliferator-activated receptors (PPARs) (Forman et al., 1995; Kliewer et al., 1995; Narumiya and FitzGerald, 2001; Narumiya et al., 1987). However, it is unlikely that PPARs participate to a significant degree in PGE_2-induced sensitization, since reduction of EP4 receptors using genetic techniques showed marked reduction in various models of sensitization, (Lin et al., 2006; McCoy et al., 2002).

Receptor subtypes, their coupling and signaling pathways

As mentioned earlier, receptors for E-series prostaglandins are termed EP receptors. EP receptors are seven-transmembrane receptors coupled to heterotrimeric G-protein under basal conditions (Sugimoto and Narumiya, 2007; Woodward et al., 2011a). Four different isoforms of the receptor exist, namely EP1-4.

As eluded to in the first section of this introduction, PGE_2-induced sensitization persists despite cell-surface receptor downregulation and that this persistence is thought to be mediated by a partial switching of the signaling pathway downstream from EP3c and EP4 (see below) which, under acute conditions, is mainly the cAMP/PKA pathway. Therefore it is important to briefly discuss the current knowledge related to EP receptors and coupled signaling pathways and their components in order to study downregulation of PGE_2-activated PKA and its underlying mechanisms.

EP1

EP1 receptors elevate $[Ca^{2+}]_i$ in Chinese hamster ovary cells and *Xenopus* oocytes (Funk et al., 1993; Katoh et al., 1995; Watabe et al., 1993). In the extravillous trophoblasts cell line HTR-8/Svneo elevation of $[Ca^{2+}]_i$ is achieved through coupling with $G_{\alpha q}$ (Nicola et al., 2005). Based on its coupling to $G_{\alpha q}$, EP1 causes activation of phospholipase Cβ (PLCβ), hydrolysis of membrane phosphatidyl inositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). DAG with calcium and phospholipids activate PKC, which phosphorylates various protein substrates. IP3 binds to IP3 receptors on the endoplasmic reticulum and causes release of calcium from intracellular stores. Both PKC-mediated phosphorylation and

IP3-mediated release of calcium are responsible for the biological effects of activation of EP1.

EP1 is expressed in sensory neurons (Fehrenbacher et al., 2005; Nakayama et al., 2004) and initially it was thought that EP1 plays a role in mediating hypersensitivity. A selective EP1 antagonist attenuated incision-induced hyperalgesia (Omote et al., 2001). The same EP1-selective antagonist also reduced carrageenan-induced hyperalgesia when perfused in the spinal cord (Nakayama et al., 2004; Nakayama et al., 2002). These observations were supported by the use of an EP1-knockout mouse in which, stretching and writhing was reduced (Stock et al., 2001). Similar findings were observed in human subjects in whom acid-induced visceral pain hyperalgesia was attenuated by a selective EP1 receptor antagonist (Sarkar et al., 2003). However in collagen-induced arthritis model, EP1 knockout mice did not demonstrate any difference from wild-type littermates in sensitization of sensory neurons (Honda et al., 2006). Not only did several studies using EP1 knockout mice show the lack of role of EP1 in mediating hyperalgesia, but some even showed a hyponociceptive effect when EP1 was deleted (Hall et al., 2007; Hosoi et al., 1999). EP1 knockout mice did not show any alteration of pain-like behaviors in an experimental model of rheumatoid arthritis, consistent with the lack of a role of EP1 in chronic inflammatory hyperalgesia. Also multiple studies show that blocking the cAMP signaling pathway inhibits PGE_2-induced sensitization (Evans et al., 1999; Hingtgen et al., 1995; Taiwo et al., 1989; Taiwo and Levine, 1991). EP1 is not known to couple to the cAMP pathway in sensory neurons. Therefore, overwhelming evidence suggests that the EP1 receptor does not contribute to PGE_2-induced sensitization of sensory neurons. Attenuation of certain pain-like behaviors (such as stretching after intraperitoneal injection of acetic acid) observed in EP1 knockout mice, can be explained by the possible contribution of other EP1-expressing cells, such as glial cells or some other cell type. For example, it was recently discovered that activation of EP1 receptors in murine astrocytes increased GDNF expression and release (Li et al., 2012). Therefore EP1 receptors on cells other than sensory neurons can indirectly mediate sensitization.

EP2

After cloning of EP2, it was found to couple to $G_{\alpha s}$ (Regan et al., 1994). This means that this receptor activates adenylyl cyclase and increases 3',5'-cyclic adenosine

monophosphate (cAMP) concentration. cAMP activates PKA, exchange proteins directly activated by cAMP (Epacs) and hyperpolarization-activated cyclic nucleotide gated channels (HCN) (Beavo and Brunton, 2002; Kopperud et al., 2003; Seino and Shibasaki, 2005). In the last decade, evidence accumulated that PKA is not the sole mediator of PGE_2-induced hyperalgesia. The discovery that both Epacs and HCNs mediate PGE_2-induced sensitization in conditions of chronic or persistent sensitization at least partly along with PKA (Eijkelkamp et al., 2010b; Emery et al., 2011b; Hucho et al., 2005; Wang et al., 2007). However, it is generally thought that of the three cAMP effectors mentioned above, PKA is the dominant effector mediating acute sensitization. The current opinion holds that both PKA-dependent and independent signaling could be downstream from EP2 (as well as EP3C and EP4, which are EP receptor subtypes that are coupled to cAMP pathway, see below) in chronic sensitization, but only PKA in acute sensitization.

EP2 receptors are expressed both in DRGs and in the dorsal horn of the spinal cord (Baba et al., 2001; Fehrenbacher et al., 2005; Kawamura et al., 1997; Kumazawa et al., 1996; Patwardhan et al., 2008; Zhao et al., 2007). There is significant evidence that the EP2 receptors mediate hyperalgesia by inhibiting glycine receptors on the second order spinal neurons in the dorsal horn of the spinal cord, leading to disinhibition of the pain pathway (Ahmadi et al., 2002; Harvey et al., 2004; Reinold et al., 2005). It is noteworthy though that EP2 is believed to mediate only the second phase of central sensitization, and not the peripheral component. Formalin test causes a biphasic hyperalgesic response in experimental animals. The immediate early short-lived phase is usually attributed to peripheral sensitization, while the second delayed and prolonged phase is thought to represent central sensitization. EP2 knockout mice showed reduction of the second but not the first phase (Hösl et al., 2006).

EP3

EP3 is unique in that it is the first GPCR to show that alternatively spliced receptors can couple to different heterotrimeric G-proteins despite being derived from the same gene and despite sharing significant homology (Namba et al., 1993a). It is now known that there are as many as 8 different splice variants of EP3 in humans (Bilson et al., 2004), 6 in the mouse (Fujino et al., 2010) and 4 in the rat (Oldfield et al., 2001; Southall and Vasko, 2001). These receptors differ in the heterotrimeric G-protein to which they are coupled, the level of constitutive activity they show (Hasegawa et al.,

1997; Negishi et al., 1996), their subcellular localization (Hasegawa et al., 2000), and their susceptibility to internalization after prolonged stimulation (Bilson et al., 2004). EP3 splice variants can couple to $G_{\alpha s}$/cAMP, $G_{\alpha i/o}$, $G_{\alpha q/11}$/PLC and $G_{\alpha 12/13}$/Rho (Woodward et al., 2011b).

In rats, EP3C, but not EP3A or EP3B, is expressed in sensory neurons and their endings, both peripherally and centrally (Beiche et al., 1998; Nakamura et al., 2000; Southall and Vasko, 2001). The difficulty of studying the biology of EP3 stems from its alternative splicing. Genetic deletion means that the entirety of all the splice variants of EP3 will cease to be expressed, which means that studying one particular splice variant and not another is a lot more difficult using this technique and alternative genetic deletion techniques are needed. Therefore different methods such as RNAi-mediated reduction of expression of a particular splice variant must be adopted. EP3 knockout mice show reduced nociception using the acetic acid-induced writhing test, only when lipopolysaccharide (LPS) is administered first as a sensitizing agent (Ueno et al., 2001). Other examples of EP3-mediated sensitization include the human immunodeficiency virus glycoprotein 120 (HIV-gp 120) which causes tactile pain in humans through an interaction between opioid κ and EP3 receptors (Minami et al., 2003). As suggested above, alternative methods that can inhibit expression of one or more splice variants may be used. For example, selective knockdown of the splice variant EP3C and EP4, both of which are coupled to $G_{\alpha s}$ (Namba et al., 1993b), showed that both EP3C and EP4 mediate PGE_2-induced sensitization of capsaicin-evoked iCGRP release in adult rat sensory neurons (Southall and Vasko, 2001). A selective ligand that can discriminate between different splice variants of EP3 exists (Zacharowski et al., 1999). Since at least one splice variant of EP3 receptor is coupled to $G_{\alpha i/o}$, it is possible to assume that selective activation of this splice variant will inhibit cAMP-mediated signaling pathways, such as those activated by PGE_2, leading to analgesia. Under acute conditions and using PGE_2 which activates all EP receptors, only hyperalgesia occurs. However, this ligand was used recently to show that selective activation of a $G_{\alpha i/o}$-coupled splice variant of EP3 can produce analgesia selectively after induction of knee-joint inflammation (Bar et al., 2004). This highlights the potential for selective activators of EP3 splice variants to act as analgesics only in patients with chronic inflammatory conditions.

EP4

EP4 is the third member of the EP receptors family that is coupled to $G_{\alpha s}$-coupled (the other two being EP2 and EP3C). EP4 is also expressed in sensory neurons (Fehrenbacher et al., 2005). EP4 is the largest of the EP receptors family; it has the longest C-terminus and third intracellular loop (Sugimoto and Narumiya, 2007). It significantly differs from EP2 in multiple ways. First, EP2 receptors are necessary for mediating PGE_2-induced sensitization of superficial dorsal horn spinal neurons (postsynaptic second-order neurons) as mentioned earlier (Harvey et al., 2004; Reinold et al., 2005), but not necessary for development of hyperalgesia in animal models of chronic inflammation (McCoy et al., 2002). Also, EP2 knockout mice showed deficiency in the second prolonged phase of the formalin test, traditionally attributed to central hyperalgesia (Hösl et al., 2006). On the other hand, reduction of expression of EP4, but not EP2, receptor attenuates sensitization induced by CFA and collagen-induced arthritis *in vivo* (Lin et al., 2006; McCoy et al., 2002). In vitro, reduction of expression of both EP4 and EP3C attenuates PGE_2-induced sensitization of sensory neurons (Southall and Vasko, 2001). EP4-selective antagonists also attenuate hypernociception in experimentally-induced arthritis (Clark et al., 2008; Murase et al., 2008). Secondly, EP2 receptors desensitize much slower than EP4 (Nishigaki et al., 1996). This difference was attributed to the larger C-terminus of EP4 (Bastepe and Ashby, 1997). Additionally, there is a greater propensity to internalization by EP4 receptors and relative resistance by EP2 receptors (Desai et al., 2000). As it will be discussed later, the processes that regulate desensitization and internalization of GPCRs after long-term exposure to their cognate ligands also regulate switching to alternative signaling pathways. After long-term exposure to PGE_2 which results in ₍eceptor phosphorylation, EP4, but not EP2 receptors couple to $G_{\alpha i/o}$ (Fujino and Regan, 2006). From these studies it becomes clear that although both EP2 and EP4 are coupled to $G_{\alpha s}$ under basal conditions, differences between both receptors, probably in the structure of the C-terminus, result in radically different downstream signaling.

EP4 receptors are expressed in DRG neurons (Fehrenbacher et al., 2005; Oida et al., 1995). Numerous studies clearly illustrated the essential role that EP4 plays in mediating neuronal hypersensitivity. Vasko's group was the first to demonstrate that EP4 receptors, along with EP3C, are essential for sensitization of capsaicin-evoked iCGRP release from adult rat sensory neuronal cultures and PGE_2-stimulated cAMP synthesis

(Southall and Vasko, 2001). Using shRNA-mediated knockdown of EP1-4, Woolf's group showed that CFA-induced sensitization is inhibited by selective knockdown of EP4, as well as by a selective antagonist of the receptor (Lin et al., 2006; Murase et al., 2008; Nakao et al., 2007). More importantly, induction of hyperalgesia in collagen antibody-induced arthritis was abolished in EP4 knockout animals. The collagen-induced arthritis model is believed to be more similar in its attributes to chronic inflammatory human diseases such as rheumatoid arthritis and osteoarthritis than CFA or carrageenan-induced arthritis. EP4 also was shown to be the major PGE_2 receptor subtype that is involved in hyperalgesia in the GRK2 heterozygous knockout model (Eijkelkamp et al., 2010b). This work strongly suggests that EP4 antagonists might be useful therapeutic agents in arthritis patients (McCoy et al., 2002). Several antagonists of EP4 were synthesized and tested in animal models of chronic inflammatory hyperalgesia and showed promise as potential therapies for chronic inflammatory hypersensitivity, thus confirming previous studies (Clark et al., 2008; Murase et al., 2008).

Nerve growth factor, its receptors and signaling

Historic background

The first observation of a substance possessing growth-promoting activity of peripheral nerves was made in 1948 (Bueker, 1948). Tumors grown in the mouse were implanted in chick embryo and leading to growth of the lumbosacral peripheral nerves in the direction of the tumor. Later it was found that these tumors secrete NGF. Levi-Montalcini and Hamburger and their co-workers confirmed that the tumor released a factor which stimulates the growth of peripheral sensory and sympathetic nerves (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini et al., 1954). The term “nerve-growth stimulating factor” was coined by Levi-Montalcini’s group when they first isolated this factor from the mouse tumors (Cohen et al., 1954). The factor was also found to be enriched in certain snake venoms (Cohen and Levi-Montalcini, 1956) as well as in the mouse salivary submaxillary gland (Cohen, 1960; Levi-Montalcini and Booker, 1960). For their discovery, Levi-Montalcini and Cohen were awarded the Nobel Prize in medicine in 1986. The DNA sequence coding for mouse NGF was isolated (Scott et al., 1983), and subsequently mice lacking the gene were developed (Crowley et al., 1994). Homozygous NGF-knockout mice had severe loss of the small and medium diameter sensory neurons as well as sympathetic neurons. These mice demonstrated markedly decreased responsiveness to pain compared to their wild-type or heterozygous littermates. These findings corroborated the hypothesis that NGF is essential for the development of peripheral sensory and sympathetic neurons.

Nerve growth factor acts through binding to two receptors, TrkA and p75NTR receptors. TrkA (also named neurotrophic tyrosine kinase receptor 1 [NTRK1]) was first discovered as a proto-oncogene that is constituted of fusion of two proteins; tropomyosin 3 and a tyrosine kinase receptor (Martin-Zanca et al., 1986). Later it was demonstrated that the TrkA receptor is essential for NGF high-affinity ($K_d \approx 10^{-11}$ M) binding with a slow ($t_{1/2} \approx 10$ minutes) rate of dissociation (Hempstead et al., 1991). Nerve growth-promoting activities attributed to NGF were also found to be dependent on TrkA, since TrkA-knockout mice suffered from severe sensory and sympathetic neuropathies (Cordon-Cardo et al., 1991; Loeb et al., 1991; Smeyne et al., 1994). A very rare heritable mutation of the TrkA receptor causes afflicted patients to suffer loss of pain perception as well as temperature sensation and hence the ability to sweat. Thus

this condition was named congenital insensitivity to pain with anhidrosis (Indo et al., 1996). The severe impairment of pain perception in patients confirmed the conclusion made from the studies using mice with mutated TrkA receptor gene that this receptor is essential for nerve growth promoting activities of NGF. As mentioned above, NGF binds with low affinity ($K_d \approx 10^{-9}$ M) and rapid ($t_{1/2} \approx 3$ seconds) rate of dissociation to another receptor known as p75NTR. p75NTR encoding DNA sequence was first cloned in 1986 (Johnson et al., 1986), and subsequently mice containing targeted mutation of the gene were generated (Lee et al., 1992). These mice demonstrated similar, but not identical, phenotype to TrkA-homozygous knockout mice. Mice with mutated p75NTR showed significant reduction of innervation of the skin by CGRP or SP-positive nerve fibers of, significant reduction of heat sensitivity, loss of hair on the paws as well as toenails loss with skin ulceration and infection. However, unlike TrkA mutated mice, p75NTR-mutated mice did not show alteration of sympathetic ganglia or sympathetic innervation of the iris and the salivary gland. Also, unlike TrkA receptor, p75NTR belongs to the TNF receptors family which are not tyrosine-kinase receptors (Nicol and Vasko, 2007). Instead p75NTR has an intracellular signaling domain that interacts with and activates different downstream signaling molecules and thus propagates the signal across the cell membrane (Gentry et al., 2004).

I studied the effects of long-term exposure of cultured sensory neurons to NGF because much evidence supports an essential role for the trophic factor in chronic inflammatory conditions in humans as well as in animal models. In this section, a brief discussion of literature and evidence supporting such role of NGF is made.

NGF production

NGF is produced as a large precursor called pro-NGF, which is processed into smaller forms. There are two forms of mature NGF; 7S and 2.5S forms (S denotes Svedberg, the unit of sedimentation co-efficient), the former has a molecular weight of 130 KDa while the latter has a molecular weight of 26 KDa (Scott et al., 1983; Yiangou et al., 2002). The 7S form of NGF consists of two molecules of each of α, β and γ subunits. The 2.5S (also known as βNGF) form is a homodimer of the β subunit only (Pezet and McMahon, 2006). Only the βNGF subunit of either 7S or the 2.5S forms of NGF possess nerve growth promoting activity (Varon et al., 1968). Many kinds of cells synthesize and release NGF including epithelial cells, smooth muscle cells and

fibroblasts (Bandtlow et al., 1987), Schwann cells (Matsuoka et al., 1991), mast cells (Leon et al., 1994) and lymphocytes (Santambrogio et al., 1994). The expression level of NGF is dynamic and increases in many different inflammatory conditions in different kinds of cells (Heumann et al., 1987; Raychaudhuri et al., 1998; Stanzel et al., 2008). It is noteworthy that the premature form of NGF, named pro-NGF, can be secreted (Lee et al., 2001). Interestingly pro-NGF has higher and more selective affinity to p75NTR than NGF (Pezet and McMahon, 2006), therefore it is possible that pro-NGF plays a role in pathological conditions. Indeed, pro-NGF was found to be the predominant form of NGF in isolates from brain tissues collected from patients with Alzeheimer's dementia, neuropathic nerve tissue and retinas from animal models of degenerative retinopathies (Pezet and McMahon, 2006; Yiangou et al., 2002).

NGF as an inflammatory mediator

Studies using animals with gene mutations in either NGF or its receptors, demonstrated that sensory and sympathetic neurons depend on NGF for survival into adulthood (Crowley et al., 1994; Smeyne et al., 1994). However NGF is not needed by adult sensory neurons for survival (Lindsay, 1988), although NGF enhances axonal regeneration of sympathetic and sensory neurons after axotomy (Pettigrew et al., 2007; Ramer et al., 2000).

Of greater importance to this dissertation is the role of NGF as an inflammatory mediator (Nicol and Vasko, 2007). In human beings, injection of NGF locally produces hyperalgesia that starts within minutes and lasts for hours, while systemic injections can cause generalized hyperalgesia for days (Svensson et al., 2003).

Numerous studies showed that in various animal models of inflammatory pain as well as in clinical studies, the levels of NGF are increased. CFA injection in rat paw caused elevation of NGF extracted from skin of the hindpaws of adult the rat (Woolf et al., 1994). NGF was also markedly increased in exudate collected from blisters produced by the application of dry ice to the plantar skin of the hind paws of anaesthetized animals (Weskamp and Otten, 1987). In mouse models of allergic asthma, elevated levels of NGF were detected in broncho-alveolar lavage fluid and in serum (Braun et al., 1998). NGF was also significantly increased in patients with allergic diseases and asthma (Bonini et al., 1996). The mRNA levels of NGF were also elevated in gastrointestinal tissues collected from patients with Crohn's disease and ulcerative colitis (di Mola et al.,

2000). Seminal plasma collected from patients with chronic prostatitis also showed elevated levels of NGF (Miller et al., 2002). Most importantly, NGF levels were elevated in synovial fluid from arthritis patients (Aloe et al., 1992; Halliday et al., 1998). These studies clearly demonstrate that increased NGF levels are correlated with experimentally-induced inflammation in animal models as well as inflammatory diseases in patients.

Acute administration of NGF induces hyperalgesia. In humans, injection of NGF into the masseter muscle caused mechanical hyperalgesia and allodynia (Svensson et al., 2003). In rats, intraplantar injection of NGF caused significant mechanical allodynia and heat hyperalgesia (Amann et al., 1996a; Lewin et al., 1993), while in mice, intravenous administration caused marked thermal hyperalgesia (Dellaseta et al., 1994). In genetically-altered mice that have increased or decreased expression of NGF in the skin showed marked hyperalgesia and hypoalgesia, respectively, to mechanical noxious stimulation (Davis et al., 1993). Mice with increased expression of NGF in the skin also showed marked thermal and mechanical neuronal hypersensitivity in C-fibers and Aδ fibers, respectively (Stucky et al., 1999). In adult rats, application of NGF to the urinary bladder caused sensitization of the innervating peripheral sensory neuronal fibers to mechanical distension of the bladder wall (Dmitrieva and McMahon, 1996). Collectively, these studies provide strong evidence supporting the ability of NGF to enhance pain-like behavior in animal models and humans.

Multiple lines of evidence provide cause-effect relationship between NGF and various forms of hypersensitivity of peripheral sensory neurons. Several biological tools were used to perturb NGF and establish such relationships. For example, injection of anti-NGF antibodies attenuated hyperalgesic behaviors induced by inflammation in CFA-injected animals (Woolf et al., 1994). On the other hand, these anti-NGF antibodies caused hypoalgesia as evidenced by reduction in acute nociceptive behaviors in the absence of sensitization (Urschel et al., 1991). A fusion molecule between the TrkA and immunoglobulin G (IgG) was also used to bind NGF (McMahon et al., 1995). The TrkA-IgG fusion molecule acts like a false receptor that sequesters NGF and prevents its interaction with the native receptors. Not only did this molecule greatly attenuate carrageenan-induced hyperalgesia, but it also caused hypoalgesia in control animals, suggesting that NGF has a role in mediating physiologic nociception under normal conditions (McMahon et al., 1995). K252a is a small-molecular weight drug that inhibits tyrosine kinases, including the TrkA receptor. Under control conditions, capsaicin-

activated TRPV1 currents exhibit rapid desensitization after the initial exposure to the vanilloid (Koplas et al., 1997). NGF also augments capsaicin-activated TRPV1 currents after the second exposure. Prevention of desensitization and augmentation of TRPV1 currents was attenuated by k252a (Shu and Mendell, 1999b). There is growing interest among pharmaceutical companies in anti-NGF agents as therapeutic tools in treatment of chronic inflammatory conditions (Cattaneo, 2010; Hefti et al., 2006; Lane et al., 2010).

Mechanisms of action of NGF as an inflammatory mediator

NGF acts as an inflammatory mediator by a variety of mechanisms. NGF can act directly on nociceptive neurons through activating its TrkA and p75 receptors or it can act indirectly through activating these receptors on other cell types which in turn enhance neuronal sensitization via the release of a secondary wave of inflammatory mediators (Nicol and Vasko, 2007; Pezet and McMahon, 2006).

Indirect actions of NGF

Both TrkA and p75NTR NGF receptors are expressed on a variety of cell types, and some of the actions of NGF in hypersensitivity are mediated by activating these receptors on cells other than nociceptive neurons. For example, activation of mast cell TrkA receptors leads to their degranulation and release of inflammatory mediators, including NGF (Horigome et al., 1993). In this manner NGF acts as an autocrine messenger, enhancing its own release (Leon et al., 1994). In analogous manner, NGF acts on TrkA receptors expressed on keratinocytes to enhance its own release (Di Marco et al., 1993).

NGF also has a direct action on neutrophils, whose viability, phagocytosis and superoxide anion production are enhanced by NGF (Kannan et al., 1991). Production of leukotriene B4 (LTB4) by neutrophils is also enhanced by NGF (Amann et al., 1996b) and inhibitors of 5-lipoxygenase, the enzyme that synthesizes leukotrienes, attenuate NGF-induced hyperalgesia (Bennett et al., 1998b). Neutrophil depletion by using an anti-neutrophil serum almost completely prevented NGF-induced hyperalgesia. This indicates that NGF-induced sensitization of sensory neurons is, at least in part, indirectly mediated by its action on neutrophils (Bennett et al., 1998b). Interestingly, neutrophils

were essential for the development of joint inflammation and swelling in a mouse model of rheumatoid arthritis (Wipke and Allen, 2001). Collectively, these studies show that NGF acts on neutrophils to augment their ability to cause inflammation.

NGF can cause hypersensitivity indirectly through the release of other inflammatory mediators known to sensitize nociceptive neurons. NGF increases TNF-α expression in the knee-joint of experimental animals (Manni and Aloe, 1998). NGF also increases the level of mRNA of several cytokines in mast cells, including TNF-α, IL-3, IL-4 and IL-10 and macrophage colony stimulating factor (Bullock and Johnson, 1996). Other studies, however, reported that the cytokine TNF-α causes the release of IL-1β and then IL-1β induces the release of NGF (Safieh-Garabedian et al., 1995; Woolf et al., 1997). The TNF-α/IL-1β/NGF cascade of cytokines delineated by Woolf and coworkers is also supported by the work of other groups (Cunha et al., 1992; Cunha et al., 2005; Hattori et al., 1993). The question remains as to whether cytokines increase NGF expression or vice versa, and thus future studies are required. However, the use of different experimental models could explain the discrepancies.

It is evident that primary and secondary immune organs, such as lymph nodes, thymus, spleen, hematopoietic bone marrow, Peyer's patches in the small intestine, and other lymphoid tissues are innervated by sympathetic neurons. Moreover it seems that immune cells in these organs form a synapse-like contact with sympathetic nerve fibers (Simone et al., 1999). Sympathetic nerve fibers strongly express TrkA receptors and are able to secrete NGF (Ciriaco et al., 1996). This suggests that there is structural and functional interaction between the immune and the nervous systems through sympathetic neurons (Simone et al., 1999). Indeed, intracerebral administration of NGF causes proliferation of lymphatic cells in the spleen (Sacerdote et al., 1996). NGF regulates synthesis and release of epinephrine, which strongly affects the function of lymphocytes (Muller and Unsicker, 1986). Therefore it is clear that NGF can affect the immune and the nervous system. On the other hand, the nervous system may also affect the function of NGF as a sensitizing agent. For example, hyperalgesia induced by administration of NGF or CFA is significantly reduced by surgical or pharmacological ablation of sympathetic neurons (Andreev et al., 1995; Woolf et al., 1996). Sympathetic denervation only delays the onset of CFA-induced hyperalgesia, which implies that sympathetic nerves mediate only the early phase (Nicol and Vasko, 2007).

Direct actions of NGF

Numerous studies suggest that NGF can directly activate sensory neurons expressing TrkA and p75 receptors. Direct effects of NGF can be broadly classified in two categories; posttranslational modification of different effectors that mediate neuronal function and effects on expression levels of these various effectors (Nicol and Vasko, 2007; Pezet and McMahon, 2006).

To date, relatively few targets for the effects of NGF that are mediated via posttranslational modification. NGF increases the number of action potentials in small diameter sensory neurons of the young adult rat (Zhang et al., 2002). Binding of NGF to its receptors can result in phosphorylation of Nav1.7 channels and augment the channel expression or trafficking. Phosphorylation of Nav1.7 alters the gating properties of the channel in a way that makes the channel open at an elevated threshold potential; i.e. makes the channel easier to open and thus augments neuronal firing of action potentials (Stamboulian et al., 2010). A much more studied target of NGF-induced sensitization is the TRPV1 channel. NGF acutely increases capsaicin-activated current (Shu and Mendell, 1999a). NGF also augments heat-mediated currents in sensory neurons (Galoyan et al., 2003). Since NGF-induced sensitization of heat-activated currents occurs within 30 seconds and since it was attenuated by PLC inhibition, it is likely that post-translational modifications of the TRPV1 channel is the underlying mechanisms rather than changes in its trafficking or expression (Galoyan et al., 2003).

NGF also increases the expression of many targets; emphasizing the “trophic” nature of NGF. NGF binds to TrkA on nerve endings in peripheral tissues, and the complex is then retrogradely transported to the cell bodies in the DRGs. In the cell body, the NGF/TrkA complex increases the expression of a multitude of proteins (Goedert et al., 1981; Hendry et al., 1974; Stoeckel et al., 1975). Early accounts of NGF-mediated upregulation of a protein that is involved in nociceptor function were made in 1980 when it was shown that NGF increases the expression of both iSP and iCGRP (Christensen and Hulsebosch, 1997; Goedert et al., 1981; Kessler and Black, 1980; Lindsay and Harmar, 1989; Otten et al., 1980; Winston et al., 2001). NGF increases the expression of the mRNA of TRPV1 (Winston et al., 2001) and TRPV1 protein as well as trafficking of the channel to the cell membrane (Zhang et al., 2005b). These increases in expression and trafficking augment hypersensitivity and thus are a likely mechanism for maintaining sensitization over time (Ji et al., 2002). The mRNA and protein of TRPA1 (transient

receptor potential ankyrin repeat 1) are upregulated by NGF which can contribute to cold hyperalgesia (Diogenes et al., 2007; Obata et al., 2005). Sodium channel expression, including Nav1.7 (Toledo-Aral et al., 1997), Nav1.8 (Dib-Hajj et al., 1998), is increased by NGF both *in vivo* and in sensory neuronal cultures (Fjell et al., 1999b; Gould et al., 2000). Expression of acid sensing ion channel 3 (ASIC3) (Mamet et al., 2003), bradykinin receptor (Petersen et al., 1998), and P2X3, another ligand-gated cation channel, (Ramer et al., 2001) are also increased in response to NGF. Moreover, NGF increases the expression of mRNA and protein of another neurotrophic factor, BDNF (Apfel et al., 1996; Michael et al., 1997). All these molecules act as effectors of nociceptive neurons, and enhancement of their function by NGF contributes to enhanced excitability of neurons and thus their hypersensitivity.

In summary, extensive literature clearly shows that NGF is a key inflammatory mediator that orchestrates the development and maintenance of hyperalgesia and sensitization of nociceptive neuron.

Signaling pathways mediating sensitization

Cyclic AMP signaling pathway

Discovery

The discovery of cAMP and the conceptual development of second messengers are attributed to Sutherland and co-workers (Rall and Sutherland, 1958; Robison et al., 1965; Sutherland et al., 1965). cAMP is celebrated as the first identified second messenger (Kresge et al., 2005; Robison et al., 1965; Sutherland et al., 1965) and it is serendipitous that it is the first signaling molecule to be identified as mediating hyperalgesia (Ferreira and Nakamura, 1979).

One of the aims of the work presented in this dissertation is to determine whether cAMP/PKA signaling pathway, which mediates acute PGE_2-induced sensitization, is altered after long-term exposure to the eicosanoid or to NGF. cAMP/PKA signaling pathway involves multiple proteins [including $G_{\alpha s}$, AKAPs (A-kinase anchor proteins), adenylyl cyclases, PDEs (phosphodiesterases) and PKA itself] each of which has several isoforms. It is therefore essential to briefly review the current knowledge of these signaling proteins and different mechanisms for their regulation.

Adenylyl cyclases

Cyclic AMP is synthesized by the adenylyl cyclase family of enzymes (ACs) (Sutherland et al., 1962). There are 10 different isoforms of ACs (Bundey and Insel, 2004; Cooper and Crossthwaite, 2006). Nine isoforms are membrane bound (AC1-AC9) and only one isoform is soluble (sAC). Soluble AC is insensitive to stimulation by $G_{\alpha s}$ and forskolin, but is activated by bicarbonate (Buck et al., 1999; Chen et al., 2000). The minimal active unit of AC is a dimer (Rodbell, 1980). Cyclic AMP synthesis by these enzymes is highly compartmentalized. ACs are compartmentalized through their interaction with various anchor proteins such as A-kinase anchor proteins (AKAP, see below) (Willoughby and Cooper, 2007). AKAPs act as scaffold proteins to anchor not only PKA, but ACs as well as many other enzymes that are essential in “sculpting” a cAMP microdomain in order to achieve a selective activation of a particular pool of PKA (Baillie et al., 2005). In this manner, cAMP can serve as a second messenger to a large

number of biological functions activated by various first messengers without spillover or cross talk. Other binding proteins also participate in establishing microdomains including snapin, a synaptic vesicle associated protein that helps organize a signaling compartment mediated by cAMP/PKA on the synaptic vesicles. This appears to be important in regulating transmitter release and its modulation by PKA activation (Chheda et al., 2001; Chou et al., 2004). Another unique and equally intriguing interaction exists between AC8 and protein phosphatase 2A catalytic subunit (PP2A) (Crossthwaite et al., 2006). As mentioned above, ACs synthesize cAMP, which in turn activates PKA, Epacs as well as HCN channels. Phosphatases like PP2A remove the phosphate group from various targets and thus help to reset the signaling system and restore its homeostasis. Therefore, the existence of ACs, PKA and PP2A in a supramolecular complex serves to channel and integrate signaling (Dai et al., 2009). An interesting phenomenon is the fact that ACs localize differentially in membrane lipid rafts. Since certain receptors are also localized to lipid rafts, simultaneous presence of ACs in these lipid rafts will place them in the vicinity of these receptors and thus facilitate signal transduction. For example, it is known that AC is localized in the same membrane rafts to which β-adrenergic (βAR), but not EP2, receptors localize. This allows for a more robust cAMP synthesis in response to receptor stimulation by β-adrenergic receptor but not EP2 receptor agonists (Ostrom et al., 2001). ACs are also subject to extensive posttranslational modifications which regulate multiple facets of their activity (Cooper, 2003; Willoughby and Cooper, 2007). It is interesting to speculate that posttranslational modification might influence ACs enzymatic activity or targeting of the enzymes to various cell membrane compartments through modulation of their association with anchor proteins or with lipid rafts.

Cyclic AMP/PKA pathway in sensory neuronal sensitization

The effect of activating the cAMP signaling pathway on sensitization of sensory neurons has been demonstrated many times using different methods and end points. Ferreira and his group first speculated about the connection between adenylyl cyclase and hyperalgesia. In their seminal work, Ferreira and Nakamura used PGE_2, isoprenaline, epinephrine and norepinephrine and demonstrated that these agents cause dose-dependent hyperalgesia (Ferreira and Nakamura, 1979). All of these compounds activate $G_{\alpha s}$-coupled receptors. Two experiments initially suggested that the cAMP pathway was involved in PGE_2-induced sensitization. The first experiment

involved the intraplantar injection of dibutyryl-cAMP, a metabolically-resistant analogue of cAMP. Ferreira and co-workers showed that dibutyryl-cAMP causes dose-dependent hyperalgesia. The second one showed that xanthines (caffeine and theobromine) enhance hyperalgesia caused by PGE_2 and dibutyryl-cAMP. The xanthines are phosphodiesterase inhibitors and thus would be expected to increase cAMP concentration if PGE_2 was coupled to the $G_{\alpha s}$/cAMP pathway (Ferreira and Nakamura, 1979). Levine and his group were the first to show that adenylyl cyclase activation using forskolin causes hyperalgesia (Taiwo and Levine, 1991). It is known now that cAMP has several intracellular effectors but at the time Ferreira and his group conducted this pioneering research, the only known effector was cAMP-activated protein kinase (PKA) (Walsh et al., 1968b). PKA is a heterotetramer made of two regulatory subunits and two catalytic subunits. There are four isoforms of the regulatory subunit (RIα, RIβ, RIIα and RIIβ) and two catalytic subunits (Cα and Cβ). One regulatory subunit binds one catalytic subunit. The two regulatory subunits are bound together to form the heterotetramer (Johnson et al., 2001; Taylor et al., 2005). As mentioned before, PKA-mediated signaling is highly compartmentalized, through interaction with multiple AKAPs. This interaction occurs between the regulatory subunits of PKA and the AKAP (Beene and Scott, 2007; Carnegie et al., 2009; Herberg et al., 2000; Welch et al., 2010).

In a subsequent work by Levine and co-workers, a causal connection was made between PKA and hyperalgesia. They showed that inhibition of PKA using Rp-cAMPS (a competitive inhibitor of and an analogue of cAMP that prevents it from activating PKA) attenuated hyperalgesia caused by a several agents that elevate cAMP levels such as forskolin, PGE_2, PGI_2 and adenosine receptor A2 agonist (Taiwo and Levine, 1991). Vasko and his group used cholera toxin (CTX) to irreversibly activate the heterotrimeric G-protein $G_{\alpha s}$ and thus increase adenylyl cyclase activity and elevate cAMP concentration (Northup et al., 1980). Vasko and co-workers demonstrated that locking the $G_{\alpha s}$ in the activated conformation in sensory neuronal cultures using CTX caused augmentation of bradykinin or capsaicin-evoked iSP and iCGRP (Hingtgen et al., 1995). They also showed that inhibition of adenylyl cyclase using 9-tetrahydro-2-furyl adenine abolished PGE_2-induced augmentation of neuropeptide release (Hingtgen et al., 1995).

In addition to the early experiments using animal behavior as an end-point for neuronal sensitization described above (Ferreira and Nakamura, 1979; Taiwo et al., 1989; Taiwo and Levine, 1991), activation of the cAMP signaling pathway was also shown to stimulate neuropeptide release from DRG cultures (Hingtgen et al., 1995),

increase the number of action potentials generated by sensory neurons in response to an activator (Cui and Nicol, 1995), sensitize small unmyelinated sensory fibers to heat (Kress et al., 1996), increase TRPV1 channels activity (Np_o, which is an integrates the total number of channels expressed on the cell membrane and the probability of opening of each channel) (Lopshire and Nicol, 1998), reduce potassium currents (Evans et al., 1999), enhance calcium conductance (Nicol et al., 1992), increase sodium channel Nav1.8 trafficking to cell membrane (Liu et al., 2010b), and increase the tetrodotoxin-resistant (TTX-R) sodium current in sensory neurons (England et al., 1996b; Gold et al., 1996). PKA produces these effects on sensitization via phosphorylation of different protein targets; TRPV1 channel (Bhave et al., 2002) and TTX-R sodium channels (Fitzgerald et al., 1999). PKA also phosphorylates and thus modulates synaptic vesicle proteins including cysteine ring protein, snapin, synapsin I and tomosyn which augments the rate at which synaptic vesicles fuse to the cell membrane and thus augments the release of neurotransmitters; i.e. sensitization of neurons (Chen et al., 2011; Chheda et al., 2001; Chou et al., 2004; Cousin and Evans, 2011; Evans and Morgan, 2003; Menegon et al., 2006). Evidence exists that PKA has a role in inflammatory hyperalgesia but not neuropathic hyperalgesia. As mentioned earlier, PKA-mediated signaling is highly compartmentalized through an interaction between regulatory subunit RI and AKAPs. Therefore mutation of RI subunit of PKA will cause the inability of a specific pool of PKA to localizes to the appropriate domains and thus inhibit the functions it mediate. Mice with a deletion of the neuronal selective isoform of RI subunit (RIβ) showed attenuated inflammatory but not neuropathic sensitization (Malmberg et al., 1997). This indicates that specific isoforms of PKA regulatory subunits mediate different functions in different types of chronic hyperalgesia.

EPAC and HCNs

Although it was thought that PKA-mediated phosphorylation was the only effector for cAMP, we now know that cAMP also can activate a group of G-protein exchange factors (GEFs), namely Epacs (de Rooij et al., 1998; Kawasaki et al., 1998). Epacs have multiple downstream effectors including the small G-proteins Rap, Ras and Rit (Lopez De Jesus et al., 2006; Roscioni et al., 2008). It is noteworthy that several groups reported that Rit is particularly important for Erk phosphorylation following stimulation of PC12 cells using NGF (Shi et al., 2006; Spencer et al., 2002). The most commonly

studied signaling protein downstream of Epacs is the small G-protein, Rap1 which in turn activates several signaling pathways (Roscioni et al., 2008). Recently, Epacs have been implicated in mediating persistent PGE_2-induced hyperalgesia (Eijkelkamp et al., 2010b; Hucho et al., 2005; Wang et al., 2007). The small molecule inhibitor of PKA, H-89, did not completely abolish PGE_2-induced hyperalgesia in models of chronic inflammation such as adjuvant-induced arthritis. Also 8-pCPT-2'-O-MecAMP, a cAMP analogue [colloquially known as 007 (Gloerich and Bos, 2010)] that selectively activates Epac but not PKA (Rehmann et al., 2003), was able to induce pain-like behavior in animal models of chronic hyperalgesia to a greater extent and for a more prolonged duration (Eijkelkamp et al., 2010b; Hucho et al., 2005). This is particularly exciting since these studies show that Epac is involved in the prolongation of the duration of hypernociception, but not in acute inflammatory pain. However none of these groups attempted to show a causal relationship between activation of Epac and chronic hyperalgesia, thus further studies are needed. To date, PKCε-mediated signaling is the most studied pathway downstream from Epac. However several other signaling pathways can be activated by Epac, thus meriting studying them.

HCN channels are another group of cAMP effectors, that recently emerged as potentially important effector of sensitization (Emery et al., 2011a; Emery et al., 2012; Takasu et al., 2010; Weng et al., 2012) long after their initial discovery (Fesenko et al., 1985). HCN2 was shown to mediate not only persistent inflammatory pain, but also neuropathic pain as well (Emery et al., 2011c). HCN2 represent an even more attractive target for development of potential therapeutic agents since selective inhibitors of the channel already exist (Shin et al., 2001).

Phosphodiesterases

The notion of the existence of cAMP phosphodiesterase (PDE) was first proposed by Rall and Sutherland in 1958 and the enzyme was subsequently isolated by Sutherland and his group (Butcher and Sutherland, 1962; Rall and Sutherland, 1958). In general, PDEs cleave the phosphodiester bond in both cAMP and 3',5'-cyclic guanosine monophosphate (cGMP) into their corresponding non-cyclic forms (Beavo et al., 1994). PDEs comprise one of the most complex enzyme superfamilies with 21 genes encoding proteins that are grouped into 11 subfamilies. Different splice variants exist for multiple isoforms, leading to a total of 50 different proteins (Boswell-Smith et al., 2006; Conti and

Beavo, 2007; Lugnier, 2006). In addition to representing a diverse group of enzymes that differ in their kinetics and sensitivity to various inhibitors, they are also localized in different subcellular compartments and are regulated by various signaling pathways (Houslay and Milligan, 1997). Cyclic AMP compartmentalization is not only linked to AKAPs, but also to PDEs. It is thought that PDEs help break down cAMP (and cGMP) in order to restrict its presence to a particular compartment and thus prevent the second messenger from activating effectors outside a specific microdomain and thus contributes to specificity of signaling (Baillie et al., 2005; Houslay, 2010). As described above, much evidence supports that cAMP mediates the signaling pathways causing hyperalgesia. Thus it seems logical that inhibition of cAMP breakdown, via inhibition of PDEs, would potentiate hyperalgesia. Indeed, rolipram which is a PDE4 inhibitor, potentiates hyperalgesia produced by a number of inflammatory mediators including PGE_2, TNF-α, IL-1β, IL-6 and inflammagens such as carrageenan (Cunha et al., 1999). Inhibition of only PDE4, but not PDE3 or PDE5 potentiated hyperalgesia (Cunha et al., 1999). In contrast, rolipram produced anti-inflammatory effects in particular aspects of other models of inflammation in experimental animals. Specifically, rolipram reduced lipopolysaccharide-induced TNF-α production, swelling in carrageenan-induced paw edema and production of TNF-α and swelling in collagen-induced arthritis (Sekut et al., 1995). The contradictory effects of rolipram may depend on the effect of elevating cAMP in different cell types. For example, elevation of cAMP in nociceptive sensory neurons is associated with their sensitization. On the contrary, cAMP elevation in immune cells results in their suppression which may explain reduction of release of TNF-α in the above mentioned study (Peters-Golden, 2009; Teixeira et al., 1997). When administered orally, rolipram attenuates hyperalgesia in chronic inflammation animal models (Francischi et al., 2000). This led to the speculation that selective PDE4 inhibitors might be an attractive path for developing novel therapies for chronic pain.

A-kinase anchor proteins

It is estimated that an average mammalian cell contains approximately 1,000,000,000 protein molecules (Sims and Allbritton, 2007). Considering the enormity of this figure, the fact that cells respond specifically and differentially to various stimuli is an astonishingly remarkable feat. Over the past two decades, evidence accumulated showing that the level of complexity of signaling is possible because of, and regulated

by, compartmentalization (Scott and Pawson, 2009). In order for cells to be able to respond specifically to external stimuli, it is logical that proteins in a signaling cascade are coupled or held together through binding to a scaffold. Indeed, multiple scaffolding and anchoring proteins have been characterized and these proteins provide platforms for various components of a signaling pathway (Wong and Scott, 2004). Among the most famous of these anchoring proteins are the AKAPs. The human genome contains 20 different genes encoding for AKAPs and there are multiple splice variants for many of these genes, which lead to the existence of approximately 75 different AKAP isoforms (Scott and Pawson, 2009). Compartmentalization by AKAPs is visualized in myocardial cells where gradients of cAMP can be visually detected using a fluorescent FRET reporter (Zaccolo and Pozzan, 2002). Furthermore cAMP compartmentalization occurs in invertebrate and mammalian neurons (Bacskai et al., 1993; Hempel et al., 1996a; Klauck et al., 1996) and appears to be essential for development of dendrites and axons in embryonic hippocampal neurons (Shelly et al., 2010).

Multiple anchor proteins are expressed in sensory neurons including yotiao, AKAP12 (AKAP250, gravin), AKAP5 (AKAP79/150) and AKAP15/18 (Irmen et al., 2008; Rathee et al., 2002a; Schnizler et al., 2008). It was found that inflammatory mediators that activate $G_{\alpha s}$-coupled GPCRs require an AKAP in order to produce sensitization. AKAP5 is essential for sensitization of TRPV1 by PGE_2 *in vitro* (Schnizler et al., 2008) and *in vivo* (Jeske et al., 2008). AKAP5 is also essential for the development of sensitization by bradykinin through activation of PKC, ligand-mediated activation of TRPV1 in the absence of any sensitizing agents and trafficking of the channel to the cell membrane (Zhang et al., 2008). AKAP5 also binds PP2B (calcineurin) in sensory neurons and thus facilitates dephosphorylation of TRPV1 channels under resting conditions. This means that not only does AKAP5 mediate sensitization, but also it helps maintain TRPV1 channel from being sensitized under basal conditions (Zhang et al., 2008). Other AKAPs, such as AKAP12 (AKAP250, gravin) (Irmen et al., 2008) and yotiao (Schnizler et al., 2008), also are expressed in DRG neurons and their functions are yet to be identified. AKAPs are also important for modulating the function of other types of neurons. For example dopamine modulates peak sodium currents through phosphorylation of sodium channels in hippocampal pyramidal neurons, and this phosphorylation requires AKAP15 (Few et al., 2007). In rat cortical neurons, AKAP5 was also important in keeping ASIC1a and ASIC2a channels quiescent through anchoring calcineurin which maintains ASIC channels in a dephosphorylated state. It is postulated

that anchoring calcineurin to ASIC channels by AKAP5 is of crucial importance during pathological conditions such as stroke. Intracellular [Ca^{2+}] occurs during a stroke as well as acidosis. Increase in protons concentration due to acidosis leads to augmentation of ASIC channels activity which increases the current of positively charged cations flowing in the neurons leading to further depolarization and further increase in $[Ca^{2+}]_i$, eventually culminating in neuronal death (Chai et al., 2007). In heterologous expression systems, it was found that AKAP5 also targets PKA and calcium/calmodulin to potassium channels thus allowing modulation of the function of these channels by the anchored signaling modules (Bal et al., 2010; Dart and Leyland, 2001). These targets are expressed in sensory neurons (Huang et al., 1998; Julius and Basbaum, 2001; Lingueglia et al., 1997) but direct physical or functional interaction with AKAP is yet to be determined.

Protein phosphatases

The human genome contains over 500 kinases, roughly 400 are serine/threonine kinases while the rest are tyrosine kinases (Manning et al., 2002; Shi, 2009). Kinases derive their specificity from the diversity in their primary structure despite the high conservation of their kinase catalytic domain. Approximately 33 percent of all the proteins in any given eukaryotic cell are post-translationally modified by phosphorylation (Mann et al., 2002). Since post-translational modification often leads to alteration of the state of the cell in response to an environmental change, it is logical to assume that a built-in mechanism must exist to allow reversal of this phosphorylation; i.e. dephosphorylation through phosphatases. Thus an a priori logical assumption is that for each kinase there is a phosphatase to reverse its function and offer the same level of selectivity. This is true for protein tyrosine phosphatases (PTPs); there are roughly 107 putative PTPs (Alonso et al., 2004). However, there are merely 30 serine/threonine protein phosphatases (PSPs) in the human genome (Shi, 2009). The imbalance in the number of the serine/threonine kinases and PSPs can be understood by the manner in which PSP holoenzymes are formed. PSPs catalytic subunits associate with an assortment of regulatory subunits, leading to formation of large number of holoenzymes that differ in their function dramatically (Shi, 2009). The holoenzyme undergoes the dephosphorylation and not the catalytic subunit. Therefore it is the combined diversity of the catalytic and regulatory subunits that determine the actual number of holoenzymes and thus the biologically functional phosphatase. For example PP1 consists of two

subunits, catalytic and regulatory. There are close to a 100 regulatory subunits and three catalytic subunits of PP1 (Shi, 2009). This means there are three hundred possible holoenzyme combinations. PP2A, on the other hand, is formed of three subunits, catalytic, regulatory and scaffolding subunits. Catalytic and regulatory subunits have two isoforms each. Moreover, regulatory subunit is made up of four families, each has several members, and some of the isoforms have multiple splice variants. This means there are potential 30-160 holoenzymes of PP2A. Thus, combinatorial association is the basis for diversity in phosphatases (Shi, 2009). This mechanism enables phosphatases to attain high selectivity utilizing relatively few phosphatase isoforms. At the same time, this is one of the reasons why the study of phosphatases is more challenging since there are few tools to functionally discriminate between these closely related holoenzymes (Sim and Ludowyke, 2002; Virshup and Shenolikar, 2009).

Owing to this remarkable complexity of phosphatases, interactions with other proteins are difficult to study and delineate. It is thought that the known interactions with phosphatases is a tiny fraction of their actual interactome (Virshup and Shenolikar, 2009). As mentioned above AKAP5 anchors calcineurin to ASIC channels which helps maintain them in the dephosphorylated state and keeps their activity low to protect cortical neurons from neuronal death subsequent to over-stimulation of ASIC channels (Chai et al., 2007). Calcineurin anchored through AKAP5 to TRPV1 channel helps desensitize the channel activation to repeated exposure to capsaicin (Zhang et al., 2008).

Protein phosphatases interact with AKAPs (Schillace and Scott, 1999) suggesting their involvement in the signaling complexes either by affecting other signaling molecules (for example PKA, PKC, ACs or PDEs), or by dephosphorylating substrates such as receptors or channels (Collas et al., 2004).

Inhibition of PSPs by okadaic acid was found to augment neuropeptide release from embryonic sensory neuron cultures in vitro (Hingtgen and Vasko, 1994a). Calcineurin inhibition leads to attenuation of TRPV1 desensitization in adult rat DRG neurons (Docherty et al., 1996) and *in vivo* (Noda et al., 2008; Sato et al., 2007). It was found that calcineurin inhibition attenuated heterologous desensitization of TRPV1 by activating TRPA1 (Ruparel et al., 2008). Cannabinoids, which are purported to have analgesic properties, induce dephosphorylation and hence desensitization of TRPV1 channel in a calcineurin dependent manner (Jeske et al., 2006; Patwardhan et al., 2006). To sum up, phosphatase inhibition in sensory neurons causes sensitization,

presumably by preventing dephosphorylation and thus enhancing phosphorylation of various effectors.

Phosphatase activity is important for axonal regeneration and sprouting, synaptogenesis and neuronal plasticity. Phosphorylation of tyrosine residues on $G_{\alpha s}$ by epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, increased its activity and enhanced cAMP synthesis by the coupled AC in the rat brain (Poppleton et al., 1996). It was found that PTP1B is essential for NGF mediated signaling (Shibata et al., 2008). Phosphatase and tensin homologue deleted on chromosome10 (PTEN), a phosphoinositide phosphatase as well as a PTP, was found to exert tonic inhibition of axons regeneration and neuronal plasticity in adult rat peripheral DRGs (Christie et al., 2010). These data suggest that inhibition of PTEN might be a useful therapeutic approach to enhance peripheral axonal regeneration. On the other hand, during embryologic development, calcineurin is required for neurotrophin mediated axonal sprouting from the DRGs (Graef et al., 2003). Another PTP, PTP receptor T, was also found to enhance synapse formation in brain neurons, one of the standard definitions of neuronal plasticity (Lim et al., 2009). MAP kinase phosphatase1 (MKP1) was also found to be essential for BDNF-induced axonal branching in CNS neurons (Jeanneteau et al., 2010).

The effects of phosphatases on synaptic plasticity were extensively reviewed by Winder and Sweatt (Winder and Sweatt, 2001). GPCRs can interact both functionally and physically, with PP2A (Pitcher et al., 1995). Furthermore, the “metastasis suppressor” GPCR, GPR54, is coupled to PP2A (Evan et al., 2008), suggesting that PP2A may dephosphorylate critical signaling molecules and thus inhibit metastasis. Another mechanism by which phosphatases might interact with GPCRs is through the adaptor protein β-arrestin, which regulates the signaling outcome of PTEN activation (Lima-Fernandes et al., 2011). PKA phosphorylates and thus inactivates an inhibitor of a PP1 isoform, myosin light chain phosphatase (MLCP), thus leading to its activation (Aslam et al., 2010). PP2A also can be activated in a cAMP-dependent but PKA-independent fashion via Epac in cell lines (Feschenko et al., 2002; Hong et al., 2008). Epac was also found to modulate the activation of MLCP (Roscioni et al., 2011). In the myocardium, Epac mediates cardiac myocyte hypertrophy through calcineurin (Metrich et al., 2008). Tyrosine phosphorylation enhances PP2A phosphatase activity (Chen et al., 1992). In the liver, this “PTP/PP2A axis” activates lipogenesis (Shimizu et al., 2003).

On the other hand agents that elevate cAMP or activate PKC signaling, activated the phosphatase activity of PTP (Brautigan and Pinault, 1991b).

PKC

It is generally accepted that PKC does not contribute to acute PGE_2-induced sensitization of sensory neurons. However, some EP receptors are coupled to PKC-mediated signaling pathway (see the section of Eicosanoids within this introduction for detailed discussion). Moreover, it was shown before that PKC can desensitize GPCRs (see section Receptor Desensitization for detailed discussion and examples). Therefore brief revision of the biology of PKC is essential.

There are three different classes of PKC with 12 different isozymes (Newton, 2001) and various PKCs mediate peripheral sensitization in inflammatory and neuropathic pain. Activation of PKC causes sensitization of adult DRG neurons in culture when activated by capsaicin (Barber and Vasko, 1996) and this is mediated in part by phosphorylation of TRPV1 channels leading to enhancement of channel activation (Bhave et al., 2003; Cesare and McNaughton, 1996; Crandall et al., 2002; Numazaki et al., 2002). PKC also can increase tetrodotoxin-sensitive (TTX-S) sodium currents (Costa and Catterall, 1984; Murphy and Catterall, 1992; Numann et al., 1991) and more substantially TTX-R sodium currents (Gold et al., 1998; Khasar et al., 1999b). It also is postulated that PKC activity may be essential for PKA modulation of these channels since selective peptide inhibitors of PKC impaired the ability of PKA to enhance TTX-R sodium channels activity (Gold et al., 1998). Involvement of PKC in hypersensitivity in animal models of inflammation has also been shown (Souza et al., 2002). PKC indirectly augments activation of NMDA receptors by its ligands in the central terminals of sensory neurons in the dorsal horn of the spinal cord. PKC phosphorylates a tyrosine protein kinase known as pyk2, which in turn phosphorylates and activates the tyrosine kinase src (Lu et al., 1999). Subsequently, src kinase phosphorylates a tyrosine residue on the NMDA receptor and increases its activation (Woolf and Salter, 2000; Yu et al., 1997). Several studies show that sensitization of sensory neurons by inflammatory mediators, including bradykinin, TNF-α and protease-activated receptor 2, is mediated by PKCε

(Amadesi et al., 2006; Cesare et al., 1999; Parada et al., 2003b). Levine and his group advanced the concept that development of persistent sensitization, which they labeled hyperalgesic priming, depends on PKCε (Hucho and Levine, 2007; Khasar et al., 1999a; Reichling and Levine, 2009).

Receptor desensitization

As mentioned above, the aims of the work presented in this dissertation include determining whether there is desensitization of PGE_2-activated PKA after long-term exposure to NGF or the prostanoid, and the mechanisms underlying such desensitization. Since PGE_2 activates PKA through a family of GPCRs (see above in the section on Eicosanoids), I considered known mechanisms of desensitization of GPCRs for the desensitization of PGE_2-activated PKA. The various mechanisms proposed for desensitization are discussed in this section.

History of discovery of the role of Grks/β-arrestins to desensitization

Following the exposure of any organism to a given stimulus, three events occur; 1) the stimulus must evoke an adaptive response in the organism to enable it to cope with its environment, 2) excessive stimulation must be avoided to prevent a detrimental maladaptation and 3) the receptor and the signaling pathway coupled to it must return to the resting baseline state in order to restore the ability of the organism to respond to subsequent stimuli. Desensitization describes the processes of inactivating receptors and shutting off their coupled signaling pathways to avoid excessive stimulation. Resensitization describes the process by which the receptor and the signaling pathway regain their resting sensitivity (Grady, 2007; Lohse, 1993; Sibley and Lefkowitz, 1985). This section will focus on the process of receptor desensitization.

GPCRs comprise a huge family of proteins; approximately 800 of known and unknown functions exist in the human genome (Lagerstrom and Schioth, 2008). These receptors serve as sensors for many different endogenous ligands such as neurotransmitters, hormones, and other intercellular signals, and thus it is not surprising that they are also the most commonly used targets for clinical applications (Ma and Zemmel, 2002).

Desensitization is classified into homologous and heterologous desensitization (Lefkowitz, 2004). Homologous desensitization is the desensitization of single receptor in response to all the ligands that bind to and activate this receptor. On the contrary, heterologous desensitization is the indirect desensitization of a receptor through activation of a completely unrelated receptor with its respective ligand. Homologous desensitization is usually thought to be mediated by Grks, whereas heterologous

desensitization by second messenger-activated kinases such as PKA and PKC (Lefkowitz, 2004).

The initial discoveries related to GPCR desensitization were first made using rhodopsin, a light-activated GPCR in the outer segments of rods in the retina which inhibits cGMP PDE when activated by its ligand, photons. In 1972 when Kuhn, Bownds and their co-workers discovered light-dependent phosphorylation of a protein isolated from membrane preparations from the outer segments of rods (Bownds et al., 1972; Kuhn and Dreyer, 1972). The phosphorylated protein was not conclusively identified as rhodopsin until six years later by Shichi and Somers who also isolated the kinase responsible for the observed phosphorylation (Shichi and Somers, 1978). Subsequently, Liebman and co-workers found that the ability of rhodopsin to inhibit cGMP PDE in membrane preparations from the outer segment of rod cells in the retina is significantly reduced after adding ATP to the isolated membranes of outer rods (Liebman and Pugh, 1980). Molecular mechanisms of desensitization of GPCRs were also first discovered while studying β-adrenergic receptors (βARs) (Galas and Harden, 1996). Similar to rhodopsin, βARs were found to be phosphorylated by PKA in turkey erythrocytes (Benovic et al., 1985). The following year, a novel kinase, initially named β-adrenergic receptor kinase (βARK, also known as G-protein coupled receptor kinase [Grk]) that selectively phosphorylates agonist occupied βARs was discovered (Benovic et al., 1986). Purified hamster lung βAR receptors reconstituted in phospholipid vesicles were first phosphorylated by Grk (βARK), which caused limited desensitization of the receptor. Then a novel protein, named β-arrestin, since it was homologous to visual arrestin, was found to bind with high-affinity to the phosphorylated receptor and potently desensitized it (Benovic et al., 1987). β-arrestin is named after the visual arrestin, which is a protein that exerts the same function in the retinal photoreceptors (Wilden et al., 1986). Initially it was thought that the only mechanism, by which β-arrestin desensitizes βAR, is through sterically hindering the interaction between the receptor and its heterotrimeric G-protein (Freedman and Lefkowitz, 1996; Goodman et al., 1998; Krupnick and Benovic, 1998). Later it was also found that β-arrestin recruits PDE to the receptor and thus brings it in close proximity to degrade cAMP and facilitate signal termination (Baillie et al., 2003). Subsequent seminal work by Lefkowitz and his group delineated the specific aspects of Grks and β-arrestins in desensitization of βARs and subsequently many more GPCRs (Pitcher et al., 1998). The role of the Grk/β-arrestin machinery in receptor desensitization, internalization and downregulation from the plasma membrane was

subsequently examined using receptor types other than βARs and using different models (Ferguson, 2001).

Grks/β-arrestins role in alternative signaling

A major discovery was made when it was found that βAR phosphorylation by PKA caused it to shift its coupling from $G_{\alpha s}$, to which it is "canonically" coupled, to $G_{\alpha i/o}$. This pioneering work ushered the concept of signaling switching at the level of the receptor (Daaka et al., 1997). An even more important discovery is that the desensitization machinery, Grks/β-arrestins and their isoforms, also engage and couple to alternative and usually more temporally prolonged signaling pathways (Perry and Lefkowitz, 2002). β-arrestins were found to channel signals from the activated, ligand-bound GPCR to a large variety of signaling pathways such as src kinases (Luttrell et al., 1999), MAPKs (Luttrell et al., 2001; McDonald et al., 2000; Sun et al., 2002), phosphatidyl inositol-3 kinase (PI3K) and protein kinase B (PKB) (McDonald et al., 2000), PP2A (Lin et al., 1997), and inhibitor of κB (IκB) (Lin et al., 1997). Several other interactions were also documented (Lefkowitz and Shenoy, 2005; Shenoy and Lefkowitz, 2005).

Interestingly, it was recently found that some pathogens utilize Grks/β-arrestins mediated internalization of GPCRs to cross cell membranes. For example *Neisseria meningitidis*, the bacterium responsible for causing meningitis in humans, crosses the blood-brain barrier and reaches the brain hijacking Grks/β-arrestins machinery. Meningitis-causing bacteria (meningiococci) possess a hair-like appendage called pilus which is made of a protein called pilin. It was found that meningiococcal type IV pili bind to β2AR and act as biased agonists (see below). This leads to selective recruitment of the Grk/β-arrestin machinery and receptor internalization along with the bound bacterium. Thus meningiococci traverse the blood-brain barrier and gain access to the meninges, their sites of infection (Coureuil et al., 2010; Tourret and Finlay, 2011).

Grks/β-arrestins and desensitization of membrane-bound non-GPCR proteins

The spectrum of membrane proteins that are desensitized by Grks/β-arrestins was expanded when it was discovered that several tyrosine kinase receptors such as insulin-like growth factor 1 (IGF1) receptor 1 (Lin et al., 1998), Na+/H+ exchanger

(Szabo et al., 2005) and TRPV4, a non-selective cation channel (Shukla et al., 2010) are desensitized by Grks/β-arrestins. Several other non-GPCR receptors and ion channels are also regulated by β-arrestins (Shenoy and Lefkowitz, 2011b).

Grks/β-arrestins, biased agonism and receptor barcoding

The concept of biased agonism of GPCRs was brought forth by Lefkowitz and co-workers through their work on Grks/β-arrestins (Rajagopal et al., 2010b). Biased agonism refers to the situation when a ligand/receptor pair which shows great preponderance to signal through heterotrimeric G-proteins versus Grks/β-arrestin-mediated signaling and vice versa (Rajagopal et al., 2010b). Biased agonism could be due to biased ligand, biased GPCR or both. Biased ligands show greater tendency to activate one of multiple possible signaling pathways downstream from a GPCR more than the others, while unbiased or balanced ligands activate these downstream signaling pathways equally (Jarpe et al., 1998; Rajagopal et al., 2010b; Reiter et al., 2012). For example, carvedilol is a βAR antagonist and it inhibits canonical $G_{\alpha s}$-mediated signaling. Yet it is capable of causing βAR receptor phosphorylation, β-arrestin recruitment and activation of the alternative MAPK signaling pathway (Wisler et al., 2007). This is one of the earliest examples of a ligand that is biased towards β-arrestin mediated signaling pathway. CXCL-12 is unique in that it is capable of behaving both as an unbiased and biased ligand. CXCL-12, a chemokine, can exist as monomer or dimer depending on its concentration under physiologic conditions (Ray et al., 2012; Veldkamp et al., 2005). The oligomerization state of the ligand determines which pathway will be activated upon receptor binding (Drury et al., 2011; Ray et al., 2012). Thus a single ligand is biased to activate two different pathways depending on whether it is a monomer or a dimer.

Biased receptors are tonically biased towards one signaling pathway, irrespective of the ligand that binds and activates them (Rajagopal et al., 2010b). Obviously, GPCRs which are phosphorylated by Grks are biased towards Grk/β-arrestins mediated signaling. Under normal conditions, some receptors were found to be biased towards Grks/β-arrestins-mediated signaling. These receptors were referred to as “decoy” since they were able to bind their cognate ligand with high-affinity, however, this binding could not significantly activate heterotrimeric G-protein signaling (Rajagopal et al., 2010a). It is now known that some of these decoy receptors are not silent; they activate alternative signaling pathways that were not considered and hence not tested before. The

chemokine receptor CXCR7 is an example of these receptors (Rajagopal et al., 2010a). Recently it was shown that GPCR heterodimerization can cause an unbiased receptor to become biased. For example, α1 adrenergic receptor, which is $G_{\alpha q/11}$-coupled under resting conditions, becomes biased towards Grk/β-arrestin-mediated signaling after heterodimerization with the chemokine receptor CXCR2 (Mustafa et al., 2012).

There are 7 different isoforms of Grks (1-7). Grk isoforms 1 and 7 are referred to as visual Grks since they are exclusively expressed in the retinal rods. Grks 2, 3, 5 and 6 are ubiquitously expressed, while Grk4 expression is restricted to the testes (Premont and Gainetdinov, 2007). Recently, Lefkowitz and his group found that different Grks phosphorylate different residues of βAR and that receptors phosphorylated on different residues recruit and activate different signaling pathways after long-term exposure to isoproterenol (Nobles et al., 2011). The phenomenon was termed “receptor barcoding” since the signaling outcome of receptor is dependent on phosphorylation of various sites by various Grks (Liggett, 2011; Nobles et al., 2011).

The classical view of any receptor is that it exists in either an active or inactive conformation or states which exist in equilibrium. Agonists shift this equilibrium towards the active conformation, while antagonists shift it in the opposite direction. Partial agonists and mixed agonists-antagonists shift the equilibrium incompletely towards the activated conformation (Kenakin, 1997). Because of the extensive work by Lefkowitz and co-workers that was briefly described above, receptors are now viewed as microprocessors with “pluridimensional efficacies” (Kenakin, 2011; Kenakin, 2009). A receptor can be activated by different ligands some of which may be biased. Ligands that are more biased towards particular one or more downstream signaling pathways than others activate these pathways more. Unbiased ligands activate all downstream signaling pathways with equal efficiency. Different ligands with bias towards different pathways will produce different biological outcomes (Kenakin, 2011; Rajagopal et al., 2010b). This novel view of GPCRs action shall deeply impact drug discovery.

Grks-dependent β-arrestin independent actions

Initially it was thought that the only function of Grks was to phosphorylate GPCRs and thus create high-affinity binding sites for β-arrestins which bind to and physically uncouple the GPCR from its heterotrimeric G-protein (Lohse et al., 1992). Thus it appears that Grks are needed but not sufficient for desensitization of GPCRs and

initiation of alternative β-arrestins-mediated signaling. However, as knowledge about Grks increase, it is clear that this notion is a drastic oversimplification of a complex and versatile signaling molecule.

In 1999, it was found that kinase-negative mutants of Grks 2, 3 and 5 were able to desensitize parathyroid hormone receptors which are both $G_{\alpha s}$ and $G_{\alpha q/11}$ coupled (Dicker et al., 1999). This observation was confirmed when it was found that these kinase-dead mutants of Grks 2, 5 and 6, were also able to desensitize GPCRs activated by follicle stimulating hormone (Reiter et al., 2001). Both of these studies clearly indicate that with certain receptors and in certain cell types, Grks are both necessary and sufficient for desensitization. Indeed a whole host of receptors were found to be desensitized in a β-arrestin independent but Grk-dependent fashion (Dhami et al., 2004; Freedman et al., 1997; Perroy et al., 2003; Willets et al., 2005; Willets et al., 2004).

Not only are Grks necessary and sufficient for mediating desensitization and uncoupling, but also for mediating internalization. Hosey and co-workers found that muscarininc receptors can get internalized independent of β-arrestins (Lee et al., 1998; Pals-Rylaarsdam et al., 1997). It was also found that Grk2 contains a “clathrin-box” that is able to bind clathrin and promote βAR1 receptors internalization (Shiina et al., 2001). In this study it was also shown that phosphorylation of the receptor might not be required for internalization. This implies that the kinase function of Grks might not be necessary. A number of receptors and their cognate ligands that can internalize in an arrestin-independent manner have since been identified (Bhatnagar et al., 2001; Fernandez et al., 2011; Giebing et al., 2005; Heding et al., 2000; Ribeiro et al., 2009; Zhang et al., 1996).

It was found that Grks can desensitize membrane proteins other than GPCRs, such as inward rectifying potassium channels(GIRK) (Raveh et al., 2010). In this study the authors showed that GIRK desensitize in a Grks/β-arrestins dependent manner if they were activated by stimulation of μ-opioid receptors. However, when stimulated by muscarinic cholinergic receptors, GIRKs desensitize in a β-arrestins-independent Grk-dependent and non-enzymatic manner in hippocampal neurons (Dang et al., 2009; Raveh et al., 2010).

Possible mechanism of action of some of Grks functions that are β-arrestin-independent

The literature review presented above clearly demonstrates that Grks can uncouple and mediate internalization of some GPCRs and that the kinase function of Grks is not always necessary for desensitization or internalization. Available evidence suggest that an important structural feature of Grks, the regulator of G-protein signaling homology (RH) domain is responsible for mediating these non-enzymatic functions of Grks (Shiina et al., 2001; Sterne-Marr et al., 2004). Grks contain three main domains, a central kinase domain, a C-terminus pleckstrin homology domain (which $G_{\beta\gamma}$ dimers) and an N-terminus RH domain (Ferguson, 2007). It was initially found that Grk2 can bind $G_{\alpha q/11}$ in bovine brain extracts as well as in live HEK293 and COS cells (Carman et al., 1999). Subsequently, many studies showed that Grks can attenuate $G_{\alpha q/11}$-coupled GPCRs signaling in a kinase-independent manner via their RH domain (Dhami et al., 2002; Freedman et al., 1997; Sallese et al., 2000; Usui et al., 2000). Moreover, $G_{\alpha s}$-coupled GPCRs were also found to be regulated by RH domain of Grks such as the serotonin 5HT4 receptor and the histamine H2 receptor (Barthet et al., 2005; Fernandez et al., 2011).

MATERIALS AND METHODS

Experimental animals

The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies.

Materials

F-12 media, FBS, glutamine, penicillin-streptomycin, fungizone, NuPAGE Novex 4-12 percent bis-tris bel (1.5 mm, 10 well), SeeBlue Plus2 pre-stained standard, NuPAGE LDS sample buffer, NuPAGE antioxidant, NuPAGE MES SDS running buffer, NuPAGE transfer buffer and Invitrolon PVDF/filter paper sandwiches were obtained from Invitrogen, Carlsbad, CA (cat nos. 21700-075, 16000-036, 25030-081, 15070-063, 15290-018, NP0335BOX, LC5925, NP0007, NP0005, NP0002, NP0006-1, LC2005) and Normocin from InvivoGen (cat no. ant-nr-2, San Diego, CA). PKA inhibitor fragment 5-24, the small molecule PKA inhibitor H-89 and its substrate kemptide (cat nos. P7739, B1427, K1127, respectively), Poly-D-lysine (cat no. P0899), laminin (cat no. L2020), collagenase (cat no. C9891), 5-fluoro-2'-deoxyuridine (cat no. F0503), uridine (cat no. U3750), capsaicin (cat no. M2028), sodium vanadate (cat no. S6508), 1-methyl-2-pyrrolidinone (MPL, cat no. 494496), cholera toxin (CTX, cat no. C8052) and other routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). PGE_2, $cPGI_2$, L902688, PTP1B and cAMP EIA kit were purchased from Cayman Chemicals, Ann Arbor MI (cat no. 14010, 18210-1, 10007712-1, 10010896 and 581001-480 respectively). Protease inhibitor cocktail Set III, EDTA free (cat no. 539134), phosphatase inhibitor cocktail set I (cat no. 524624), okadaic acid (cat no. 495604) and anti-AKAP 150 antibody (cat no. 07-210) were obtained from EMD Millipore, Darmstadt, Germany. IBMX was obtained from Tocris Bioscience, Minneapolis, MN (cat no. 2845). Mouse NGF 7S was obtained from Harlan (cat no. BT.5023). Calcineurin autoinhibitory peptide (cat no. 1891) and rat CGRP (cat no. 1161) were purchased from Tocris. (Tyr27)-α-CGRP (27-37) was acquired from Bachem (cat no. H-5504). AG 1-X8, P-4 resins, Bio-Rad protein assay dye reagent concentrate and protein standard I (bovine γ-globulin) were purchased from Bio-Rad (cat no. 140-1441, 150-4114, 500-0006, 500-0005). Peroxidase-AffiniPure donkey anti-goat IgG (H+L) was purchased from Jackson

laboratories, Bar Harbor, ME (cat no. 705-035-003). Protein phosphatase inhibitor 1 (I-2) was purchased from New England Biochemicals (cat no. 50811860). Microcystin-LR (MCS-LR) was acquired from Enzolife (cat no. ALX-350-012). [γ-P^{32}]-ATP, $Na^{125}I$ and Western lightning plus-ECL were purchased from Perkin-Elmer (cat no. Blu502A, NEZ033, NEL104001EA). Phosphatase assay kit was purchased from Sciencell Research Laboratory (cat no. 8108). The transfecting reagent, Lipofectamine RNAi/Max was acquired from Invitrogen. Rat AKAP5, AKAP12, EPs 1-4, Grks 2, 3, 5 and 6 gene expression assay (assay IDs Rn01786021_m1, Rn00588999_m1, Rn00565349_m1, Rn00579419_m1, Rn00562282_m1, Rn00583420_m1, Rn00562822_m1, Rn00563688_m1, Rn00578086_m1, Rn00581369_m1 respectively), GAPDH endogenous control (P/N 4352338E) and TaqMan Universal PCR Master Mix (P/N 4304437) and other real-time PCR supplies were obtained from Applied Biosystems (Carlsbad, CA). Total RNA extraction kit PrepEase Spin Kit (P/N 78766) was purchased from Affymetrix (Santa Carla, CA). Whatmann P81 filter paper discs were acquired from Fisher Scientific, Hampton, NH (cat no. 05-717-2B) and ATP γ-P32 from PerkinElmer Waltham, MA (cat no. BLU502A001MC). B-plus full blue radiographic films were purchased from RPS imaging, Michigan City, IN (cat no. EBA45). Non-fat dry milk was purchased from LabScientific, Livingstone, NJ (cat no. M0841). Throughout the study, PGE_2 was used at 1 µM except when mentioned otherwise. The vehicle for capsaicin, PGE_2, okadaic acid, MCS-LR, $cPGI_2$, L902688, BIM-I, IBMX and forskolin was MPL, for H-89, isoproterenol and sodium vanadate was phosphate-buffered saline and for CTX was a buffer consisting of 0.05 M Tris buffer salts, pH 7.5, 0.2 M NaCl, 0.003 M NaN_3 and 0.001 M sodium EDTA as per Sigma-Aldrich product information.

Cell culture

Preparation of sensory neuronal cultures was performed as described previously with few modifications (Burkey et al., 2004). Male Sprague-Dawley rats (140-145 g) were euthanized by placing them in CO_2-filled chambers for no more than one minute followed by decapitation. Dorsal root ganglia (DRG) were harvested in Puck's solution containing fungizone (250 µl fungizone / 40 ml Puck's solution). Time taken from animal euthanasia to completion of harvest was kept ≈ 1 hour. Puck's solution was removed by aspiration and replaced with 3 ml F-12 media containing collagenase for one hour at 37°C. DRG's were centrifuged at low speed, collagenase-containing F-12 was aspirated and replaced

immediately with fresh F-12 containing normocin and with or without 30 ng/ml NGF. DRGs were mechanically dissociated using a fire polished glass pipette. Approximately 30,000 cells were plated into each well of 12-well culture plates, or 60,000 cells into 35-mm dishes, pre-coated with poly-D-lysine (overnight) and laminin (overnight). Cells were maintained with or without added NGF as indicated, in F-12 media containing normocin and supplemented with 10 percent horse serum, 2 mM glutamine, 100 µg/ml Normocin, 50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µM 5-fluoro-2'-deoxyuridine and 150 µM uridine in saturated humidity and 3 percent CO_2-incubator at 37°C.

Neuropeptide release

Neuronal cultures were washed with HEPES buffer (25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM D-glucose, and 0.1 percent bovine serum albumin, pH 7.4) at 37°C. Afterwards, cultures were exposed to one 10-min incubation in 0.4 ml HEPES in the presence and absence of vehicle or the drug. A second incubation to 0.4 ml of HEPES buffer included 30 nM capsaicin in the presence or absence of vehicle or the drug to stimulate peptide release. This concentration of capsaicin was chosen because it lies within the linear region of the capsaicin concentration vs. immunoreactive CGRP (iCGRP) release curve. A third incubation with HEPES for ten minutes was also performed to assure that after treatment(s) were used, neurons remained viable. At the end of each release, cells were hypotonically lysed by incubation for 10 minutes in 0.4 ml of 0.1 M HCl, to extract total remaining iCGRP in the culture. After each incubation, the buffer was removed, aliquoted, and assayed for iCGRP by radioimmunoassay.

Iodination of CGRP for radioimmunoassay

AG 1-X8 and P-4 resins were swollen for 24 hours in buffers containing 1 M acetic acid and 0.1 percent BSA and 2.19 M Na acetate, pH 5.0 respectively. The P-4 resin was packed in a plastic column plugged with a small piece of glass wool to a height of 15 cm while the AG 1-X8 resin was packed in a Pasteur pipette plugged with a small glass bead to a height of 5 cm. P-4 column was washed and kept moistened by the 1 M acetic acid 1 percent BSA solution while the AG 1-X8 column was washed and kept moistened by a 200 mM Na acetate solution. At the time each column is used, the buffer

was allowed to flow through so that the surface of the resin was exposed. (Tyr27)-α-CGRP (27-37) was dissolved in 1 ml water. In a glass tube, the following was combined; 90 µl of 250 mM buffer pH 4.0, 10 µl reconstituted (Tyr27)-α-CGRP (27-37), 20 µl 4.4 mM chloramine-T solution and 10 µl $Na^{125}I$ solution. After 40 seconds 40 µl of 26.3 mM $Na_2S_2O_5$ to terminate the reaction. The reaction mixture is then loaded on the AG 1-X8 resin column after all the buffer wetting it was allowed to flow through. This step removes the excess unreacted iodide by binding it to the anion-exchange AG 1-X8 resin. After the loaded reaction mixture is absorbed, the column is washed with 0.5 ml Na acetate four times and the flow through is collected every time. Radioactivity in aliquots each of 10 µl of the 4 collected fractions was counted. The fraction with the highest counts was loaded on the P-4 column after the entire wetting buffer on its top was allowed to flow through. This step further purifies the iodinated (Tyr27)-α-CGRP (27-37) from other reaction products. The P-4 column is eluted 16 times using 2 ml of 1 M acetic acid with 1 percent BSA solution and the eluate fractions are collected at 5 minutes intervals. A 10 µl of each of the 16 fractions is counted and the fraction with the highest counts and the two subsequent fractions are selected. Non-specific binding was tested for these three fractions. This was done by first diluting an aliquot from each fraction till it contains 10,000 counts/100 µl using a buffer containing Tris base (2.42 g) BSA 0.1g and dextran (T70) 0.06 g in 100 ml, pH 7.4. A 100 µl aliquot of this solution is then added to 400 µl of the same buffer, 0.5 ml of 1 percent Norite charcoal suspension in 0.1 M phosphate buffer that also contains 50 mM NaCl and 1 percent BSA. Charcoal bound to CGRP peptide (radioactive or not) was centrifuged at 3000 rpm (≈2060 x *g*) for 15 minutes. The supernatant is decanted and counted along with the sediment. Fractions that had radioactivity remaining in the supernatant no more than 1 percent of the total radioactivity was diluted to 1,000,000 cpm/100 µl, aliquoted and stored at -20°C. Radioactive (Tyr27)-α-CGRP (27-37) aliquots can be used until maximum specific binding falls to approximately 30 percent of the total counts.

iCGRP radioimmunoassay

iCGRP was assayed by radioimmunoassay (RIA) as described previously (Chen et al., 1996; Duarte et al., 2011). Briefly, CGRP (Tocris, cat no. 11671) for standard curve was reconstituted in 50 mM Tris-HCl buffer pH 4.0. Further dilutions were done in a buffer containing 0.2 mM Tris base, 0.1 percent BSA, 0.06 percent dextran (T70) at pH 7.4. CGRP was diluted 0-250 fmol in duplicates. A new standard curve was prepared each time an experiment was conducted. An aliquot of 25 µl of 1:65,000 of anti-CGRP antibody (generous gift from M. Iadorola, NIH) were added to each tube of standard and sample and tubes were incubated at 4°C overnight. Another aliquot of 25 µl of ^{125}I-(Tyr^{27})-α-CGRP (27-37) containing approximately 3000 cpm was added to each tube of standard and sample and all tubes were incubated for another overnight at 4°C. Antibody-bound iCGRP was separated by adding charcoal (see above for recipe). Unbound peptide (radioactive or not) adsorbed to charcoal particles was separated by centrifugation at 3000 rpm (≈2060 x *g*) in a centrifuge with swinging bucket rotor. Supernatant containing antibody-bound peptide (radioactive or not) was then decanted in a fresh tube and radioactivity was quantified using gamma scintillation spectrometry. Values of unknown iCGRP released from cultures were calculated using the standard curve assayed alongside the experiment using four point non-linear least-squares regression analysis.

Quantitative PCR

Real-time quantitative PCR was done as described previously (Fehrenbacher et al., 2005). Briefly, 12-day old cultures were washed once in sterile PBS and total RNA was extracted using the PrepEase RNA Spin Kit according to manufacturer's instructions. Two hundred and fifty nanograms RNA were converted to cDNA using iScript cDNA synthesis kit (cat no. 170-8891, Bio-Rad, CA). Quantitative real time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan gene expression assays according to manufacturer's instructions. The real time PCR reaction was run using 7500 fast Real-Time PCR System (cat no. 4351106, Applied Biosystems, CA). A validation experiment for the TaqMan gene expression assays was conducted by running standard curves for the all target transcripts versus GAPDH expression assays and efficiencies were determined. C_T for GAPDH in sensory neuronal cultures did not

change significantly whether the cultures were grown in the absence or presence of NGF (19.49 ± 0.13, N = 3 and 19.45 ± 0.31, N = 3, respectively) or exposed to vehicle or 1 μM PGE_2 for 5 days (19.87 ± 0.03, N = 3 and 10.74 ± 0.08, N = 3, respectively). It was judged that $\Delta\Delta C_T$ method (folds change of expression) is a valid and feasible method of analysis for relative quantification since differences between efficiencies of amplification of different target transcripts versus that of GAPDH, were within acceptable range (Livak and Schmittgen, 2001). Folds change of expression ($\Delta\Delta C_T$) was calculated as follows (Ling et al., 2012; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008):

$$\Delta C_T \text{ treated sample} = C_T \text{ gene of interest} - C_T \text{ GAPDH}$$

$$\Delta C_T \text{ control sample} = C_T \text{ gene of interest} - C_T \text{ GAPDH}$$

$$\Delta\Delta C_T = \Delta C_T \text{ treated sample} - \Delta C_T \text{ control sample}$$

$$\text{Folds change of expression} = 2^{-\Delta\Delta C_T}$$

Western Blot

Cells were scraped in cold PBS using a cell scraper and centrifuged at 14,000 x g at 4°C for 10 minutes. The pelleted cells were resuspended in modified RIPA lysis buffer containing 50 mM Trizma base, 150 mM sodium chloride, 1 percent NP-40, 0.25 percent sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium vanadate, and 25 mM sodium fluoride. The cells were sonicated on ice for 10 seconds. The lysate was centrifuged at 14000 x g at 4°C, and the pellet containing membrane fragments was discarded. The supernatant was assayed for protein content by the Bradford assay according to manufacturer's instructions using bovine γ-globulin for standard curve (0-500 μg/ml). Approximately 50 μg of the protein was loaded on precast bis-tris polyacrylamide gels and run at 200 mV for 30 minutes. The proteins on the gel were transferred to a PVDF membrane at 30 mV for 1 hr. The membrane with transferred proteins was washed once with ddH2O and blocked for 1 hour with 5 percent non-fat dry milk in tris-buffered saline containing 0.1 percent tween-20. The membrane was incubated overnight at 4°C with a 1:500 dilution of the AKAP150 antibody in 5 percent non-fat dry milk in tris-buffered saline with 0.1 percent tween-20. The blot was washed 3 times with tris-buffered saline with 0.1 percent tween-20, each time for 10 minutes. Next, the blot was incubated with a 1:10,000 dilution of a goat anti-donkey antibody coupled to horseradish peroxidase for 1 hour at room

temperature. After washing off the secondary antibody 3 times with tris-buffered saline with 0.1 percent tween-20 for 10 minutes per wash, the blot was incubated with Western Lightning chemiluminescent substrate solution, exposed to light sensitive radiographic film, and the film developed.

Measurement of PKA activity

When DRG cultures were grown in the absence of NGF and incubated for long period of time with a drug treatment (PGE_2, L902688, forskolin or CTX), the media was initially replaced with drug-free fresh media for 20 minutes and cultures kept in the incubator at the time of doing the experiment. In experiments using cultures grown in the presence of NGF, this step was omitted. DRG cultures were exposed to different treatments at 37°C for 10 minutes, followed by two washes in ice-cold PBS. Cultures were lysed in ice-cold 250 µl lysis buffer that contains β-glycerophsophate 25 mM, EGTA 1.25 mM, $MgCl_2$ 10 mM, dithiothrietol 1 mM, protease inhibitors cocktail 2X, NaCl 100 mM and triton-X 100 1 percent. Phosphatase inhibitors cocktail 2X was included both in lysis buffer and in PKA activity assay buffer in all experiments that does not examine the effects on NGF. In experiments involving the use of cultures grown in the presence of absence of NGF, phosphatase inhibitors cocktail 2X, or a single phosphatase inhibiting agent was included both in lysis and PKA activity assay buffers when indicated. Cells were scraped and snap-frozen in liquid nitrogen and then stored at -80°C and assayed 24 hours later. Cell lysates were briefly sonicated three rounds each of 10 one-second bursts at 60 percent of the power and for 60 percent of the time in cup horn sonicator followed by centrifugation for 30 minutes at the maximum speed and then the supernatants were separated. During and in between different manipulations cell lysates and supernatants were constantly kept on ice. Aliquots of 10 µl were added to 40 µl aliquots of PKA activity assay buffer and the reaction was incubated at 30°C for 5 min. In this buffer, kemptide (10 µM), acts as a substrate that is selectively phosphorylated by PKA. At the end of the 5 minutes incubation, 20 µl of this reaction were spotted on P81 filter paper discs, which were washed 5 times, each for 5 minutes, in dilute phosphoric acid and the bound radioactivity was measured. PKA activity was measured as follows; treatment-activated PKA was measured in the presence or absence of PKI (5 µM) and the difference represented selective PKA activation by that treatment. Total PKA activity

was measured also in the presence or absence of PKI (5 µM) after exposure to cAMP (10 µM) and the difference represented selective total PKA activity. PKA data are represented as the ratio of the former to the latter. The following equation describes the calculation.

$$\text{PKA activity} = \frac{\text{Treatment-activated PKA - its nonspecific activity [with PKI 6-24 (5 µM)]}}{\text{Maximum PKA activity [with cAMP (10 µM)]-its nonspecific activity [with PKI 6-24 (5 µM)]}}$$

siRNA treatment

At the time of treating DRG cultures with siRNA, F-12 media was removed and replaced with equal volume of Opti-MEM I. Meanwhile, siRNA-Lipofectamin complexes were prepared as described previously (Vasko et al., 2011). Briefly, siRNA (custom synthesized by Dharmacon) was dissolved in siRNA buffer at the concentration of 20 µM (cat no. B-002000-UB-100, Dharmacon), aliquoted and stored in -80 °C till the time of the experiment. Two solutions are to be prepared; siRNA-Opti-MEM I solution and Lipofectamine/RNAiMAX-Opti-MEM I solution. The siRNA solution in Opti-MEM I solution is composed of either 5 µl or 2.5 µl of the 20 µM siRNA stock solution and the volume q.s.ed to 50 µl with Opti-MEM I, to obtain a final concentration of 100 or 50 nM of siRNA in the 35 mm culture dish, respectively. The Lipofectamine/RNAiMAX solution was kept at 6 µl/50 µl Opti-MEM I. Then both solutions were combined into a one 100 µl mixture, which was left for 20 minutes at room temperature to form the siRNA-transfecting agent complexes. When the siRNA complexes were ready, medium in the cultures grown in 35 mm dishes was replaced again with a 900 µl of fresh Opti-MEM I. The siRNA complexes suspension was added to these dishes and cells were returned to the incubator for 24 hours. At the end of the first 24 hours, the Opti-MEM I was replaced with regular F-12 media and cells were returned to the incubator for another 24 hours, before the second siRNA treatment was applied. It is noteworthy that the when Opti-MEM I was used, Normocin was not added in accordance with the manufacturer's instructions to avoid toxicity. The GRk2 siRNA sequences used is as follows: 5'-GCAGGUACCUCCAGAUCUC-3' [nucleotides 417-435 relative to start codon, accession no. NM_012776.1] (Morris et al., 2010). An overhanging 3' dTdT was added on both complimentary strands. A control siRNA was obtained from Applied Biosystems (cat no. AM4611).

Assays of cAMP concentration and tyrosine phosphatase activity

Cyclic AMP assay was done using 12-day old cultures exposed to vehicle of 1 μM PGE_2 for 5 days. Cyclic AMP was assayed using enzyme immunoassay kit obtained from Cayman Chemical. The assay procedures were carried out according to manufacturer's instructions except that 50 μM IBMX was added to the lysis buffer.
Tyrosine phosphatase assay was done using the ability of phosphatase to break down p-nitrophenyl phosphate into a colored product the concentration of which can be measured colorimetrically. The assay was done using a kit purchased from Sciencell according to manufacturer's instructions.

Data analysis

Data are expressed as mean ± the standard error of the mean for at least three independent experiments from separate harvests. Data were analyzed using one-way ANOVA followed by appropriate post hoc test to determine statistically significant differences between treatment groups or using student t-test as indicated. GraphPad Prism 4.02 was used to conduct the statistical analysis. A $p < 0.05$ was considered statistically significant in all experiments.

RESULTS

Much evidence supports that acute sensitization of sensory neurons induced by PGE_2 is mediated by the $G_{\alpha s}$/cAMP/PKA pathway (Ferreira and Nakamura, 1979; Hingtgen et al., 1995; Taiwo et al., 1989). However, whether PGE_2-induced sensitization is mediated by PKA under chronic inflammatory conditions was not directly investigated before. Moreover, direct measurement of activation of PKA in sensory neurons was never performed. PKA activation by PGE_2 in sensory neurons was usually inferred from studies using inhibitors such as H-89 or PKI.

Also, PGE_2-induced sensitization in models of chronic inflammation was reported to be partially mediated by PKA (Hucho et al., 2005; Wang et al., 2007). However, in these studies, contribution of PKA-mediated signaling to PGE_2-induced sensitization was inferred from usage of PKA inhibitors that can be non-selective depending on concentration. Because of this poor selectivity, attenuation of PGE_2-induced sensitization by these PKA inhibitors can be attributed to non-selective inhibition of kinases other than PKA.

Therefore the aims of the work presented in this dissertation are:

1- To determine whether long-term exposure to NGF or PGE_2 alters PKA activated by the eicosanoid.

2- To determine the mechanism of such alteration.

The studies outlined below can be divided in those involving DRG cultures grown in the presence of NGF and those involving cultures that were exposed to PGE_2 for long-term.

Characterization of PKA activity assay

In order to use PKA-activity assay, I asked two questions; 1) whether the reaction conditions used allow for linear relationship between the duration of incubation of the substrate with PKA, and 2) whether phosphorylation of the substrate by PAK was selective. As mentioned earlier, PKA activity was measured as a function of incorporation of radioactive phosphate in kemptide, which is a peptide that is selectively phosphorylated by PKA (Demaille et al., 1979; Kemp et al., 1977). As shown in figure 1, purified bovine PKA catalytic subunit increased phosphorylation of kemptide by more than 650-fold and PKI, a specific PKA inhibitor, inhibited approximately 99 percent of

PKA-induced phosphorylation. Under the assay conditions used, PKA-induced phosphorylation exhibited linear relationship (r^2 = 0.99) with duration for which the reaction was allowed to proceed (up to 10 minutes which is two times longer than the standard assay conditions used in all subsequent experiments involving PKA activity assay) (Fig. 2). This indicates that in the enzyme assay conditions used the substrates, ATP and kemptide, are in excess. Depletion of the substrates would cause the quantity of PKA-induced phosphorylation to reach a maximum and thus makes the reaction non-linear and non-quantitative. Therefore, the conditions of the PKA activity assay used ensure linearity of the reaction and hence quantitation.

Measurement of PKA activation by PGE_2 in DRG cultures showed concentration-dependence (Fig. 3). The duration of exposure of the culture to PGE_2 was kept constant at 10 minutes to match the duration of exposure to the eicosanoid in release experiments. No significant PKA activation, compared to vehicle [1-methyl-2-pyrrolidinone; (MPL)], was evident at 100 nM PGE_2. However, 0.3, 1, 3 and 10 µM PGE_2 caused significantly increasing PKA activity. The extent to which PKA is activated by the aforementioned concentrations of PGE_2 was significantly different from vehicle and from each other at all concentrations, except 10 µM PGE_2 which activated PKA to a similar extent as 3 µM PGE_2. The relationship between concentration of PGE_2 and PGE_2-activated PKA was non-linearly fitted to the sigmoid curve and had an $EC_{50} \approx 0.8$ µM PGE_2. The correlation coefficient r^2 was 0.95 showing strong positive correlation.

The question that arises is whether this effect is selective for PGE_2 or that other agents that activate PKA can do so in sensory neuronal cultures? To address this question, sensory neuronal cultures were exposed to such drugs and PKA activation was subsequently assayed. Since these drugs were dissolved in different vehicles, the data is presented normalized to vehicle (Fig. 4). Compared to vehicle, PGE_2 (1 µM, non-selective EP receptors agonist), L902688 (300 nM, selective EP4 agonist), $cPGI_2$ (1 µM, stable selective IP agonist), forskolin (1 µM, adenylyl cyclase activator), cholera toxin (1.5 µg/ml, locks $G_{\alpha s}$ in the activated conformation) and isoproterenol (10 µM, selective βAR agonist) activated PKA 9.4, 8.9, 3.5, 5.3, 8.5 and 1.2 folds, respectively. It is noteworthy that isoproterenol at a relatively high concentration caused the least activation of PKA. This is a surprising finding since isoproterenol, a selective βAR agonist, is frequently used as a sensitizing agent in experimental animals (Hucho et al., 2005; Khasar et al., 1999b; Levine et al., 1988). Attempts to establish concentration-PKA activation relationship were not successful, since a range of isoproterenol concentrations

from 1 µM - 100 µM activated PKA to a similar extent (the ratio of isoproterenol-activated PKA to total PKA activity were 0.063 ± 0.005, 0.119 ± 0.007, 0.100 ± 0.004, 0.108 ± 0.009, 0.129 ± 0.011, 0.113 ± 0.003, for 1, 3, 10, 30 and 100 µM, respectively). A possible explanation for the lack of robust concentration-dependent PKA activation by isoproterenol is that βARs are expressed at a very low level in sensory neuronal cultures and hence the smallest isoproterenol concentration that I used is sufficient to saturate them and produce maximal PKA activation.

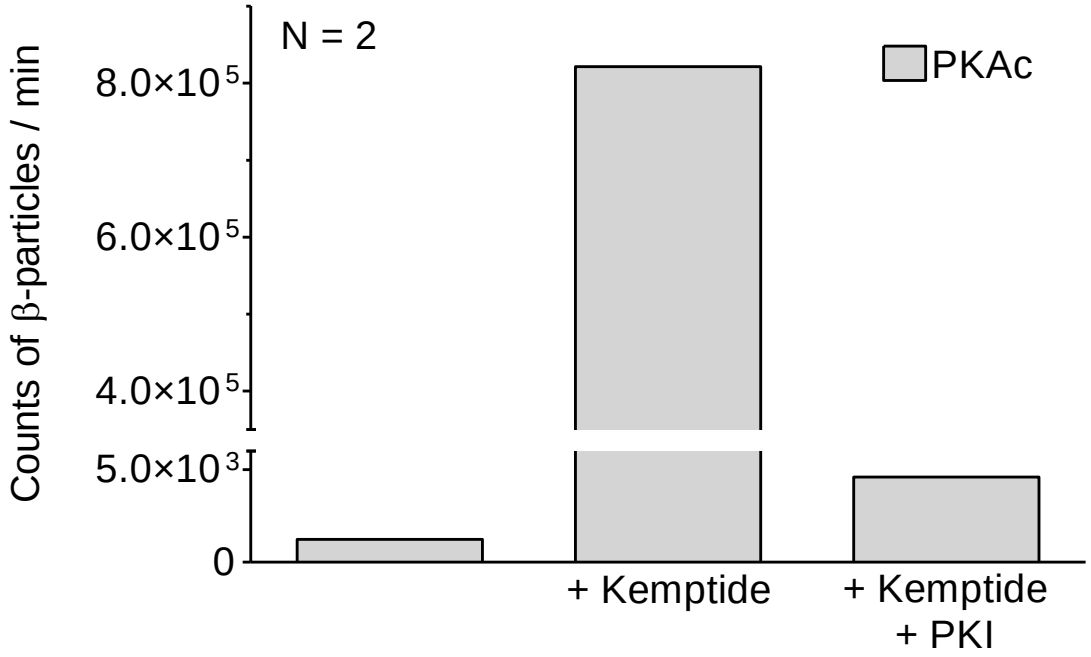

Figure 1. Phosphorylation of kemptide by bovine heart catalytic subunit is highly selective. The ordinate shows the number of β-particles emitted by P^{32} incorporated as phosphate group in the presence of vehicle, kemptide or kemptide and the specific PKA inhibitor, PKI. Each column represents the mean of two trials.

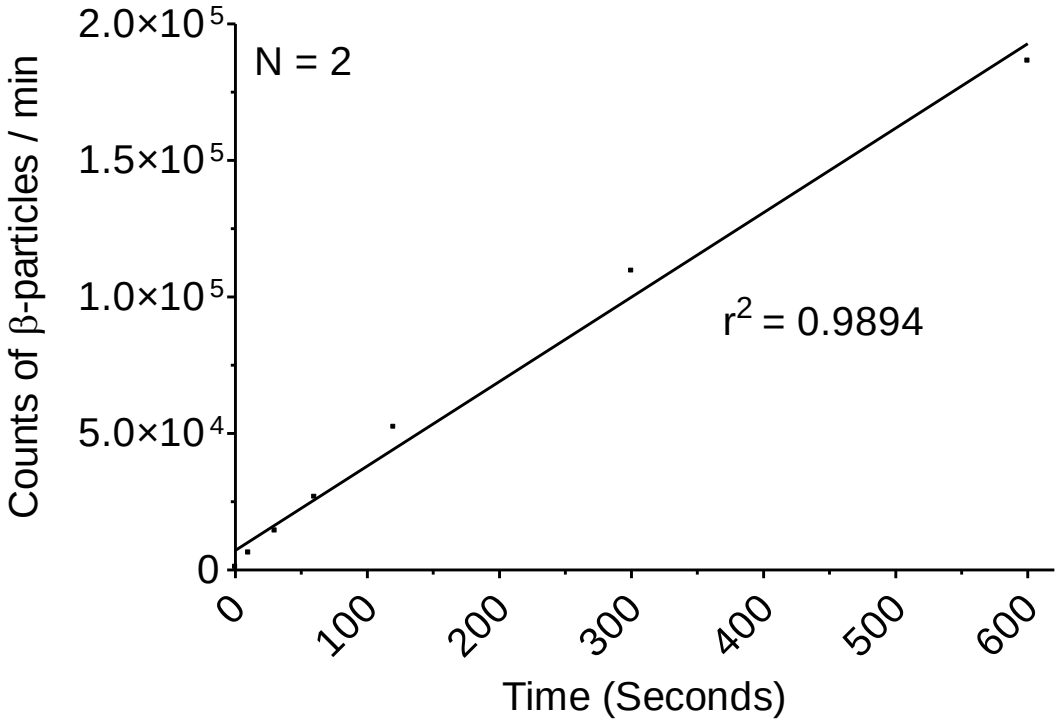

Figure 2. Phosphorylation of kemptide by bovine heart PKA catalytic subunit is highly linear under the conditions of the assay used. The ordinate shows the number of β-particles emitted by P^{32} incorporated as phosphate group in kemptide. Under conditions of the assay used to determine PKA activity, sufficient substrates (kemptide and ATP) are present allowing the reaction to proceed with high degree of linearity for 10 minutes. Each point represents the mean of two trials.

Table 1. PGE_2 activates PKA in a concentration-dependent manner in adult rat sensory neuronal cultures.

Concentration of PGE_2 (μM)	Ratio of PGE_2-activated PKA to vehicle-activated PKA, both normalized first to total PKA activity (mean ± standard error)	n
Vehicle	0.07 ± 0.02	4
0.1	0.06 ± 0.01	6
0.3	0.23 ± 0.04[a,b]	4
1.0	0.48 ± 0.1[a,b,c]	4
3.0	0.71 ± 0.05[a,b,c,d]	4
10	0.78 ± 0.1[a,b,c,d]	4

[a] signifiucanlt different from vehicle
[b] significantly different from PGE_2 0.1 μM
[c] significantly different from PGE_2 0.3 μM
[d] significantly different from PGE_2 1.0 μM

Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post-test.

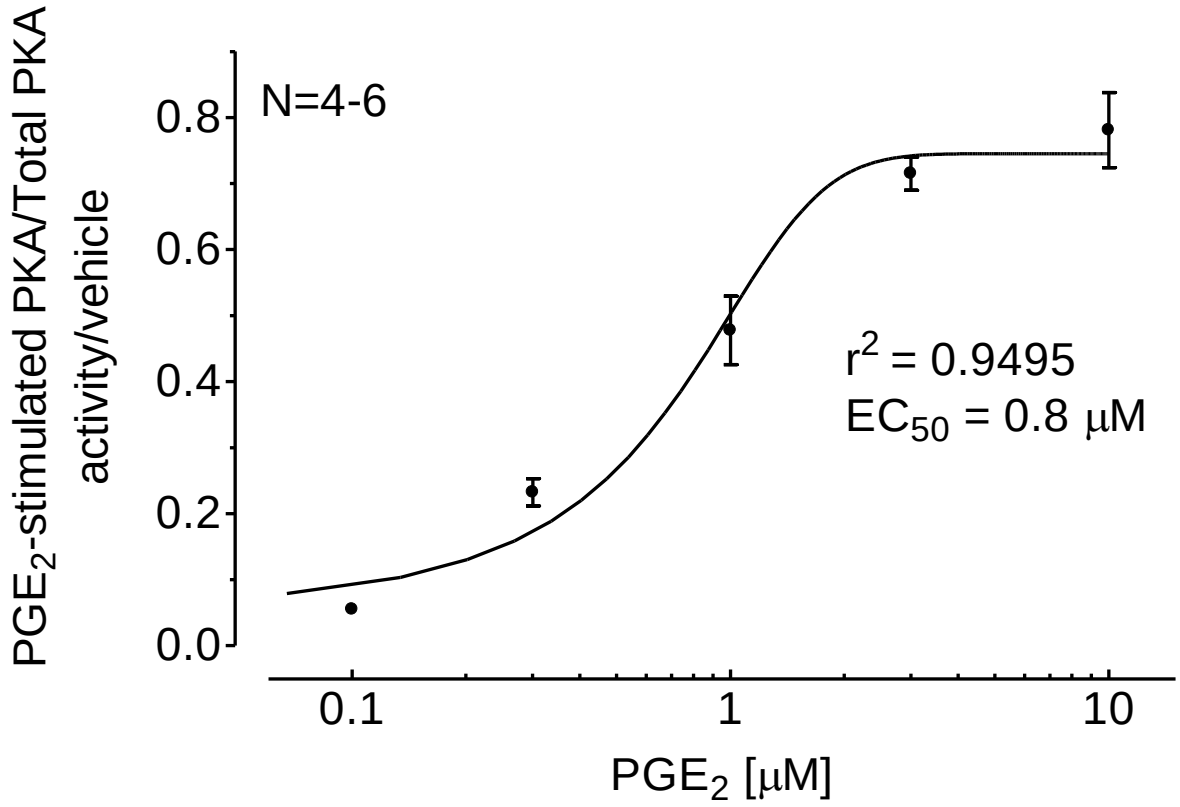

Figure 3. Concentration-response curve for PGE_2-induced activation of PKA. Each point represents mean ± SEM of PKA activity after 10-minutes exposure to PGE_2 normalized to total PKA which is measured after exposure to cAMP 10 μM. The abscissa shows the log concentration of PGE_2. PKA activity at each concentration is significantly different from all others, $p < 0.05$, except that 3 and 10 μM are not significantly different. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post-test.

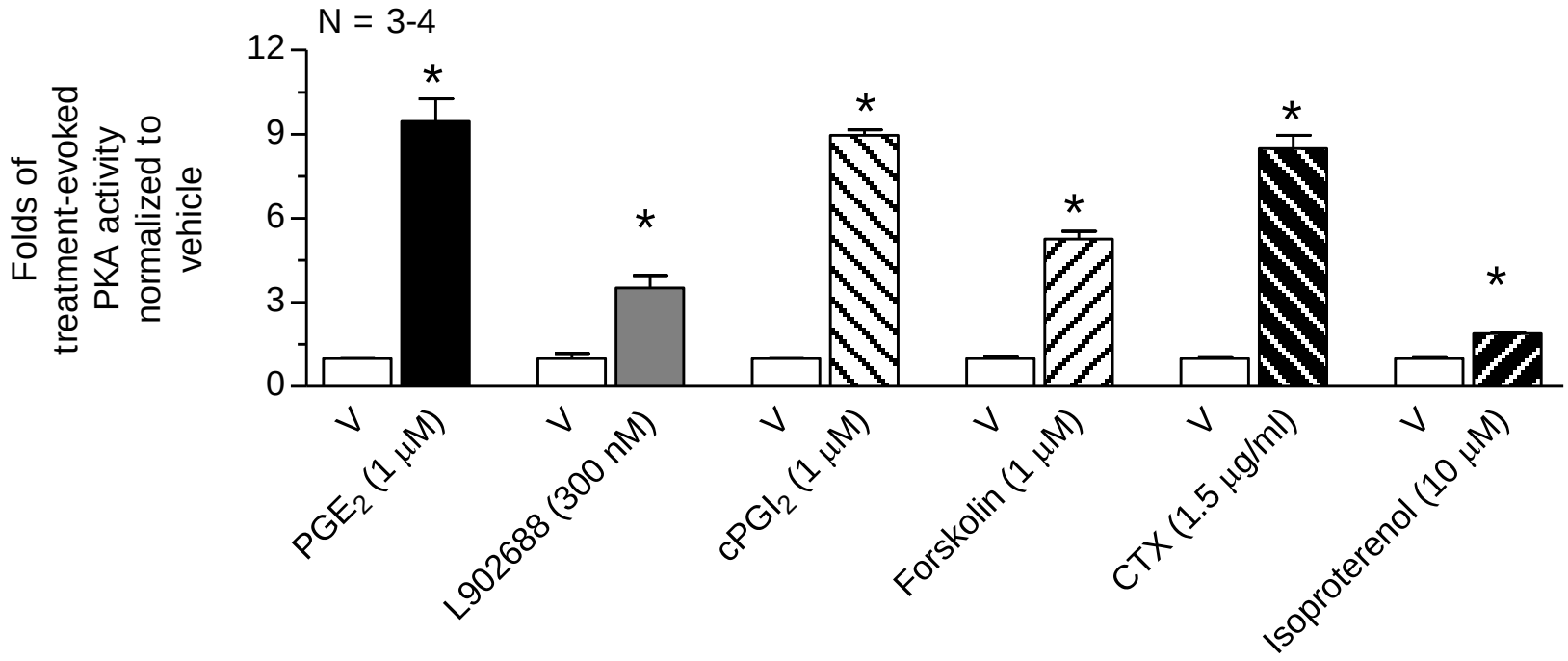

Figure 4. Treatments-induced activation of PKA normalized to its respective vehicle. Each column represents mean ± SEM of PKA activity after 10-minutes exposure to the indicated treatment at the various concentrations stated. PKA activity was normalized to total PKA which is measured after exposure to cAMP 10 μM. An asterisk indicates a statistically significant difference between PKA activation by each treatment compared to its respective vehicle using student's T-test.

Inhibition of PKA reversed acute prostaglandin-induced sensitization of adult rat sensory neurons grown without NGF

As discussed in detail in the introduction, acute PGE_2-induced sensitization, as measured by hypernociception, is mediated by PKA (Ferreira and Nakamura, 1979; Taiwo and Levine, 1991). The question that presents itself is whether inhibition of PKA in sensory neuronal cultures, attenuate PGE_2-induced sensitization? This was confirmed in experiments conducted by Chunlu Guo in which PGE_2 (1 μM) augmented capsaicin-evoked iCGRP release from 10.5 ± 1.34 to 15.56 ± 1.44 as percent of total content. H-89 (10 μM), the PKA inhibitor, attenuated acute PGE_2-mediated augmentation of capsaicin-evoked iCGRP release, thus confirming previous findings (Fig. 5).

It is noteworthy that H-89 had no direct effects on basal release of iCGRP when compared to basal release in the presence of vehicle (3.4 ± 0.65 versus 2.1 ± 0.58 iCGRP released as percent of total content, respectively) nor did it alter release evoked by 30 nM capsaicin when compared to capsaicin-evoked release in the presence of vehicle (32.8 ± 4.2 versus 33.0 ± 3.2 iCGRP released as percent of total content, respectively). This substantiates the role of PGE_2 as an agent that sensitizes sensory neuronal response to an algogen (capsaicin) rather than a direct activator as well as the role PKA plays in augmentation of evoked but not basal iCGRP release.

It is possible that H-89 at 10 μM may inhibit kinases other than PKA (for review of H-89 selectivity, refer to the discussion section). However, it was found that the only receptors that are important for PGE_2-induced sensitization are coupled to PKA (Southall and Vasko, 2001). Therefore this data strongly suggests that acute PGE_2-induced sensitization is mediated by PKA.

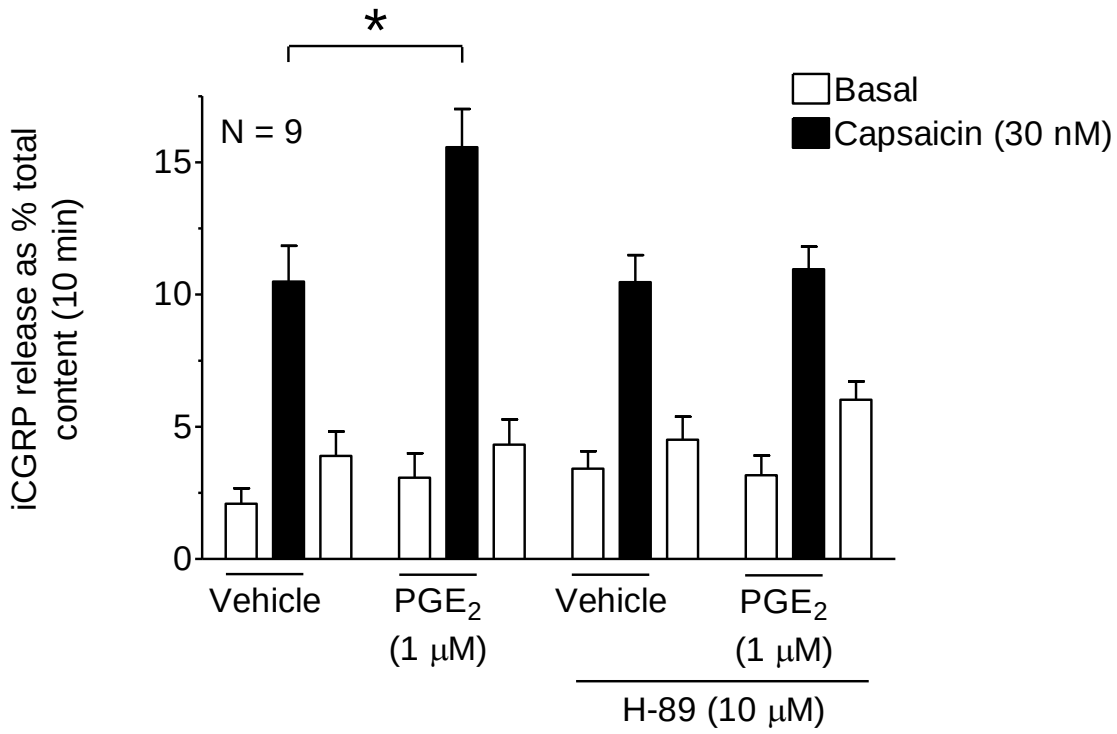

Figure 5. The PGE_2-induced increase of capsaicin-evoked iCGRP release from sensory neurons is attenuated by H-89. Each column represents the mean ± SEM of iCGRP release as percent of total iCGRP content. Open columns indicate basal release whereas closed columns represent capsaicin-evoked iCGRP release. An asterisk indicates a statistically significant difference between capsaicin-evoked iCGRP release after exposure to vehicle versus after a 20 minute exposure to PGE_2 (1 μM) using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$. Experiment was performed by Chunlu Guo, MD.

Acute PGE_2-induced sensitization is not attenuated by PKA inhibition in adult rat sensory neurons are grown in the presence of NGF

Growing adult rat sensory neuronal cultures in the presence or absence of the 7S form of NGF for 12 days was used as a model of chronic inflammation, since NGF is produced in humans with chronic inflammatory pain disorders as well as in animal models of chronic inflammatory hyperalgesia (see introduction, section on Nerve Growth Factor and discussion).

Surprisingly, when DRG cultures were grown in the presence of NGF for 12 days (30 ng/ml), augmentation of capsaicin-evoked iCGRP release by PGE_2 was resistant to attenuation by 10 µM H-89 (Fig. 6). In the absence of H-89, capsaicin-stimulated iCGRP release was augmented by PGE_2 (1 µM) from 109.3 ± 12.09 to 207.5 ± 22.52 fmol/well/10 min. PGE_2 also augmented iCGRP release from 171.1 ± 19.44 to 250.9 ± 24.8 fmol/well/10 min even with 10 µM H-89 was included in the release buffer. H-89 by itself did not cause significant augmentation of capsaicin-evoked iCGRP release (109 ± 12.9 and 171 ± 19.4 fmol/well/10 min in the absence or presence of 10 µM H-89, respectively). Thus, in sensory neuronal cultures grown in the presence of NGF, PGE_2-induced augmentation of capsaicin-evoked iCGRP release was not attenuated by H-89.

It is possible that PKA still mediates PGE_2-induced sensitization after chronic NGF but the activity of the kinase is increased so that full inhibition by 10 µM H-89 is not achieved. Total specific PKA activity (measured by adding 10 µM cAMP) was not statistically different in neurons grown for 12 days in the presence or absence of NGF (30 ng/ml) suggesting that exposure to NGF does not increase PKA activity (Fig. 7). This cAMP concentration was chosen to ensure activation of all the PKA available in lysates from the DRG cultures since cAMP concentration was a supramaximal one (higher than the concentration sufficient to maximally activate PKA) (Smales and Biddulph, 1985; Walsh et al., 1968a). Another possibility is that PKA no longer mediates PGE_2-induced sensitization due to a signaling switch caused by growing sensory neurons in NGF.

Thus, in neurons grown in the presence of NGF, PKA inhibition does not attenuate PGE_2-induced sensitization and there is no increase in the activity of the kinase, suggesting a signaling switch.

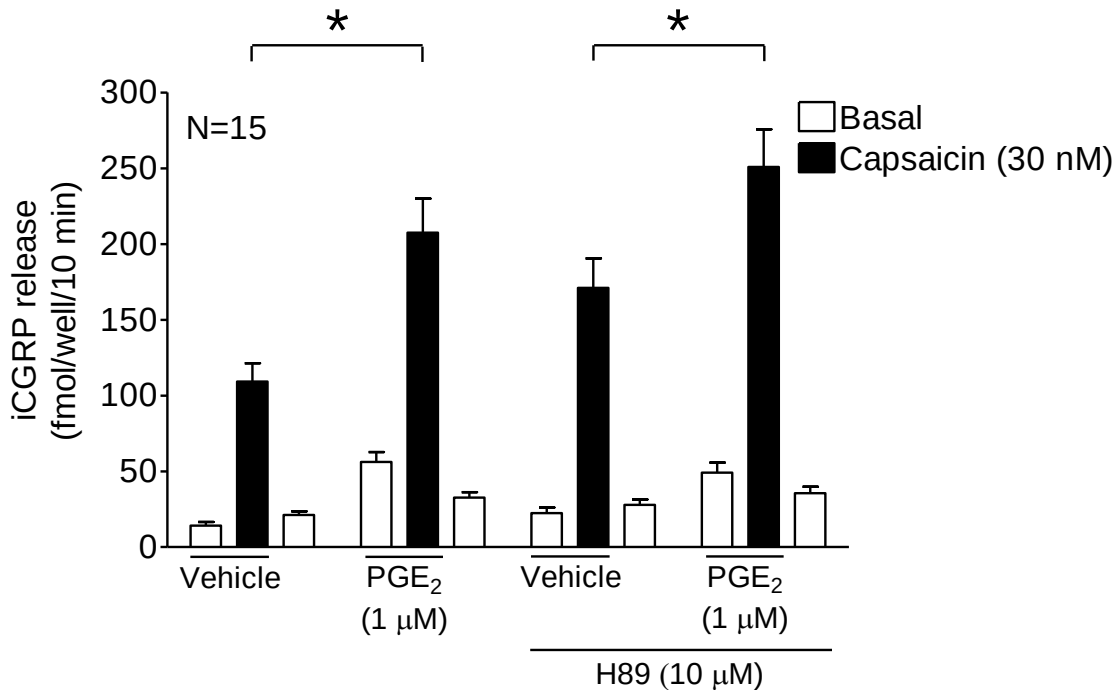

Figure 6. The PGE_2-induced augmentation of capsaicin-evoked iCGRP release from adult rat sensory neuronal cultures grown in the presence of added NGF is not attenuated by H-89. Each column represents the mean ± SEM of iCGRP release as fmol/well/10 minutes. Open columns indicate basal release whereas closed columns represent capsaicin-evoked iCGRP release. An asterisk indicates a statistically significant difference between capsaicin-evoked iCGRP release after exposure to vehicle versus after 20 minute exposure to PGE_2 (1 μM) using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$. Experiment was performed by Chunlu Guo, MD.

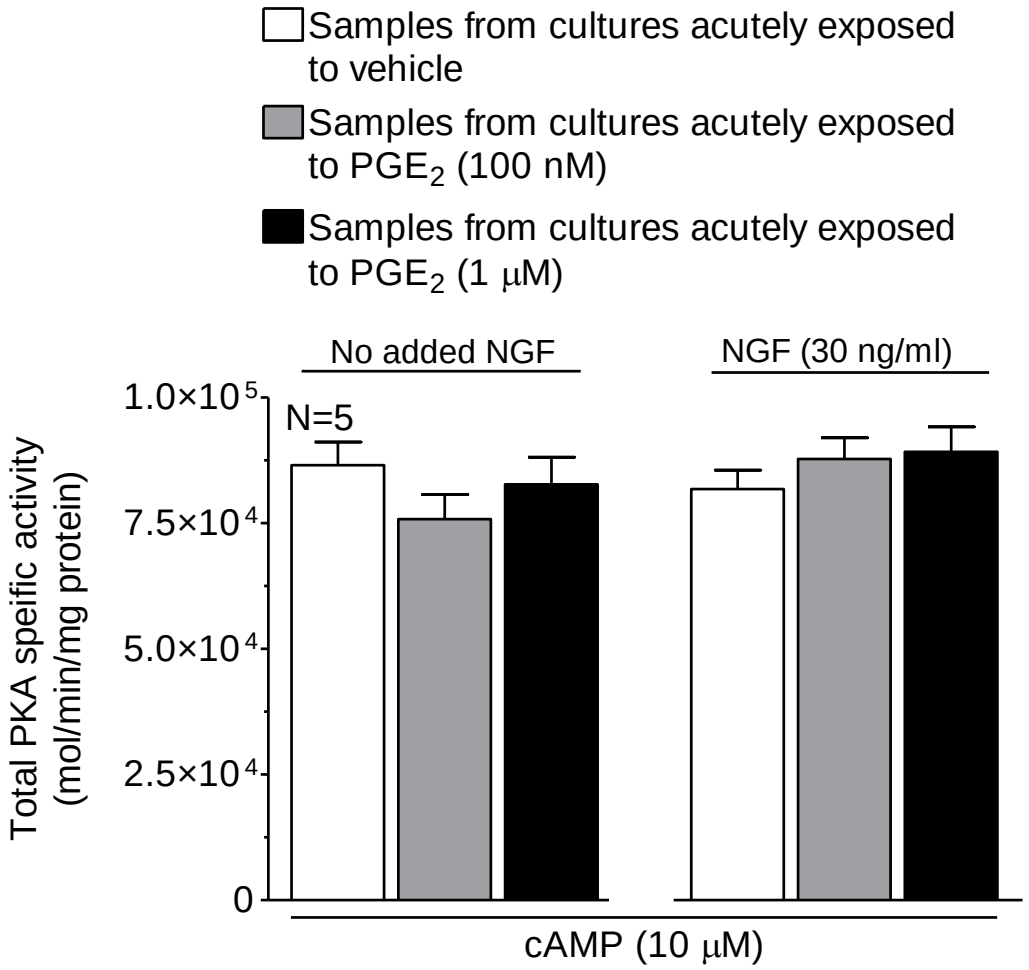

Figure 7. NGF does not alter total PKA activity in adult rat sensory neuronal cultures. Each column represents mean ± SEM of specific total PKA activity calculated after exposure to 10 μM cAMP. No statistical significance was detected using one-way ANOVA.

NGF does not reduce mRNA levels of AKAP, β-arrestins or Grks

PKA signaling is directly coupled to A-kinase anchor proteins (AKAPs), which localizes PKA and relevant (partner) signaling molecules (such as $G_{\alpha s}$, adenylyl cyclases and PDEs) to signaling compartments important for mediating effects of PGE_2 (Zhang et al., 2008). Therefore, it is possible that reduction in the expression of the anchor protein would cause uncoupling of PKA from $G_{\alpha s}$/adenylyl cyclase in compartments specifically important for PGE_2 signaling. To investigate the validity of this hypothesis, I measured mRNA levels of AKAP5 and 250 which were shown to be expressed in sensory neurons and/or mediating PGE_2-induced sensitization (Fan et al., 2001; Irmen et al., 2008; Jeske et al., 2008; Rathee et al., 2002b; Schnizler et al., 2008; Tao et al., 2007; Willoughby et al., 2006; Zhang et al., 2008). Relative levels of mRNA of *Akap5* (AKAP79/150) and *Akap12* (AKAP250, gravin) did not change in DRG neurons grown in the presence of NGF compared to control cultures (Fig. 8). This suggests that the loss of the function of PKA in mediating PGE_2-induced sensitization after chronic exposure to NGF does not depend on changes in mRNA levels of these AKAPs.

As discussed in the Introduction, GPCR desensitization machinery is based on β-arrestins 1 and 2 as well as Grks2, 3, 5 and 6. These molecules play pivotal role not only in desensitization of GPCRs, but also in recruitment of alternative non-canonical signaling pathways. Alteration of the level of expression of these molecules is correlated with rheumatoid arthritis, hypertension, schizophrenia and other pathological conditions (Bychkov et al., 2011; Gros et al., 1997a; Gros et al., 2000; Kleibeuker et al., 2008b; von Banchet et al., 2011; Vroon et al., 2004; Wu et al., 2012). It is plausible that NGF switches PGE_2 signaling by altering the level of expression of one or more of β-arrestins or Grks. However, as shown in figure 8, NGF has no effect on the level of expression of the mRNA of β-arrestins or Grks.

Levels of mRNA do not always correlate very well with the proteins they encode (for detailed review of the topic, see the discussion). Despite the lack of statistically significant effect of growing sensory neuronal cultures in the presence of absence of 30 ng/ml NGF on the levels of mRNA for genes of interest when (Fig. 9), there is a possibility that the protein levels of these genes are more strongly modulated by NGF. In data presented in figure 8 AKAP5 seems a likely case where the above scenario takes place. Therefore, Chunlu Guo conducted an experiment in which the protein level of AKAP 5 was measured using western blot. Surprisingly, growing adult rat sensory

neuronal cultures in 30 ng/ml NGF reduced the expression of AKAP 5 significantly by more than 50percent. Therefore, one such possible mechanism by which NGF reduces PKA mediated signaling is reducing AKAP 5 which anchors PKA in the proper compartment to allow receptor-bound ligand to activate it through $G_{\alpha s}$/adenylyl cyclase.

These data suggest that the loss of mediation of PGE_2-induced sensitization by PKA is not caused by alterations in the mRNA levels of the AKAPs, β-arrestins or Grks expressed in sensory neurons.

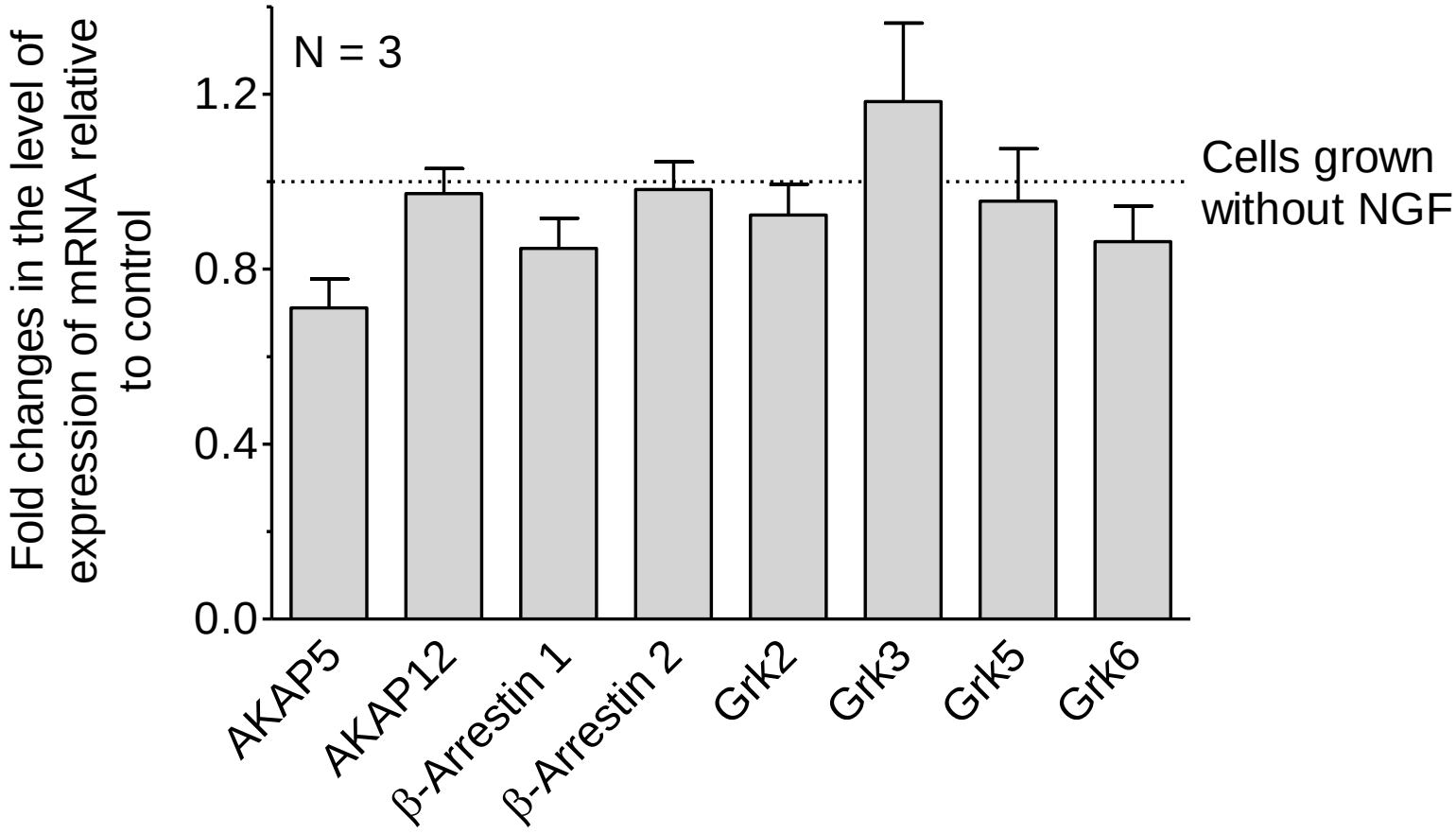

Figure 8. NGF does not alter the relative mRNA level of AKAPs, β-arrestins or Grks in adult rat sensory neuronal cultures. Each column represents the mean ± SEM of the relative level of mRNA normalized to that of control cultures. Grey columns represent sensory neuronal cultures grown in the presence of added NGF (30 ng/ml) for 12 days while the dotted line indicates control cultures grown in the absence of NGF. There was no statistical significance using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

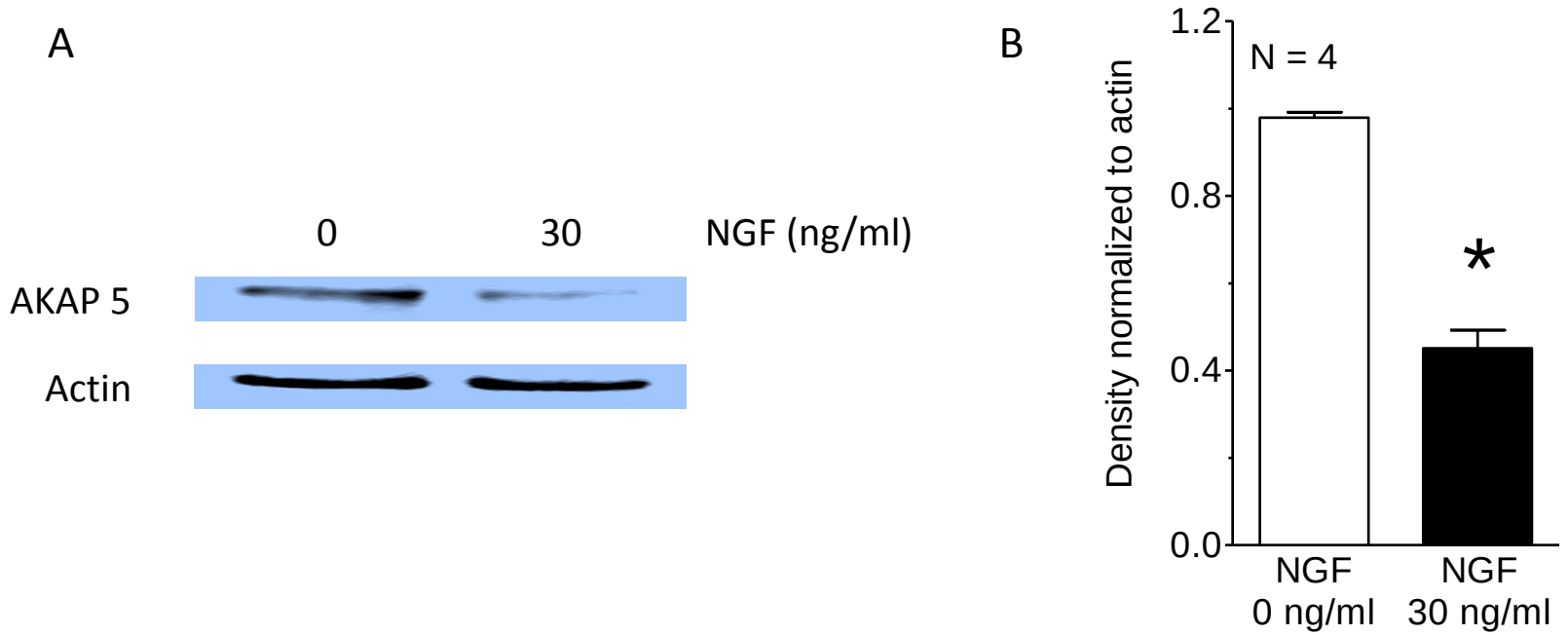

Figure 9. NGF reduces expression of AKAP 5 in adult rat sensory neuronal cultures. Each column represents the mean ± SEM of the ratio of the densitometry values of AKAP 5 protein to that of actin, normalized to control cultures. Open and closed column represents values from sensory neuronal cultures grown in the absence or presence of added NGF (30 ng/ml) for 12 days, respectively. An asterisk indicates a statistically significant difference between AKAP 5 in cultures grown in the presence or absence of 30 ng/ml NGF, using student t-test, $p < 0.05$. Experiment was performed by Chunlu Guo, MD.

Attenuation of PGE_2-induced activation of PKA in sensory neuronal cultures grown in NGF is dependent on phosphatase inhibition

Since inhibition of PKA had no effect on PGE_2-mediated augmentation of iCGRP release from sensory neurons grown in the presence of NGF, it is intriguing to determine whether PGE_2 activates PKA in neurons grown in NGF versus those grown without NGF. As evident from the literature, PKA activity has not been directly measured in DRG neurons in response to stimulation by PGE_2. Rather, the role of PKA in mediating PGE_2-induced sensitization is inferred from the ability of inhibitors to attenuate PGE_2-induced sensitization. I asked whether PGE_2 activates PKA in cultured sensory neurons after acute exposure. To this end, PKA activation was assayed after exposure to PGE_2 (1 µM) for 10 minutes in sensory neuronal cultures grown in the presence or absence of NGF (30 ng/ml). As described in the materials and methods section, PKA activity is expressed as the ratio between treatment-activated PKA to total PKA. In order to measure PKA activity, a cocktail of phosphatase inhibitors was included in the lysis buffer and the PKA-activity assay buffer. Under these conditions, PKA activity in cultures that were exposed to vehicle for 10 minutes (basal PKA activity) was not significantly different whether the cultures were grown in the absence or presence of NGF (0.16 ± 0.03 and 0.16 ± 0.02 of treatment-activated PKA/total PKA activity, respectively). After a 10 min exposure to 1 µM PGE_2, PKA became significantly activated to a similar extent (approximately 3 folds when compared to vehicle) in cultures grown in the presence or absence of 30 ng/ml NGF (0.53 ± 0.06 and 0.45 ± 0.04, Fig. 10). In cultures grown with or without NGF, PGE_2 at 100 nM noticeably, although not significantly, increased PKA activity (0.2 ± 0.02 and 0.2 ± 0.03, respectively) compared with vehicle (0.16 ± 0.03 and 0.16 ± 0.02, respectively).

As mentioned in the methods section, kinase activity was measured by quantifying the amount of radioactive phosphate incorporated into a peptide substrate (kemptide) that was selectively phosphorylated by PKA. Since the quantity of a phosphorylated substrate in a given cell lysate is determined by the balance of addition of the phosphate by kinases and removal of phosphate by phosphatases, it is important to inhibit phosphatases in order to study kinases. This way, phosphatase activity is minimized while kinase activity is left uninhibited allowing it to be quantified without interference. As described in the previous experiment, when phosphatases were

inhibited using a cocktail of inhibitors, PKA activation by PGE_2 was not directly altered by growing sensory neuronal cultures in NGF. Because of this observation, it is reasonable to assume that removal of phosphatase inhibition will allow any differences in phosphatase activity to be observed. When the cocktail of phosphatase inhibitors was left out of the lysis and assay buffers and the PKA activity assay was repeated, the impact of phosphatase activity on PKA-induced phosphorylation became evident (Fig. 11). Under these conditions, basal PKA activity (after 10 minutes exposure to vehicle) was reduced to approximately one third of its value when phosphatases were inhibited in cultures grown in the absence or presence of NGF (0.05 ± 0.01 and 0.04 ± 0.003, respectively). Importantly, activation of PKA induced by 1 µM PGE_2 was significantly less in cultures grown in the presence of NGF (0.16 ± 0.024) than in cultures grown in the absence of NGF (0.28 ± 0.037). Therefore NGF indirectly attenuates PKA activation by PGE_2 in a phosphatase-dependent manner.

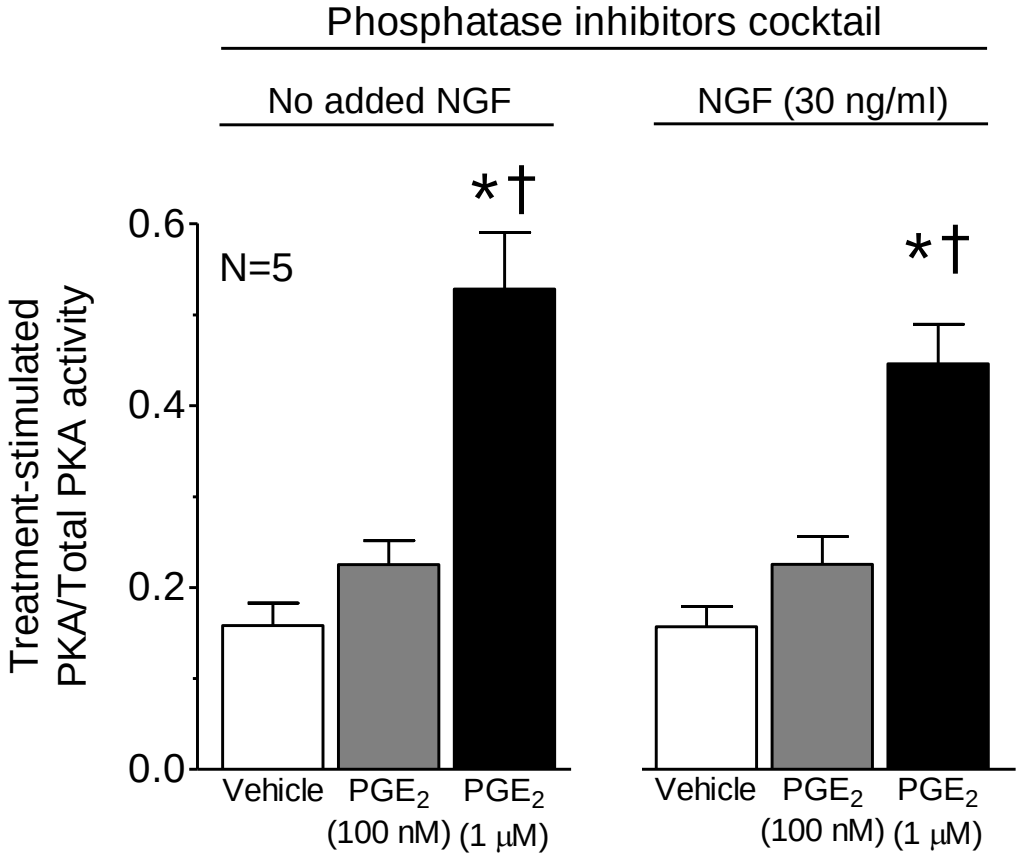

Figure 10. In the presence of phosphatase inhibitors cocktail, PGE_2-induced activation of PKA is NGF-independent. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF (30 ng/ml) for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells exposed to PGE_2 (1 μM) for 10 minutes. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

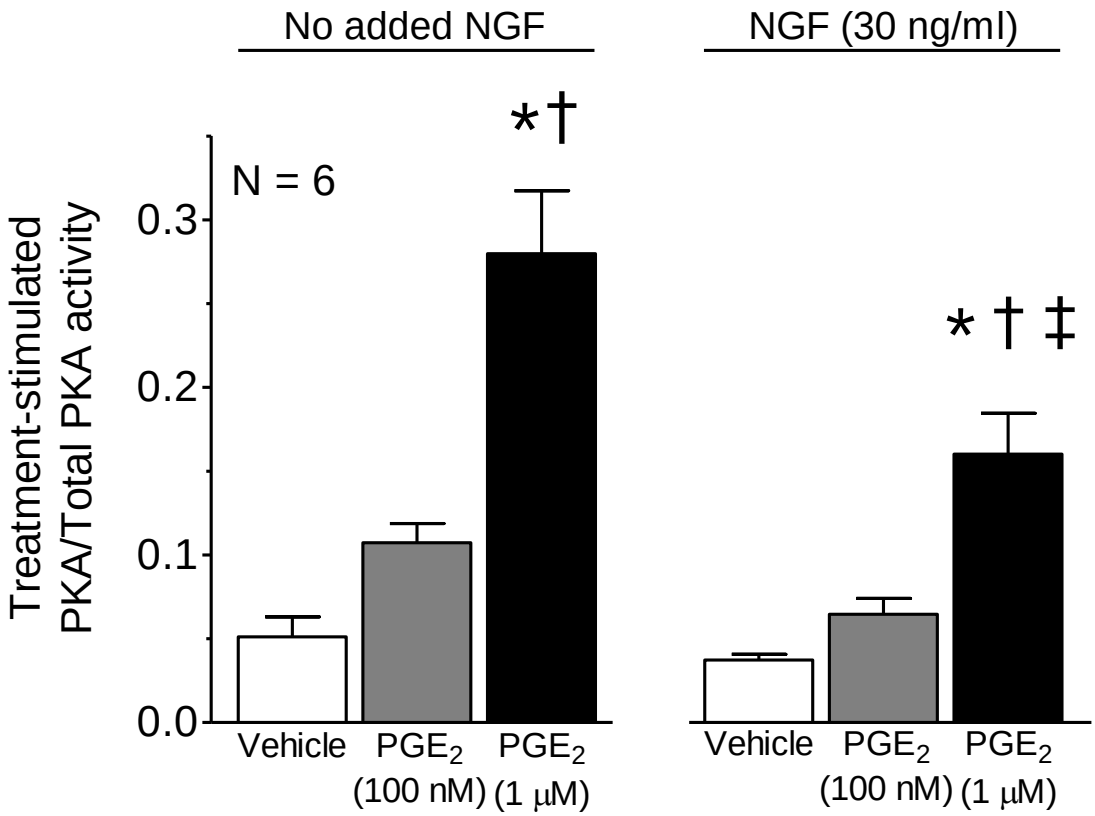

Figure 11. In the absence of phosphatase inhibitors cocktail, PGE_2-induced activation of PKA is NGF-dependent. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells exposed to PGE_2 1 μM for 10 minutes. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 and a double dagger represents significant difference from cells grown in the absence of added NGF and acutely treated with PGE_2 (1 μM) for 10 minutes using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Protein tyrosine phosphatases inhibition using sodium vanadate does not reverse in NGF-induced attenuation of PGE_2-induced activation of PKA

Since the absence of phosphatase inhibitors results in significant attenuation of PGE_2-inuduced PKA activation, I next attempted to determine which phosphatases account for this action. There are two major classes of protein phosphatases, protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSPs). PTPs can be selectively inhibited by sodium vanadate (Swarup et al., 1982). When added to both the lysis and PKA-activity assay buffers, sodium vanadate (10 µM) did not reverse NGF-induced attenuation of PGE_2-activated PKA (Fig. 12). Ten-minute exposure to 1 µM PGE_2-activated PKA in sensory neurons grown in the absence of NGF significantly higher than in neurons grown in the presence of NGF (0.13 ± 0.01 versus 0.08 ± 0.01, respectively). It is worth noting that baseline PKA activities in the presence of sodium vanadate, were similar to those obtained in the absence of phosphatase inhibitors (0.05 ± 0.004 and 0.03 ± 0.006 in cultures grown in the absence or presence of NGF respectively). This finding suggests that PTPs do not exert significant effect on baseline PKA activity.

Interestingly, in the absence of phosphatase inhibitors, PGE_2-activated PKA was more than 2 fold higher than PGE_2-activated PKA when sodium vanadate was included (0.28 ± 0.037 versus 0.13 ± 0.01, Fig. 8 and Fig. 9 respectively). One potential explanation is that sodium vanadate inhibits PKA activity. However, previous work demonstrated that sodium vanadate does not inhibit purified PKA catalytic subunit activity (Pluskey et al., 1997). One form of vanadate, named decavanadate, can inhibit PKA activity by binding to kemptide, the peptide substrate selectively phosphorylated by PKA. Nevertheless, formation of this form of vanadate is significant only at pH ≤ 5.5, which is far below the pH of the buffer used here (Goddard and Gonas, 1973).

In order to confirm that the sodium vanadate was effective in inhibiting tyrosine phosphatase under the experimental conditions employed, the purified protein tyrosine phosphatase PTP1B was used. As shown in figure 13, 10 µM sodium vanadate inhibited purified PTP1B by 91 ± 2.3 percent, yet it had no effect on NGF-mediated attenuation of PGE_2-activated PKA signaling as mentioned above.

Collectively, these experiments suggest that PTPs do not mediate the attenuation of PGE_2-activated PKA by NGF. Also, data presented in figures 11 and 12

(in comparison with data from figures 8 and 9) suggest that PTPs play a role in regulating PGE_2-activated PKA. The mechanisms and purpose of such modulation are beyond the scope of this thesis.

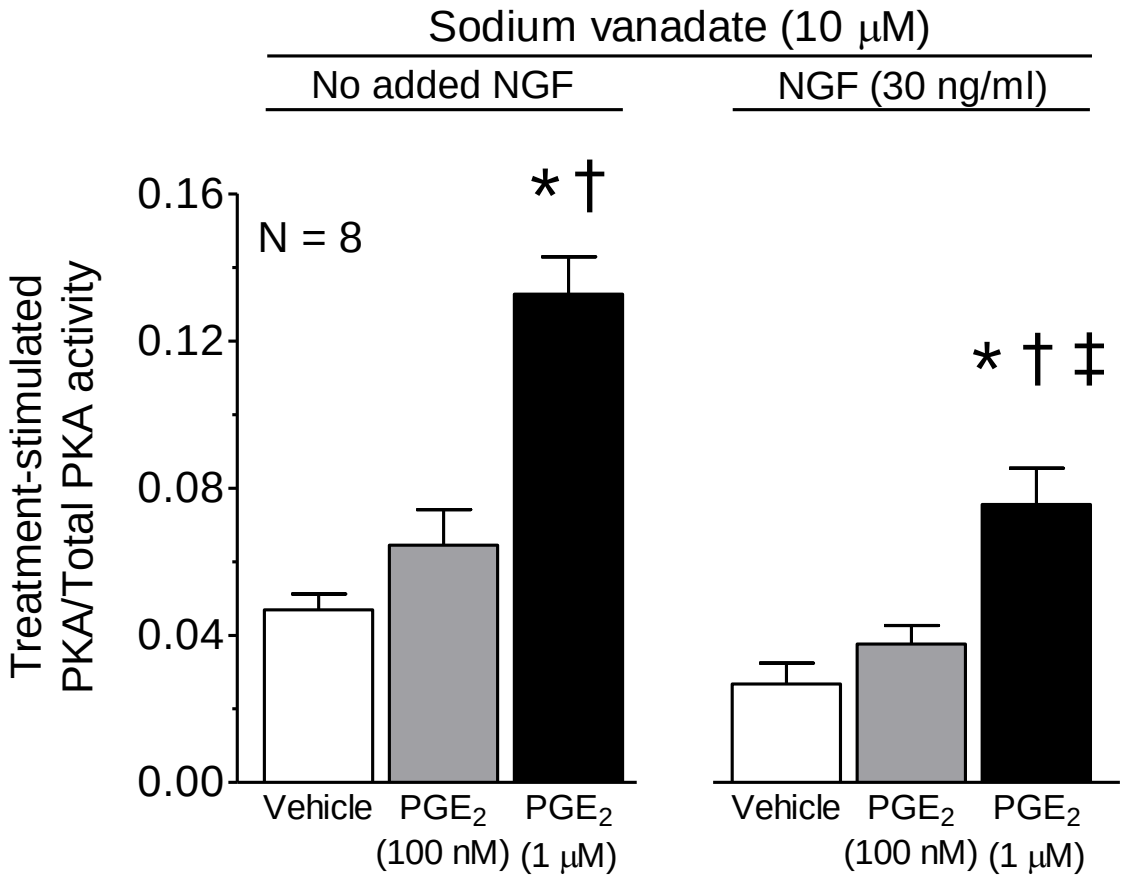

Figure 12. Sodium vanadate does not reverse NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells exposed to PGE_2 (1 μM) for 10 minutes. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 and a double dagger represents significant difference from cells grown in the absence of added NGF and acutely treated with PGE_2 (1 μM) for 10 minutes using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

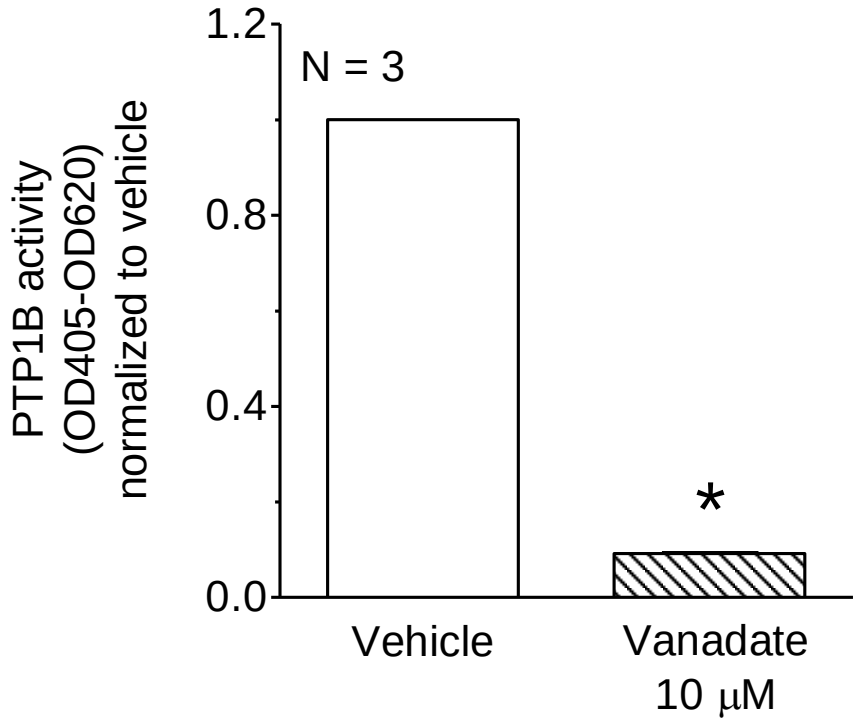

Figure 13. Sodium vanadate inhibits purified PTP1B activity. Each column represents mean ± SEM of phosphatase activity normalized to vehicle. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Calcineurin does not mediate NGF-induced attenuation of PGE_2 activation of PKA

The most well studied members of PSPs include PP1, PP2A, PP2B (calcineurin), PP2C, PP4 and PP6 (Virshup and Shenolikar, 2009). Calcineurin plays a role in multiple functions in sensory neurons, including mediating the desensitization of TRPV1 channel (Docherty et al., 1996). Calcineurin also regulates NGF-mediated activation of the transcription factor nuclear factor of activated T-cells (NFAT) in sensory neurons (Groth et al., 2007). Therefore it is possible that calcineurin mediates the ability of NGF to reduce PKA-activation by PGE_2. To investigate this possibility, I used the selective inhibitor calcineurin autoinhibitory peptide. Dephosphorylation of peptide substrates was prevented by calcineurin autoinhibitory peptide with an $IC_{50} \approx 10$ μM (Hashimoto et al., 1990). When 40 μM of calcineurin autoinhibitory peptide was included in the lysis and PKA assay buffers. This concentration was chosen since it was reported to inhibit calcineurin-induced modulation of threshold stimulus (stimulus at which at the axons of cortical neurons) (Chen et al., 2008). Under these conditions, calcineurin autoinhibitory peptide did not reverse NGF-induced attenuation of PGE_2-activated PKA (0.26 ± 0.04 in grown in the absence of NGF versus 0.12 ± 0.03 in cultures grown in the presence of NGF, Fig. 14). This suggests that calcineurin (PP2B) does not mediate the reduction of PGE_2-activated PKA by NGF.

It also is noteworthy that basal PKA activity (after 10-minute exposure to vehicle) measured with calcineurin inhibitor is very similar to that measured without phosphatase inhibitors (0.05 ± 0.008 in cultures grown without NGF and 0.02 ± 0.001 in cultures grown in the presence of NGF). This suggests that calcineurin does not contribute to PKA signaling under basal conditions. Since calcium is essential for activation of calcineurin, it is to be expected that its phosphatase activity is low in cells under resting conditions, since intracellular calcium is maintained at very low concentrations. When the lysis and PKA activity assay buffers contained calcineurin inhibitory peptide, PGE_2-induced PKA activity after 10 minute exposure was also similar to values obtained in the absence of phosphatase inhibitors altogether (see discussion of Fig. 11), suggesting that calcineurin does not contribute to PKA signaling even after stimulation with PGE_2.

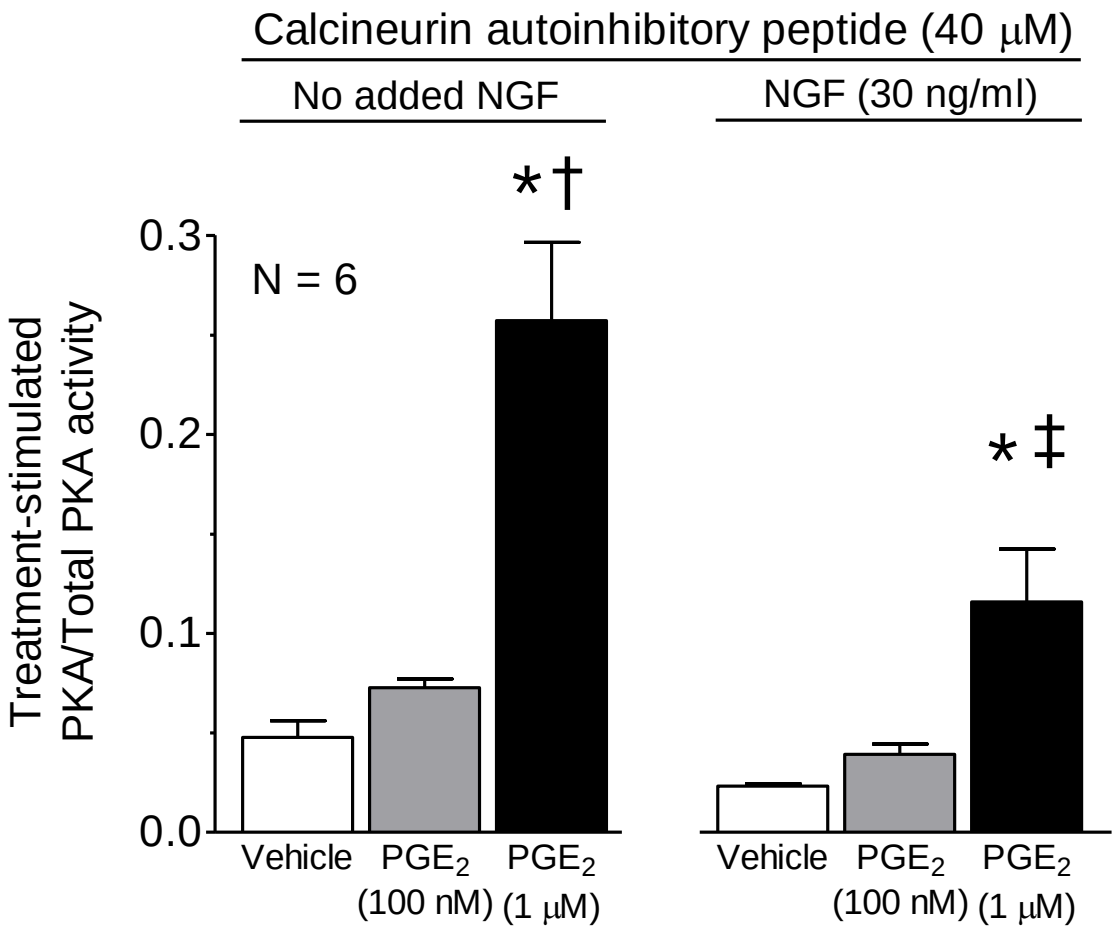

Figure 14. Calcineurin autoinhibitory peptide does not reverse NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. Asterisks indicate statistically significant differences from vehicle, while a dagger represents significant difference from 100 nM PGE_2 and a double dagger represents significant difference from cells grown in the absence of added NGF and acutely treated with PGE_2 (1 μM) for 10 minutes using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

The non-selective ser/thr phosphatase inhibitor okadaic acid, but not the specific PP1 inhibitor-2, reverses NGF-induced attenuation of PGE_2-induced activation of PKA

The remaining serine/threonine phosphatases include several members such as PP1, PP2A, PP2C, PP4, PP5, PP6 and PP7. Unfortunately, there are no selective inhibitors of these phosphatases. However, by employing multiple inhibitors, it is possible to narrow down candidate phosphatases (Swingle et al., 2007) that may contribute to the observed attenuation of PGE_2-activated PKA by NGF.

Okadaic acid is a potent but only somewhat selective serine/threonine phosphatase inhibitor. At relatively high concentration (2 µM), okadaic acid inhibits most serine/threonine phosphatases including PP1 (IC_{50} ≈ 15-50 nM), PP2A (IC_{50} ≈ 0.1-0.3 nM), PP4 (IC_{50} ≈ 0.1-0.3 nM), PP5 (IC_{50} ≈ 3.5 nM) (Swingle et al., 2007) and PP6 (IC_{50} ≈ 0.1-0.3 nM) (Prickett and Brautigan, 2006). As shown in figure 15, at this concentration okadaic acid mitigates the NGF-induced attenuation of PKA activation induced by 1 µM PGE_2 (0.16 ± 0.01 in cultures grown with NGF versus 0.21 ± 0.02 in control cultures).

At a lower concentration (low nanomolar range), okadaic acid is more selective for PP2A, PP4 and PP6 than PP1 (Swingle et al., 2007). As shown in figure 16, when okadaic acid was added to both lysis and PKA-activity assay buffers at 2 nM, the effect of NGF on activation of PKA signaling by 1 µM PGE_2- was also attenuated (0.164 ± 0.028 in cultures grown with NGF versus 0.231 ± 0.028 in control cultures). Collectively, the experiments using high- and low-concentration okadaic acid, point to the possibility that the observed NGF-mediated attenuation of PGE_2-activated PKA signaling is mediated by (a) phosphatase(s) that is sensitive to inhibition by okadaic acid in the low nanomolar range. The ability of low concentration of okadaic acid to attenuate the effect of NGF suggests that this phosphatase may belong to the PP2A/PP4/PP6 family, although PP1 cannot be affirmatively ruled out.

It is noteworthy that at 2 µM, okadaic acid affected baseline PKA activity as well as PKA activation by 100 nM PGE_2 in the presence or absence of NGF. Baseline PKA activity (after exposure to vehicle for 10-minutes) was significantly increased from 0.06 ± 0.005 to 0.09 ± 0.007 in cultures grown in the absence of NGF and also increased but without significance from 0.04 ± 0.009 to 0.06 ± 0.007 in cultures grown in the presence of NGF. More importantly, PKA activation by 10-minutes exposure to 100 nM PGE_2 was nearly significantly doubled from 0.08 ± 0.017 to 0.14 ± 0.02 in cultures grown in the

absence of NGF and from 0.06 ± 0.008 to 0.1 ± 0.01 in cultures grown in the presence of NGF (Fig. 15 and Fig. 16). This may be explained by the fact that PP1, which is more strongly inhibited by the higher concentration of okadaic acid, is more active while the cells are in the baseline unstimulated state. This is not surprising as PP1 was originally thought to exert a braking effect on cellular signaling and this prevents aberrant phosphorylation and the subsequently activated signaling (Roadcap et al., 2007).

To further investigate whether PP1 mediates the NGF effect on PGE_2-activated PKA, I added 100 nM of inhibitor-2 (I-2) to the lysis and assay buffers. I-2 is a highly selective inhibitor of PP1 and 100 nM was shown to inhibit approximately 95 percent of the phosphatase activity of purified PP1 (Park and DePaoli-Roach, 1994). Another advantage is that unlike inhibitor-1, another PP1 inhibitor, I-2 does not require prior phosphorylation to activate its inhibitory properties (Oliver and Shenolikar, 1998). As shown in figure 17, I-2 (100 nM) was unable to reverse NGF-induced attenuation of PGE_2-activated PKA. This is analogous to the results obtained with low-concentration okadaic acid (Fig. 16) and supports the notion that PP1 does not influence the action of NGF on PGE_2-activated PKA (0.136 ± 0.017 in cultures grown with NGF versus 0.219 ± 0.036 in control cultures).

Baseline PKA activity was not significantly different in lysates obtained from cultures grown in the absence of NGF without phosphatase inhibitors compared to when 100 nM I-2 was added (0.05 ± 0.012 vs 0.03 ± 0.002). In lysates from cultures grown in the presence of NGF, however, PKA activity was significantly lower when I-2 was added (0.037 ± 0.003 vs 0.027 ± 0.002). Despite that PP1 represents > 90 percent of the total serine/threonine phosphatase activity in cells in general (Shi, 2009; Virshup and Shenolikar, 2009), PP1 does not appear to play a significant role in inhibiting baseline phosphorylation by PKA in sensory neuronal cultures grown in the absence of NGF. Surprisingly, PP1 seems to enhance baseline PKA signaling in sensory neuronal cultures grown in the presence of NGF.

Collectively, the experiments using okadaic acid and I-2 suggest that the observed effect of NGF is mediated, at least in part, by a serine/threonine protein phosphatase, which is insensitive to I-2 and is sensitive to okadaic acid at low concentration, possibly a member of the PP2A/PP4/PP6 family of phosphatases.

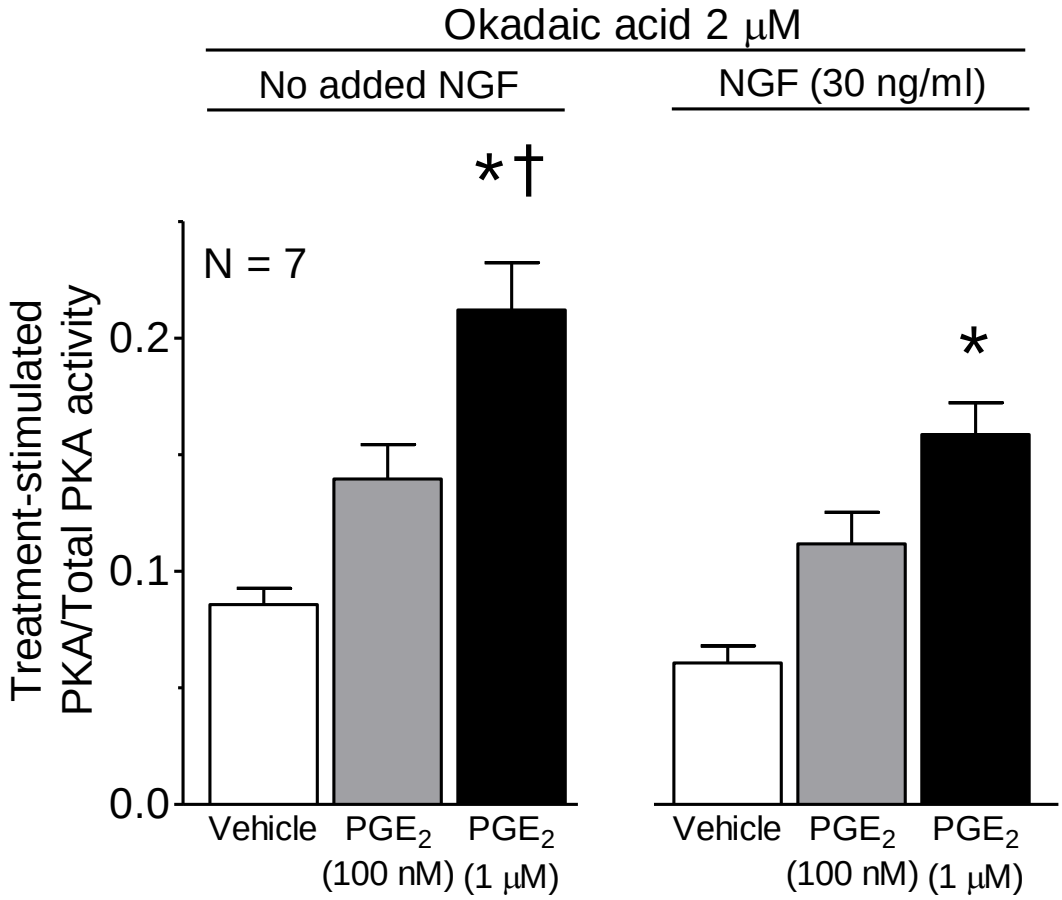

Figure 15. Okadaic acid (2 μM) reverses NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. Asterisks indicate statistically significant differences from vehicle, while a dagger represents significant difference from 100 nM PGE_2 using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

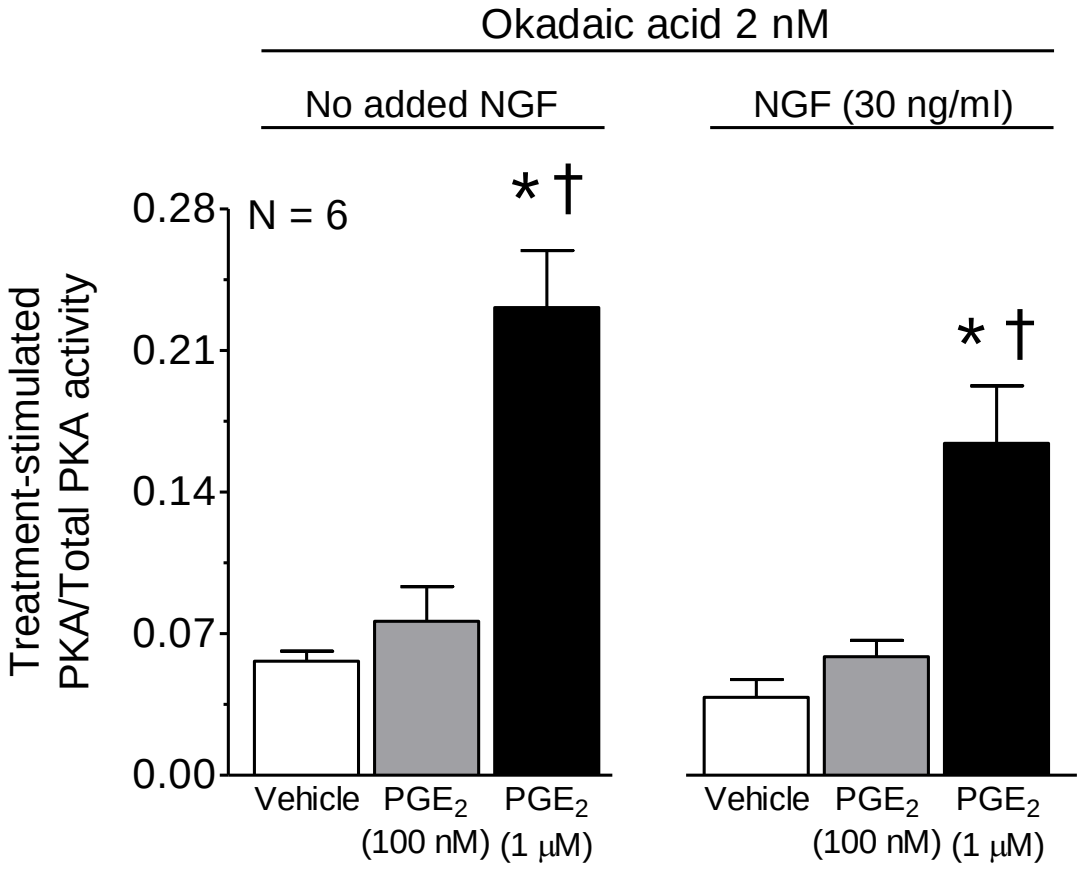

Figure 16. Okadaic acid (2 nM) reverses NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

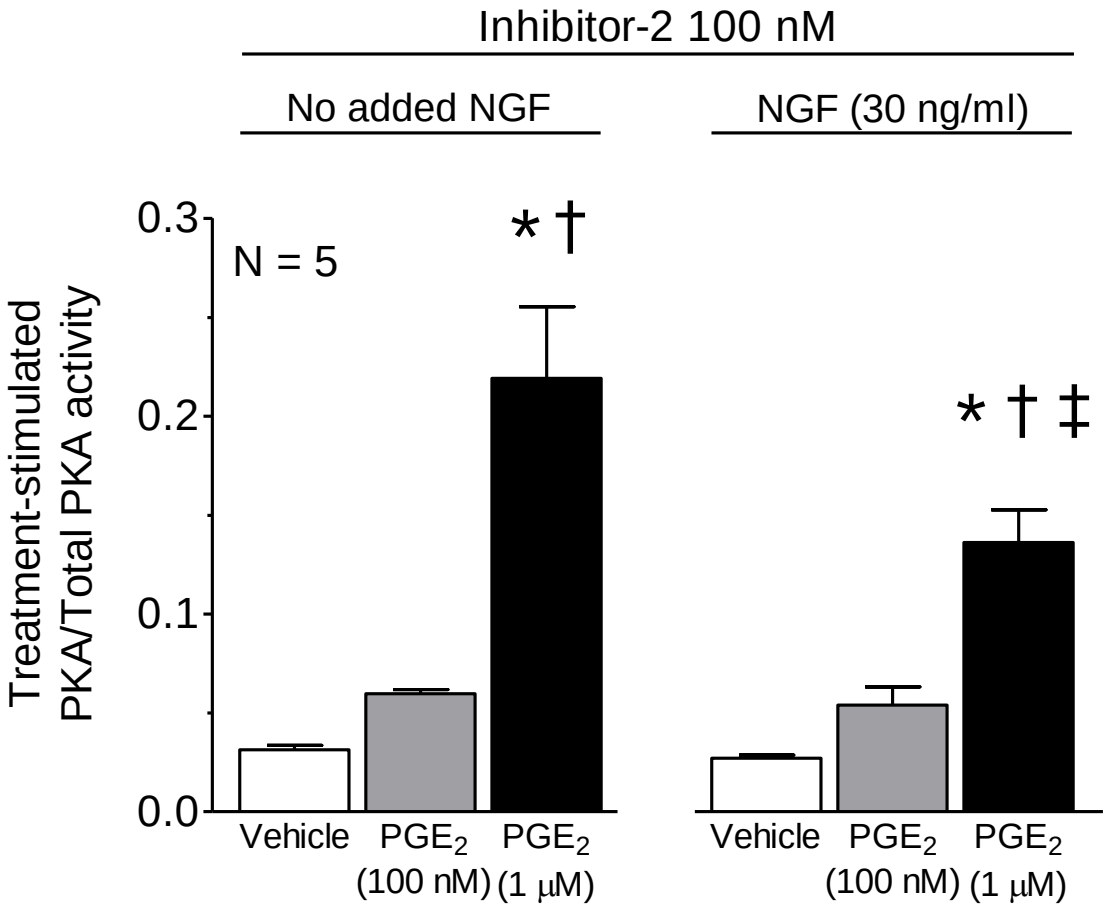

Figure 17. I-2 does not reverse NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 and a double dagger represents significant difference from cells grown in the absence of added NGF and acutely treated with PGE_2 (1 μM) for 10 minutes using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Microcystin- LR reverses NGF-induced attenuation of PGE_2-induced activation of PKA

As mentioned above okadaic acid has a potent inhibitory effect on several serine/threonine phosphatases. Microcystin-LR (MCS-LR) is a potent inhibitor of serine/threonine phosphatases PP1, PP2A, PP4, PP5 and PP6 (IC_{50} ≈ 0.3-1, < 0.1-1, 0.15 and 1 nM, respectively) (Honkanen et al., 1990). As shown in figure 18, MCS-LR (100 nM) completely reversed the attenuation of PGE_2-activated PKA caused by growing adult rat sensory neuronal cultures in NGF (0.2 ± 0.02 in cells grown without NGF versus 0.2 ± 0.03 in cells grown in 30 ng/ml NGF).

MCS-LR did not significantly increase basal PKA activity in lysates obtained from cultures grown in the absence of NGF when compared to those without phosphatase inhibitors (0.07 ± 0.004 compared to 0.05 ± 0.01). However, when grown in the presence of NGF, basal PKA activity in the lysates is significantly higher when MCS-LR is added (0.06 ± 0.007 versus 0.04 ± 0.003). Therefore NGF increases the activity of a MCS-LR-sensitive phosphatase that appears to reduce PKA-mediated phosphorylation in sensory neuronal cultures. These experiments suggest that NGF indirectly attenuates PGE_2-induced activation of PKA signaling via a MCS-LR sensitive serine/threonine phosphatase, possibly a member from the PP2A/PP4/PP6 family.

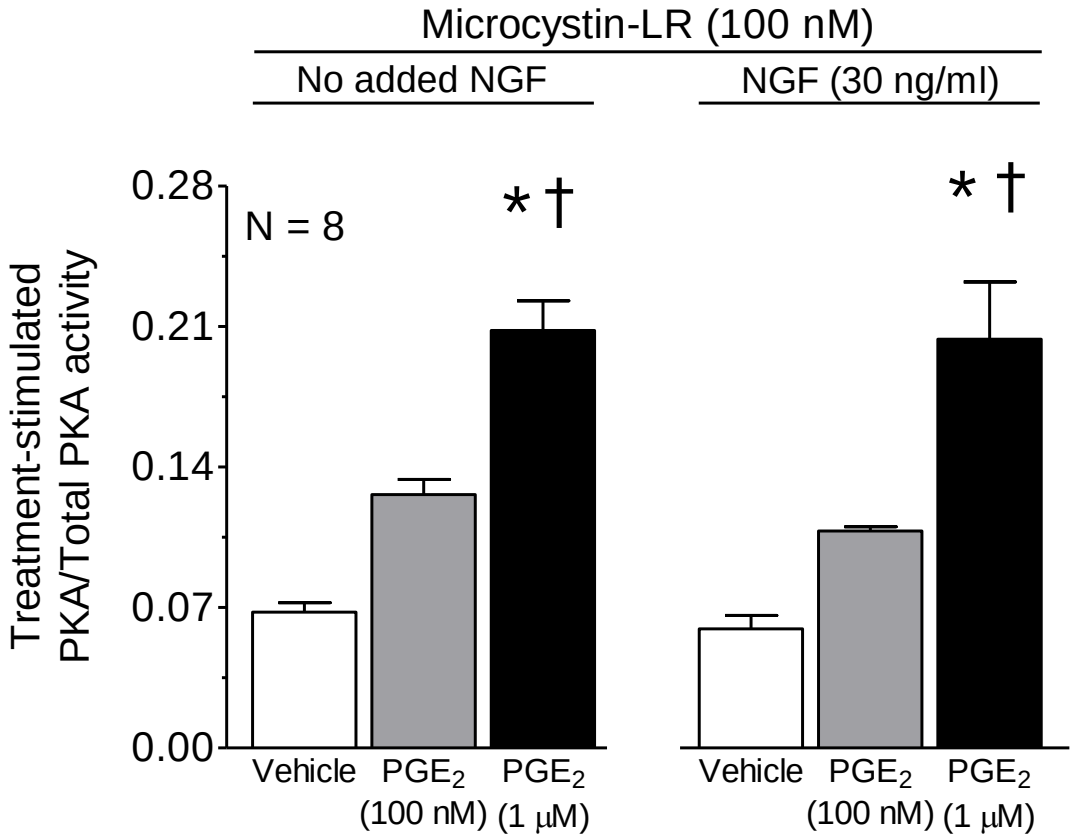

Figure 18. MCS-LR reverses NGF-induced attenuation of PGE_2-stimulated PKA activation. Each column represents the mean ± SEM of the treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells grown in the absence of added NGF while the right panel represents PKA activity from cells grown in the presence of added NGF 30 ng/ml for 12 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. Asterisks indicate statistically significant differences from vehicle, while daggers represent significant difference from 100 nM PGE_2 using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Prostaglandin-induced sensitization of adult rat sensory neurons becomes PKA-independent after long-term exposure to PGE_2

Previously, it was shown that PGE_2-induced sensitization of sensory neurons was mediated mainly by the GPCRs, EP3c and EP4 (Southall and Vasko, 2001). Classical GPCR activation is associated with desensitization and internalization, which are multistage phenomena, involving uncoupling of the receptor from its downstream signaling cascade (Ferguson, 2001; Gainetdinov et al., 2004; Pierce and Lefkowitz, 2001) and altering signaling (DeWire et al., 2007; Ferguson, 2001; Lefkowitz and Shenoy, 2005). However, it is well known that prostaglandins continue to sensitize sensory neurons under chronic inflammatory conditions, suggesting no physiological downregulation (Morlion, 2011; Sarzi- Puttini et al., 2010; Shah and Mehta, 2012b). To confirm the ability of PGE_2-induced sensitization to persist after chronic exposure to the eicosanoid, I exposed adult rat sensory neurons to PGE_2 (1 µM) from day 7 to day 12 in culture (total 5 days) and assayed capsaicin-evoked iCGRP release as a measure for sensitization. At the time of the experiment, the cells were washed twice with normal release buffer. The cultures were sequentially exposed to plain buffer, buffer containing PGE_2 or vehicle, buffer containing capsaicin with PGE_2 or vehicle and buffer alone for 10 minutes each. Cultures were then hypotonically lysed in 0.1N HCl. I observed that sensitization of capsaicin-evoked iCGRP release by re-exposure to PGE_2 is maintained even after continuous 5-day exposure to the prostanoid (Fig. 19). Indeed, in sensory neurons exposed to vehicle for 5 days capsaicin-evoked release was increased by approximately 49 percent when 1 µM PGE_2 was included in the buffer (from 10.5 ± 0.33 to 15.6 ± 0.5 percent of total content). Similarly, when exposed to 1 µM PGE_2 for 5 days, capsaicin-evoked iCGRP release increased by approximately 54 percent due to PGE_2-induced augmentation (from 10.9 ± 0.46 to 16.7 ± 0.47 percent of total content). This suggests that dissociated DRG cultures faithfully reproduce the phenomena observed *in vivo* in animal models of chronic inflammatory hyperalgesia and human clinical studies.

As mentioned earlier, the second fraction of buffer collected from DRG neurons contained vehicle or 1 µM PGE_2. In cells exposed to vehicle for 5 days, then re-exposed to 1 µM PGE_2 at the time of the experiment, second basal fraction contained significantly larger iCGRP (1.6 ± 0.08 percent of total content) when compared to iCGRP in first basal fraction collected from same cells (1.0 ± 0.1 percent) or when compared to the iCGRP contained in the second basal fraction collected from cells exposed to vehicle for

5 days then re-exposed to vehicle once more at the time of the experiment (0.9 ± 0.06 percent of total content). When cells were exposed to 1 μM PGE_2 for 5 days, iCGRP in the second basal fraction (1.12 ± 0.07 percent of total content) was significantly higher than iCGRP in the second basal fraction collected from cells exposed only to vehicle (0.8 ± 0.06 percent of total content) but not significantly higher than iCGRP from its corresponding first basal fraction (1.1 ± 0.09). It was shown previously that neurotransmitter release occurs in the absence of clear stimulation of synapses of CNS neurons (Chavez-Noriega and Stevens, 1994a; Chavez-Noriega and Stevens, 1994b; Maximov and Sudhof, 2005; Otsu and Murphy, 2003; Sara et al., 2005). It also was shown that this basal spontaneous release can be enhanced by activation of adenylyl cyclase or PKA (Chavez-Noriega and Stevens, 1994a; Maximov et al., 2007). The significant PGE_2-induced increase in iCGRP outflow in the absence of stimulation by capsaicin (basal outflow) may result from direct enhancement of spontaneous random fusion of neurotransmitter-containing synaptic vesicles in sensory neuronal endings.

It was previously reported exposure to 100 μM dimethyl-PGE_2 (a PGE_2 analogue) caused significant increase in total CGRP content (Ma, 2010). In this experiment total iCGRP content in naïve cultures exposed to vehicle for 5 days (497.5 ± 67.8 and 486.4 ± 57.5 fmol/well in cells exposed acutely to vehicle or PGE_2 respectively) was not significantly different from that in cultures exposed to 1 μM PGE_2 for five days (515 ± 74 and 540 ± 66.6 fmol/well in cells exposed acutely to vehicle of PGE_2 respectively).

I then asked if H-89 attenuates PGE_2-induced sensitization of sensory neurons after long-term exposure to the eicosanoid akin to the attenuation of the acute sensitizing actions of PGE_2 by H-89. In sensory neuronal cultures grown in the absence of NGF and exposed to 1 μM PGE_2 for 5 days, re-exposure to the eicosanoid at the same concentration augmented capsaicin-evoked iCGRP release from 6.2 ± 0.4 to 11.6 ± 0.6 percent of total content and from 8.5 ± 0.7 to 11.3 ± 0.5 percent of total content with and without 10 μM H-89, respectively (Fig. 20). Since it is possible that H-89 in these cells is not inhibiting PKA, the ability of H-89 to inhibit this kinase was confirmed using purified bovine PKA catalytic subunit under the same assay conditions used throughout the dissertation. At 1 and 10 μM, H-89 inhibited 82.63 percent and 96.23

percent respectively of the total PKA activity of the control (Fig. 21). These findings suggest that PGE_2 induced-sensitization is not mediated by PKA after long-term exposure to the prostanoid therefore providing the first clue in my work for the existence of a signaling switch.

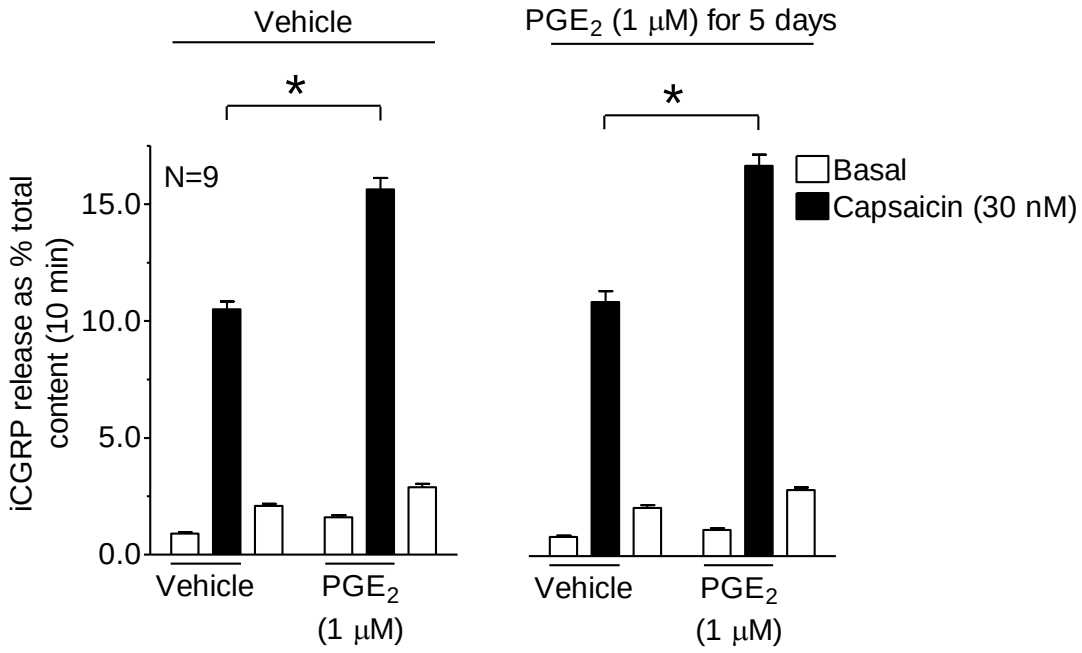

Figure 19. PGE_2-induced augmentation of capsaicin-evoked iCGRP release is maintained after long-term exposure to the prostanoid. Each column represents the mean ± SEM of iCGRP release as percent of total content. Left panel represents release from cells exposed to vehicle for 5 days, while right panel represents release from cells exposed to PGE_2 (1μM) for 5 days. Open columns indicate basal release whereas closed columns represent capsaicin-evoked iCGRP release. Asterisks indicate statistically significant difference as indicated between vehicle-treated versus PGE_2-treated cells (1 μM). Statistical analysis was done using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

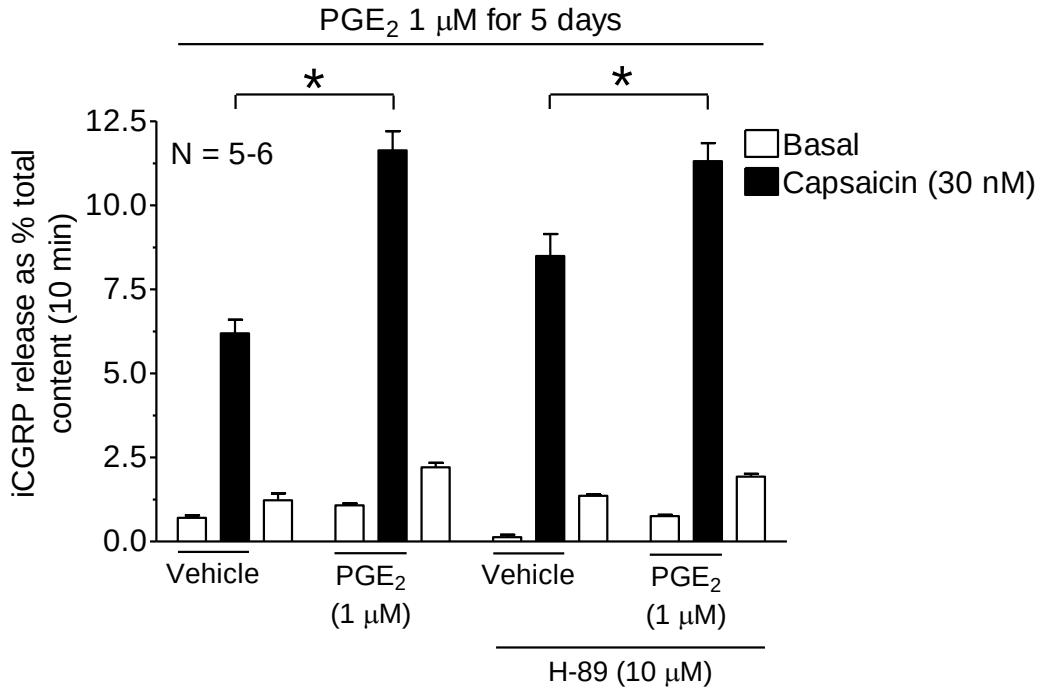

Figure 20. PGE_2-induced augmentation of capsaicin-evoked iCGRP release is not attenuated by H-89 after long-term exposure to the prostanoid. Each column represents the mean ± SEM of iCGRP release as percent of total content from sensory neuronal cultures treated with 1 μM PGE_2 for 5 days. Left panel represents release from cells exposed to vehicle for 5 days, while right panel represents release from cells exposed to PGE_2 (1μM) for 5 days. Open columns indicate basal release whereas closed columns represent capsaicin-evoked iCGRP release. Asterisks indicate statistically significant difference as indicated between iCGRP released from vehicle-treated versus that from PGE_2-treated cells (1 μM). Statistical analysis was done using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

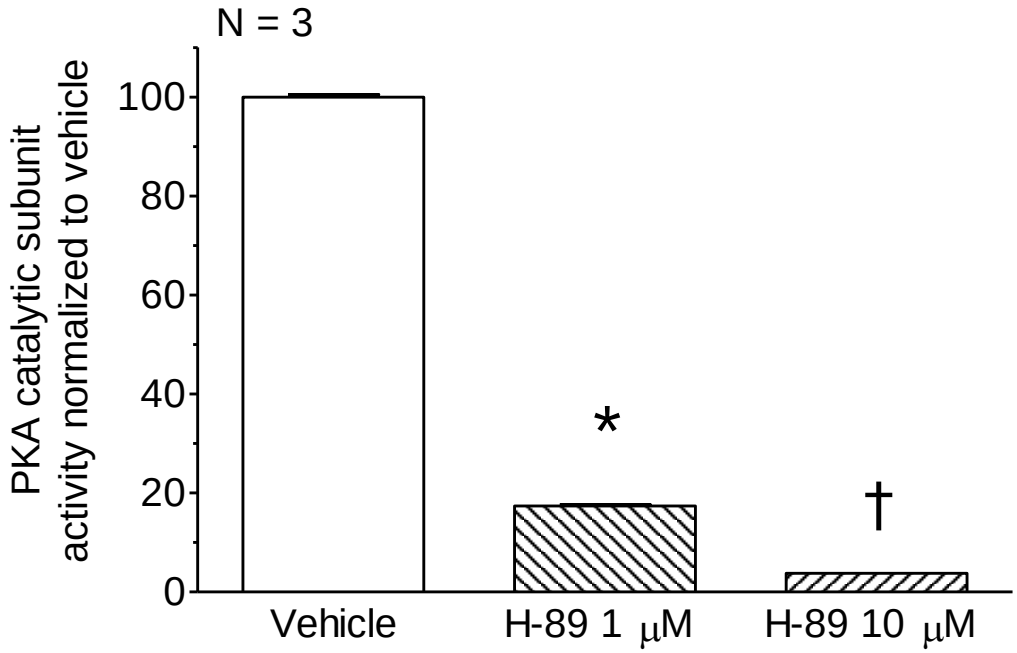

Figure 21. H-89 inhibits purified PKA catalytic subunit activity in a concentration-dependent manner. Each column represents mean ± SEM of PKA catalytic subunit activity normalized to vehicle. An asterisk represents statistically significant difference from vehicle, whereas a dagger represent statistically significant difference from 1 μM H-89 using one-way ANOVA followed by Bonferroni's post-test.

Long-term exposure to PGE_2 inhibits PKA

It is possible that PKA does not mediate PGE_2-induced sensitization of sensory neurons after long-term exposure to the eicosanoid, since the observed sensitization was not attenuated by H-89. Consequently, I asked whether re-exposure to PGE_2 activated PKA after long-term exposure to the prostanoid. As shown in figure 3, acute exposure of sensory neuronal cultures to PGE_2 causes concentration-dependent activation of PKA. After 5-days exposure to 1 µM PGE_2, re-exposure to the eicosanoid failed to activate PKA (PKA activities were 0.06 ± 0.003 and 0.52 ± 0.1 for cultures exposed acutely to vehicle and PGE_2 respectively, in cells not pre-treated with PGE_2 and 0.07 ± 0.0003 for culture exposed acutely to PGE_2 after pre-exposure to the eicosanoid for 5 days, Fig. 22). Total specific PKA activity was not altered after 5-day exposure to 1 µM PGE_2 (Fig. 23). A higher concentration of PGE_2 (10 µM) did not activate PKA after long-term exposure to 1 µM of the eicosanoid. In naïve neurons that were exposed to vehicle for 5 days, 1 µM PGE_2 stimulated PKA activity to 0.57 ± 0.08, while in neurons exposed to 1 µM PGE_2 for five days, PKA activated by re-exposure to 10 µM PGE_2 was 0.14 ± 0.01 (Fig. 24). Thus the observed desensitization cannot be overcome by increasing the ligand concentration.

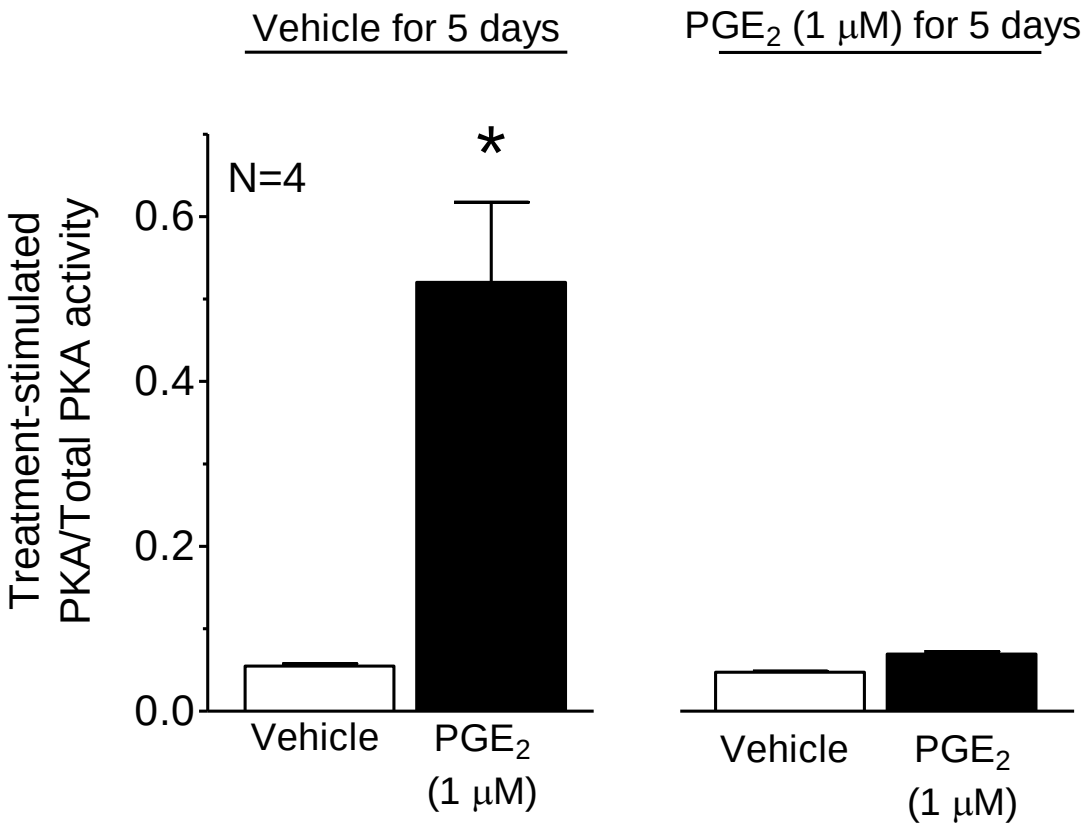

Figure 22. Five-day exposure to PGE_2 inhibits PKA activation by re-exposure the eicosanoid. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to 1 μM PGE_2. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

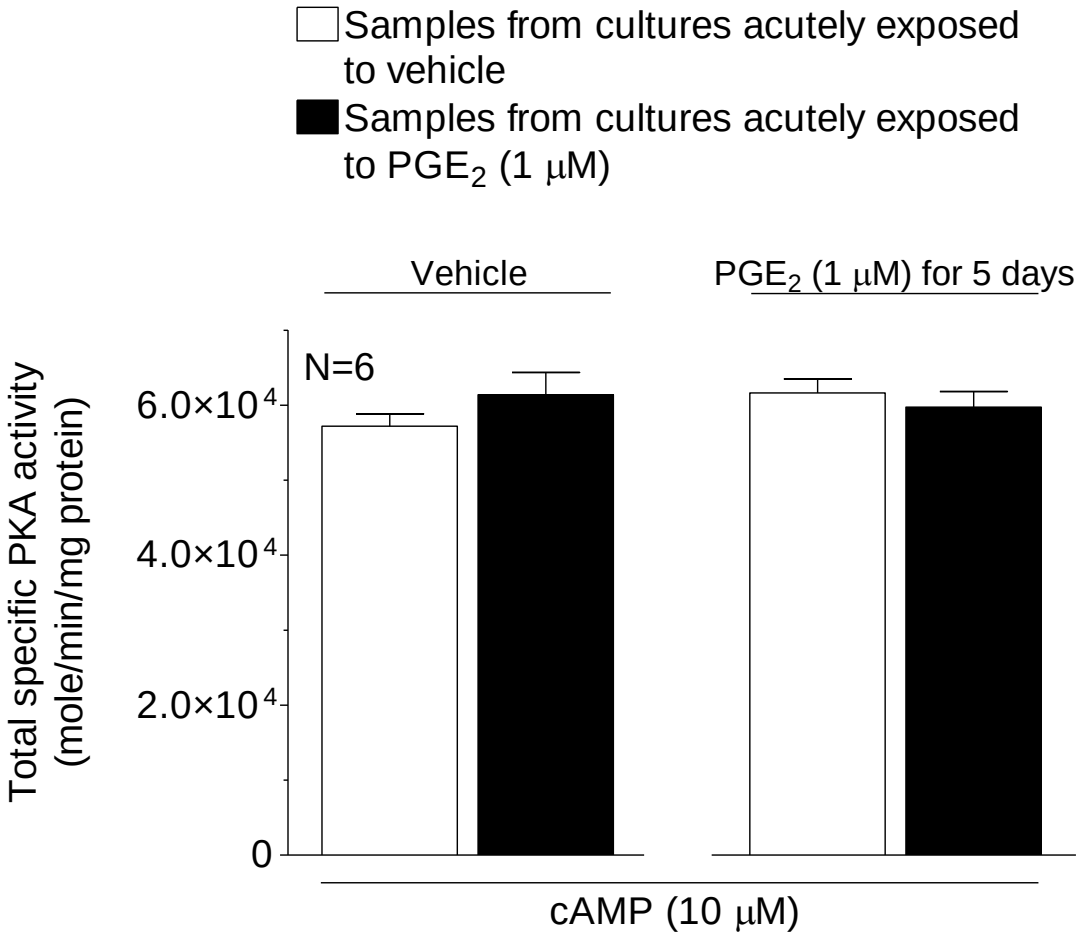

Figure 23. Five-day exposure to PGE_2 (1 μM) does not alter total PKA activity in adult rat sensory neuronal cultures. Each column represents mean ± SEM of total specific activity of PKA calculated after exposure to 10 μM cAMP. No statistical significance was detected using one-way ANOVA.

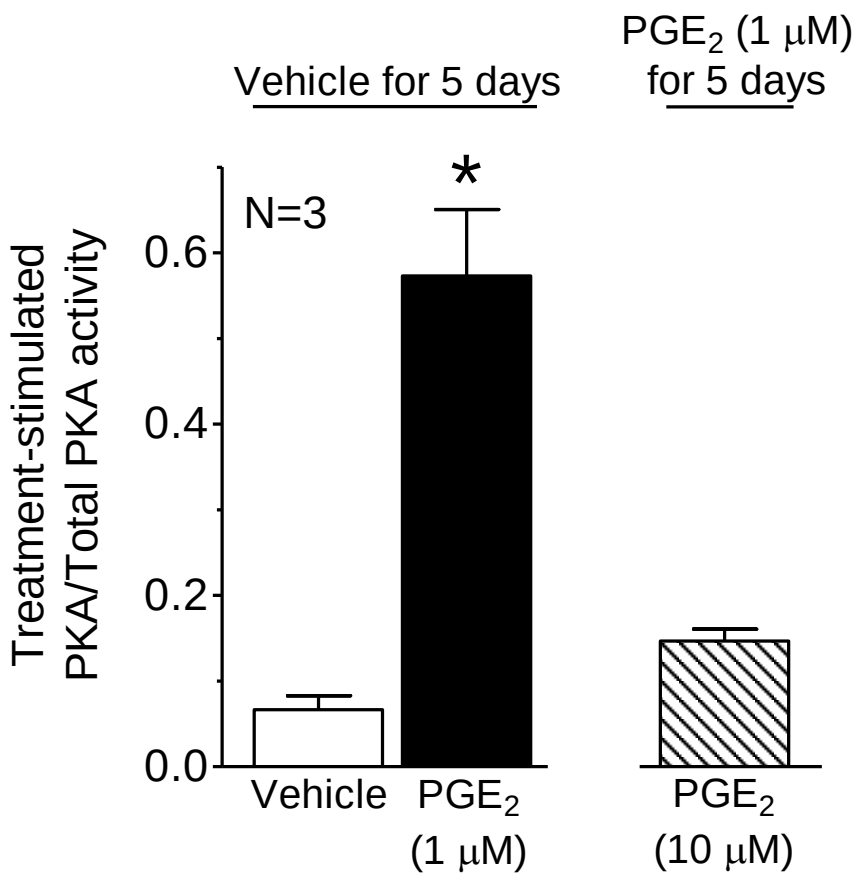

Figure 24. Exposure to 10 μM PGE_2 does not overcome inhibition of PKA activation after five-day pre-exposure to the eicosanoid. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open column represents cells treated acutely with vehicle, while closed and hatched columns represent cells acutely exposed to PGE_2 as indicated. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

PGE_2-induced cAMP synthesis is inhibited after long-term exposure to the prostanoid

Historically, PKA activity was correlated with an increase in cAMP synthesis. Therefore, I asked whether the loss of PKA activation by PGE_2 after long-term exposure to the eicosanoid, is accompanied by reduction or loss of cAMP synthesis (Fig. 25). In experiments done by Djane B. Duarte, PhD, cAMP synthesis was increased from 68 ± 6.8 to 183 ± 40 pmol/ml after 10-minutes exposure of naïve sensory neuronal cultures to 1 µM PGE_2. In cultures exposed to PGE_2 (1 µM) for 5 days, however, re-exposure to PGE_2 did not significantly increase cAMP synthesis compared to vehicle (61.3 ± 4 and 76 ± 10.3 pmol/ml for vehicle and PGE_2, respectively). This indicates that EP receptor-mediated cAMP synthesis was lost after long-term exposure to 1 µM PGE_2 for 5 days. Reduction in cAMP synthesis can be caused by several mechanisms, one of which is reduction of adenylyl cyclases expression or activity (El-Haroun et al., 2004; Matsumoto et al., 2005). Forskolin-activated cAMP synthesis was not significantly different in cultures exposed for 5 days to vehicle (530 ± 34 pmol/ml) or 1 µM PGE_2 (501 ± 46 pmol/ml). Thus, despite the loss of PGE_2-induced cAMP synthesis, adenylyl cyclase activity remained unchanged.

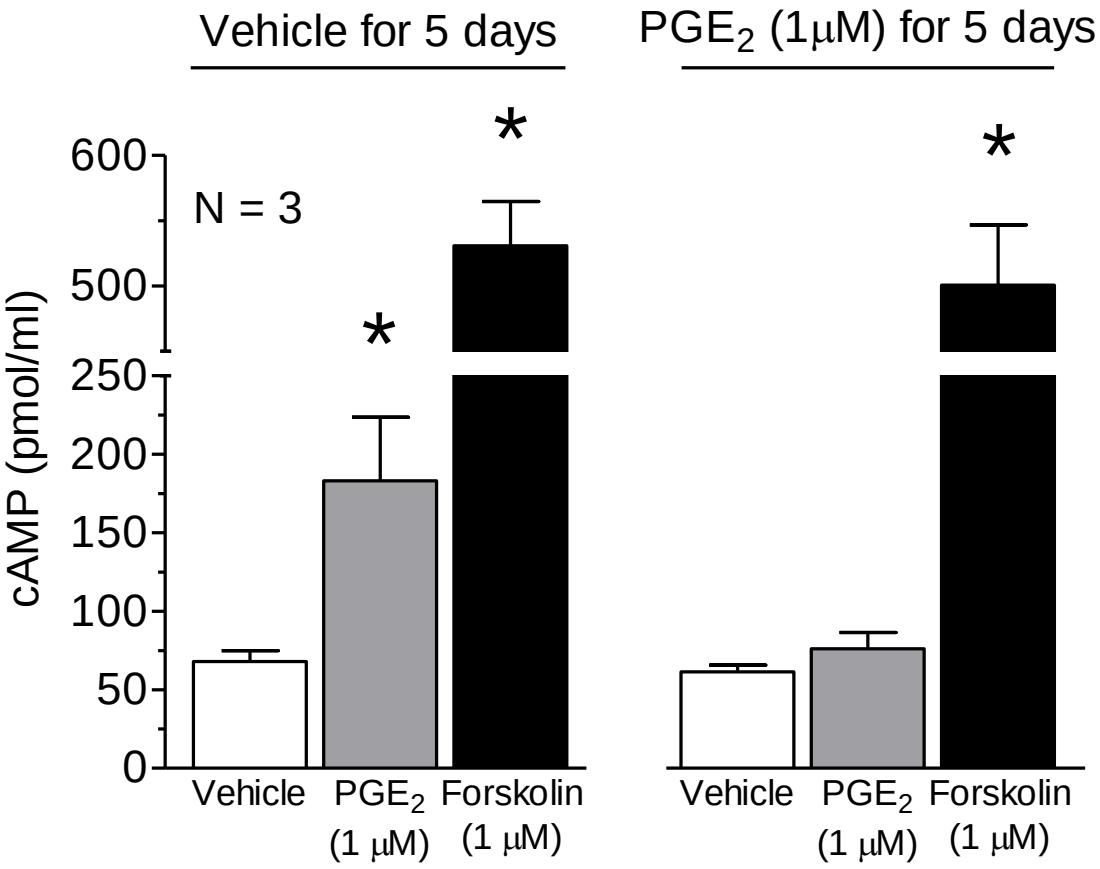

Figure 25. Five-day exposure to PGE_2 inhibits cAMP synthesis by re-exposure the eicosanoid. Each column represents the mean ± SEM of the treatment-stimulated cAMP synthesis. The left panel represents cAMP synthesis from cells exposed to vehicle for 5 days while the right panel represents cAMP synthesis from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, gray columns represent cells treated acutely with 1 μM PGE_2, while closed columns represent cells acutely exposed to 1 μM forskolin. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$. Experiment done by Djane B. Duarte, PhD.

Desensitization of PGE_2-activated PKA correlates with time of exposure to the eicosanoid and is reversible

Previous experiments show that 5-day exposure to 1 µM PGE_2 causes inhibition of PKA activation by the eicosanoid. However, the minimum duration needed to cause this inhibition of PKA activation by PGE_2 cannot be determined from these previous experiments. Therefore I asked what the minimum duration necessary for significant inhibition of PGE_2-activated PKA after long-term exposure to the eicosanoid is. In order to determine the time-course for the development of this observed desensitization, I measured PKA activation after exposure to PGE_2 for various lengths of time. In sensory neuronal cultures derived from the same harvest, wells were exposed to vehicle for the last 5 days of the 12-day long duration of the culture. Other wells got exposed to 1 µM PGE_2 for the last 3 hours, 6 hours, 12 hours, 72 hours or 5 days. All wells were 12 days old when the assay was conducted. Surprisingly, PKA desensitization was observed at all tested time points. Three-hour long exposure to PGE_2 was sufficient to reduce PKA activation by approximately 48 percent of PGE_2-activated PKA in naïve neurons. After 5 days of exposure to PGE_2, PKA activation was reduced by more than 94 percent of PGE_2-activated PKA in naïve cultures (Fig. 26).

Desensitization of PGE_2-activated PKA after long-term exposure to the eicosanoid was reversible upon removal of PGE_2 from the media. Briefly, some wells from the same cultures were exposed to either vehicle or 1 µM PGE_2 for the last 36 hours of the 12-day period for which the culture was kept. In the same cultures other wells were exposed to 1 µM PGE_2 33, 24, or 12 hours and then to vehicle for 3, 12 or 24 hours, respectively. After 12 and 24 hours of removal of PGE_2, PKA activation by re-exposure to the eicosanoid recovered to approximately 42 percent and 78 percent of PGE_2-activated PKA in naïve cultures (Fig. 27). This indicates that PKA desensitization after long-term exposure to PGE_2 is reversible and is not due to damage of neurons that is caused by prolonged exposure to the prostanoid.

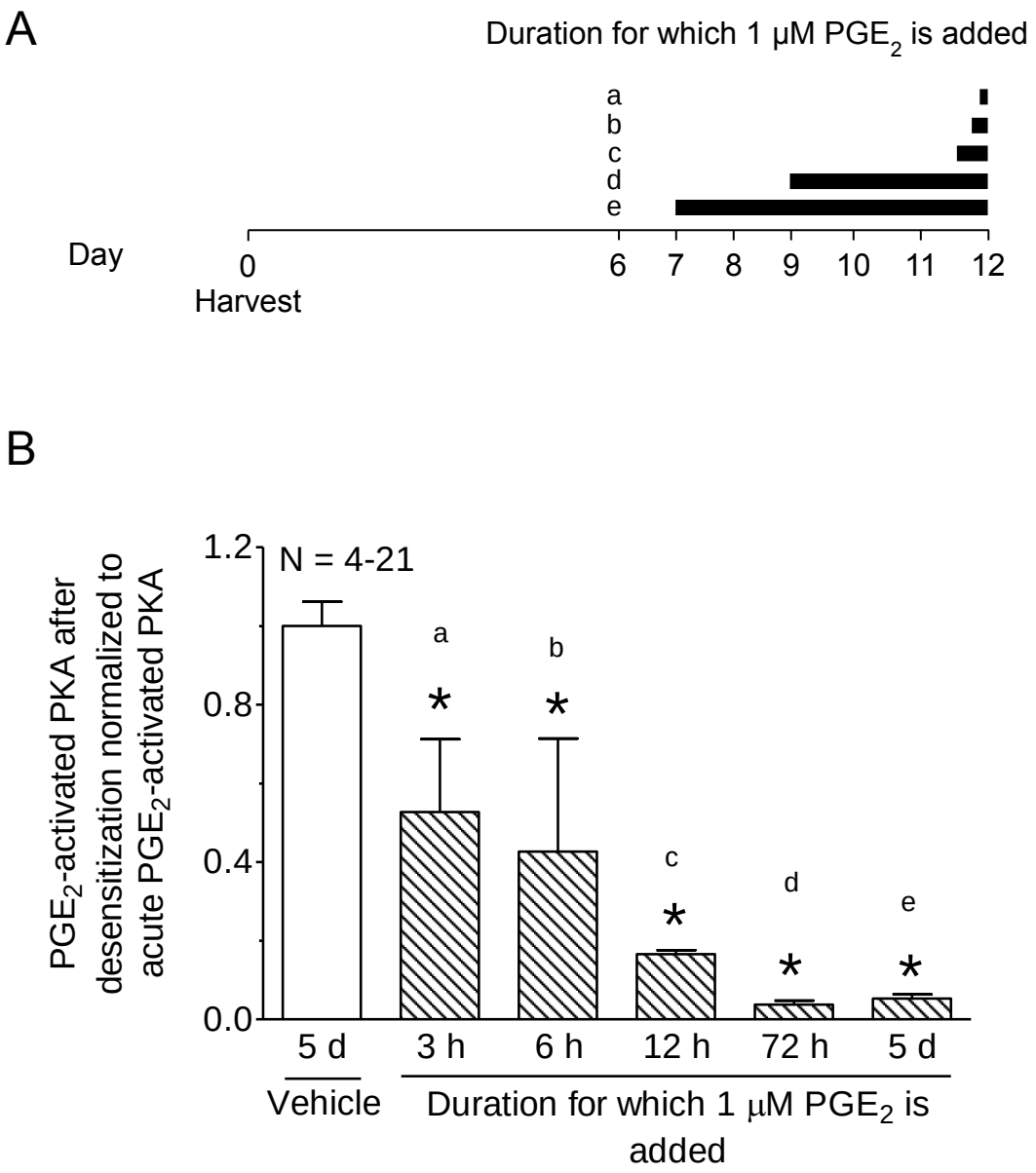

Figure 26. Desensitization of PGE_2-induced activation of PKA is correlated with the duration of exposure to the eicosanoid. (A) Protocol of long-term exposure to 1 μM PGE_2. (B) Each column represents the mean ± SEM of the ratio of PGE_2-induced PKA activation after pre-exposure as indicated normalized to neurons exposed to vehicle for 5 days. An asterisk indicates statistically significant difference between PGE_2-treated sensory neuronal cultures and vehicle-treated cultures using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

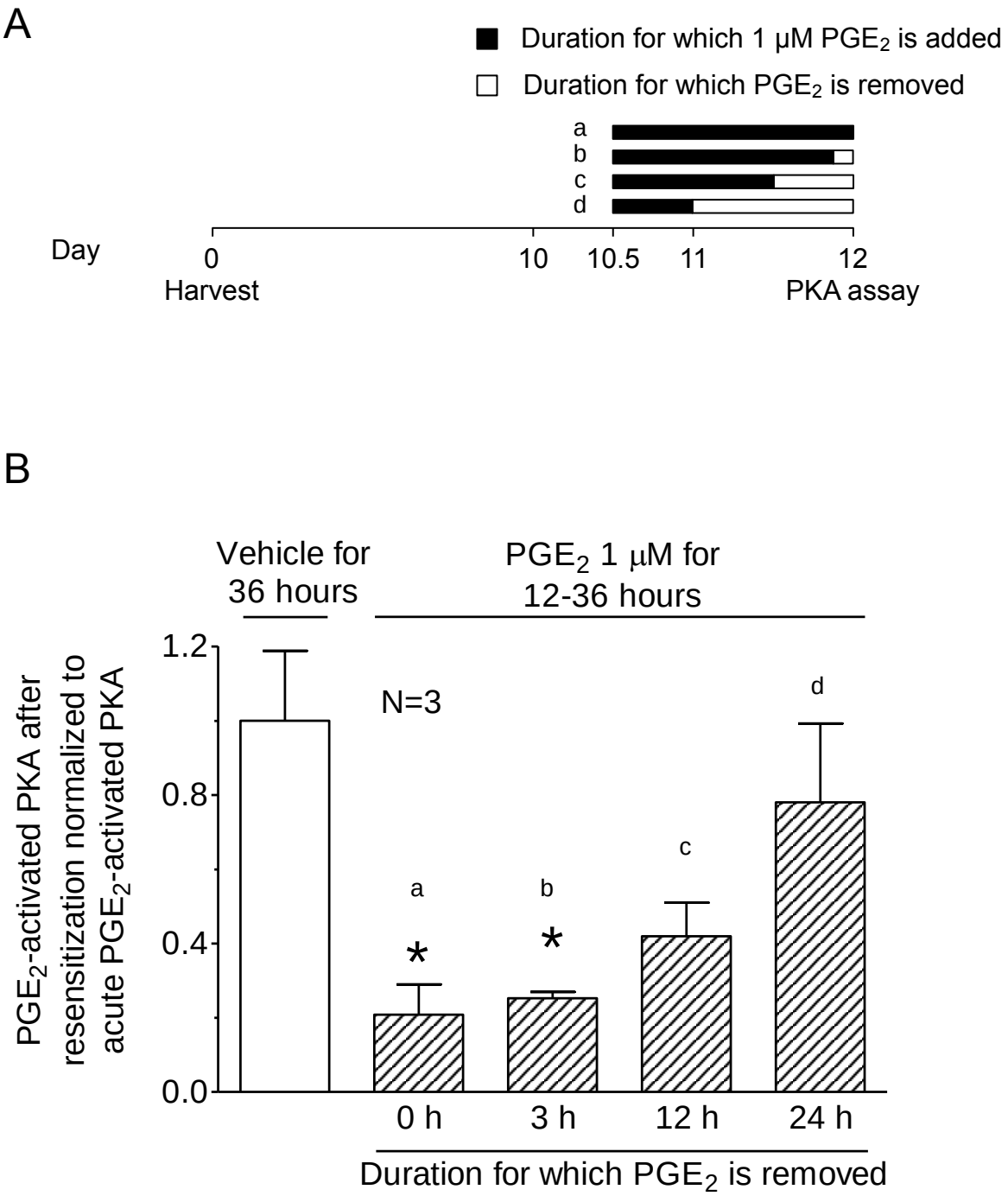

Figure 27. Resensitization of PGE_2-induced activation of PKA is dependent on the length of withdrawal from exposure to the eicosanoid. (A) Time line of long-term exposure to and removal of 1 μM PGE_2. (B) Each column represents the mean ± SEM of the ratio of PGE_2-induced PKA activation after pre-exposure and withdrawal as indicated to neurons pre-exposed to vehicle for 36 hours. An asterisk indicates statistically significant difference between PGE_2-treated sensory neuronal cultures and vehicle-treated cultures using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Homologous desensitization of PKA signaling after long-term exposure to PGE_2

To address the question whether PKA desensitization after long-term exposure to PGE_2 is homologous or heterologous, I used different ligands that act on receptors known to be coupled to the $G_{\alpha s}$/PKA pathway. I chose the stable prostacyclin analogue, carbaprostacyclin ($cPGI_2$), because it activates its cognate $G_{\alpha s}$-coupled receptor (IP receptor) which is expressed in sensory neurons (Hingtgen and Vasko, 1994b; Hingtgen et al., 1995). In sensory neurons grown in culture for 12 days in media alone, 1 µM $cPGI_2$ activated PKA to the same extent as in 5-day PGE_2 or vehicle treated DRG cultures (0.544 ± 0.04 and 0.475 ± 0.047, respectively) (Fig. 28). In contrast to $cPGI_2$ (Fig. 23), after cells were treated with 1 µM PGE_2 for 5 days, subsequent acute exposure to PGE_2 itself failed to activate PKA suggesting that the observed desensitization is not heterologous.

I also used L902688, a selective agonist of EP4 receptor, which is also $G_{\alpha s}$-coupled. L902688 has an approximate 7,000-32,000 higher affinity of binding to EP4 when compared to other EP receptor subtypes (Young et al., 2004). EC_{50} of PGE_2 is 1 µM in DRG cultures using PKA activation (Fig. 3). The EC_{50} for L902688 was not determined in DRG cultures. It was found, however, in EP4-expressing HEK293 cells, that the EC_{50}s for PGE_2 and L902688 were 3 and 0.6 nM, respectively (Young et al., 2004). I decided to use L902688 at a concentration of 300 nM so that the ratio between the EC_{50} for PGE_2 and L902688 is similar to that reported by Young *et al*, assuming that EP4 receptors in DRG cultures and in EP4-expressing HEK cells have similar affinities. As shown in figure 29, L902688 significantly increased PKA activity in naïve sensory neuronal cultures (0.12 ± 0.005 and 0.04 ± 0.007 for L902688 and vehicle respectively) and 5 day exposure to L902688 inhibited PKA activation by subsequent exposure to itself by approximately 92 percent (0.06 ± 0.01 and 0.06 ± 0.005 for L902688 and vehicle respectively). Similarly, long-term exposure to 1 µM PGE_2 significantly inhibited L902688-activated PKA (0.06 ± 0.007 and 0.04 ± 0.01 for L902688 and vehicle respectively), a 72 percent inhibition compared to L902688-activated PKA in naïve cultures (Fig. 30) Collectively, these results substantiate the model that long-term exposure to PGE_2 causes desensitization of PKA activation that is homologous.

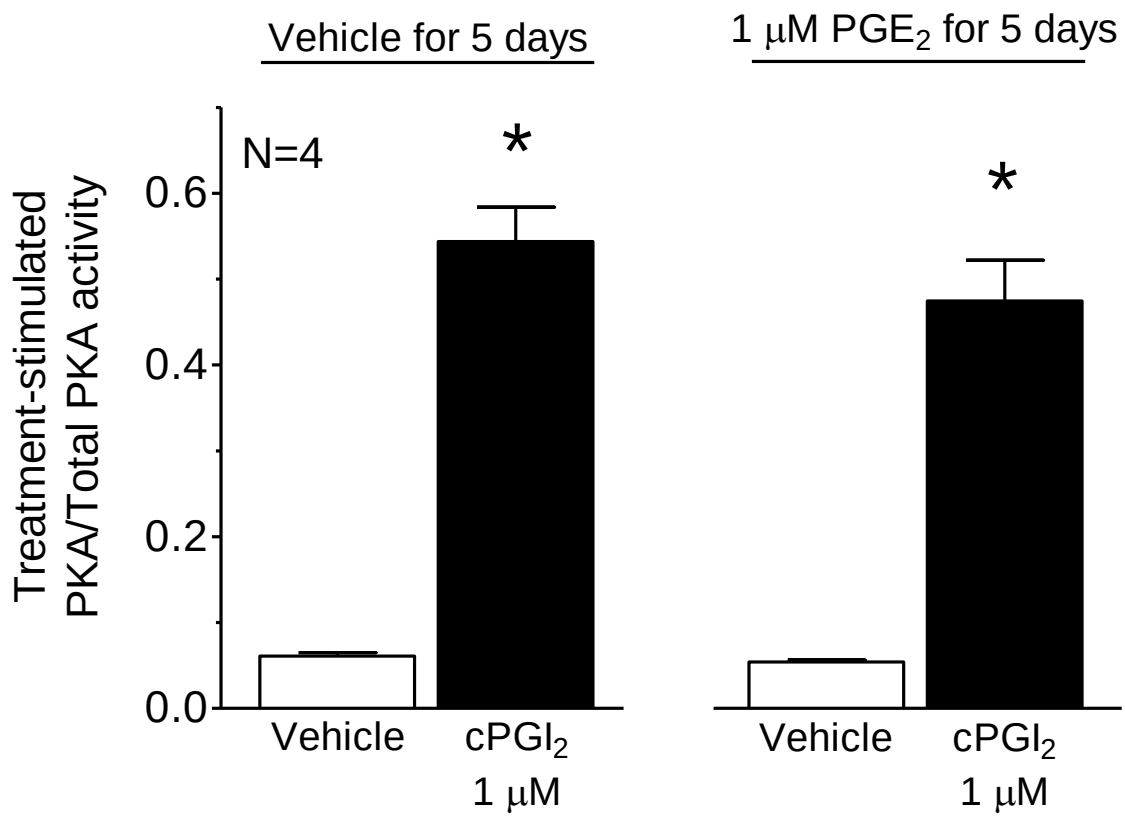

Figure 28. Five-day exposure to PGE_2 does not inhibit PKA activation by $cPGI_2$. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to $cPGI_2$ (1 μM). An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

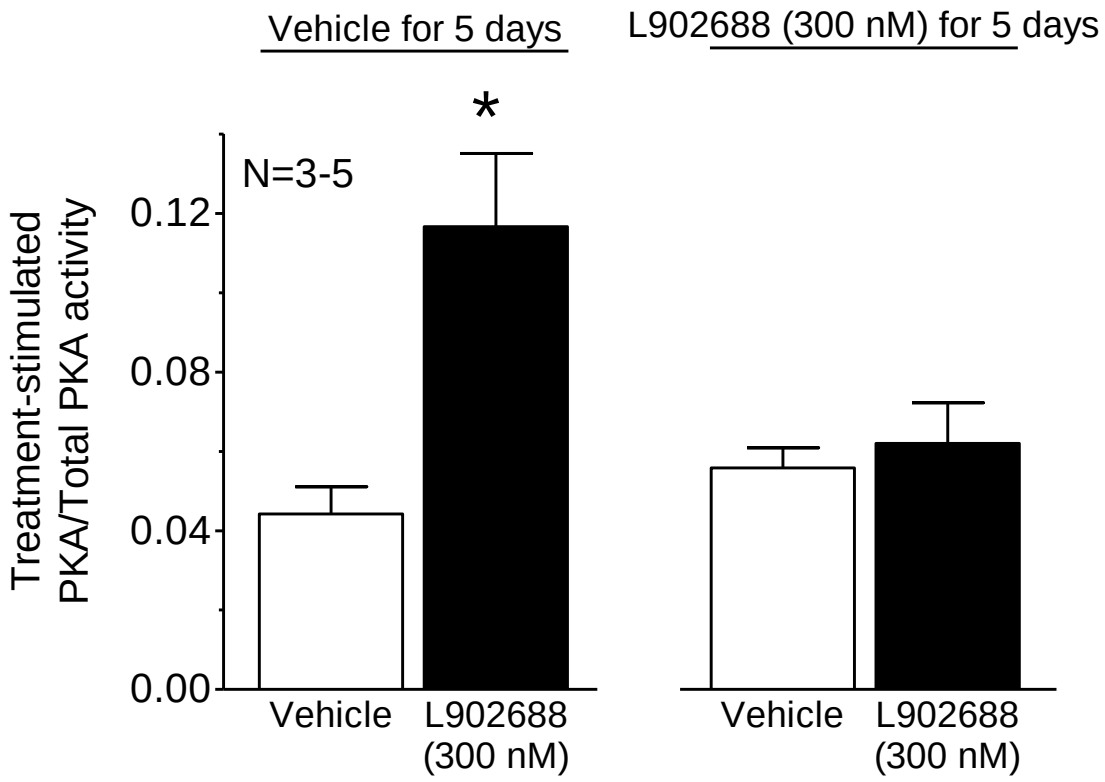

Figure 29. Five-day pre-exposure to L902688 inhibits PKA activation by itself. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 µM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to L902688 (300 nM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to L902688. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

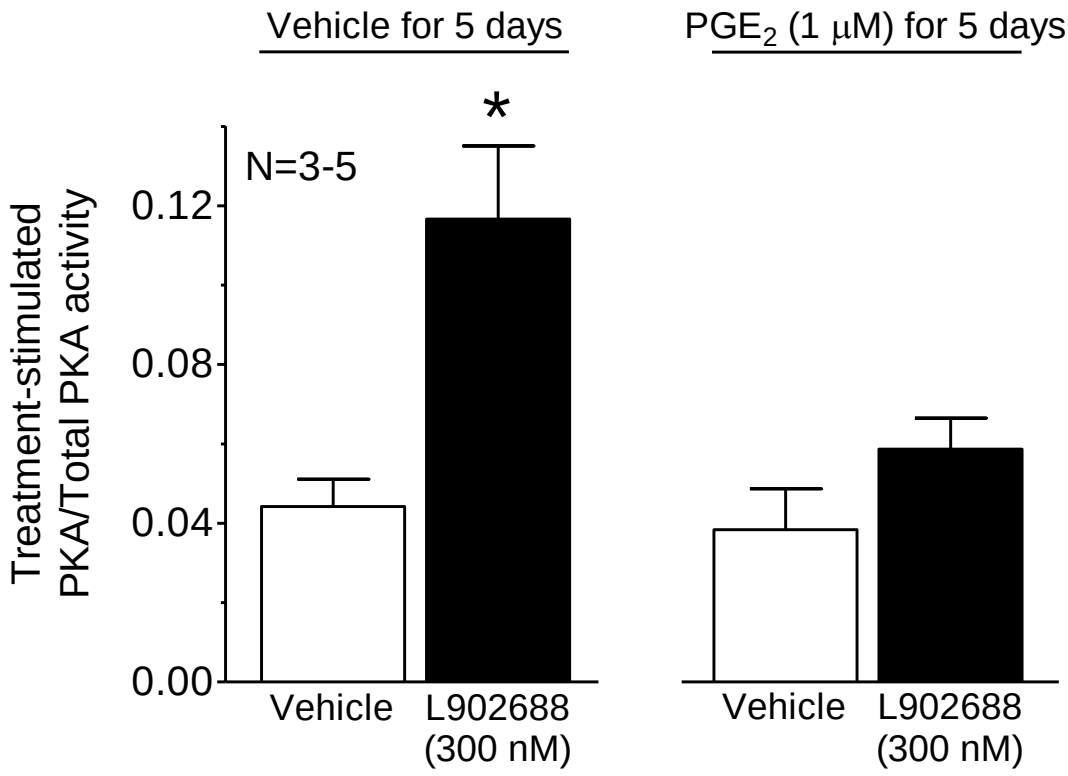

Figure 30. Five-day exposure to PGE_2 inhibits PKA activation by L902688. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to L902688. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Long-term exposure to PGE_2 does not reduce mRNA levels of EP receptors or AKAPs

One possible explanation for the loss of PKA activation after chronic exposure to PGE_2 is reduction of the expression of EP receptors. As mentioned earlier, there are 4 different isoforms of the receptors and EP3 has multiple different splice variants. Due to the potential of poor selectivity of antibodies against these receptors, I used real time PCR to examine the level of expression of receptor mRNA in sensory neuronal cultures after 5 days of exposure to vehicle or PGE_2. As shown in figure 31, the mRNA levels of any of the EP receptors did not change after long-term exposure to PGE_2.

Because PKA signaling is intimately coupled to A-kinase anchor proteins (AKAPs), I also measured mRNA levels of AKAP5 and 250 which were shown to be expressed in sensory neurons and mediate PGE_2-induced sensitization of TRPV1 (Fan et al., 2001; Irmen et al., 2008; Jeske et al., 2008; Rathee et al., 2002b; Schnizler et al., 2008; Tao et al., 2007; Willoughby et al., 2006; Zhang et al., 2008). Theoretically, loss of PKA activation after long-term exposure to PGE_2 could be mediated by reduction of expression of AKAP. Real time PCR was used to measure the mRNA levels of AKAP5 and 250 and I did not observe any changes after long-term exposure to PGE_2 (Fig. 4a). This suggests that PKA desensitization does not depend on changes in mRNA levels of AKAP5, AKAP12, EPs 1-4, Grks2, 3, 5 and 6 and β-arrestins 1 and 2.

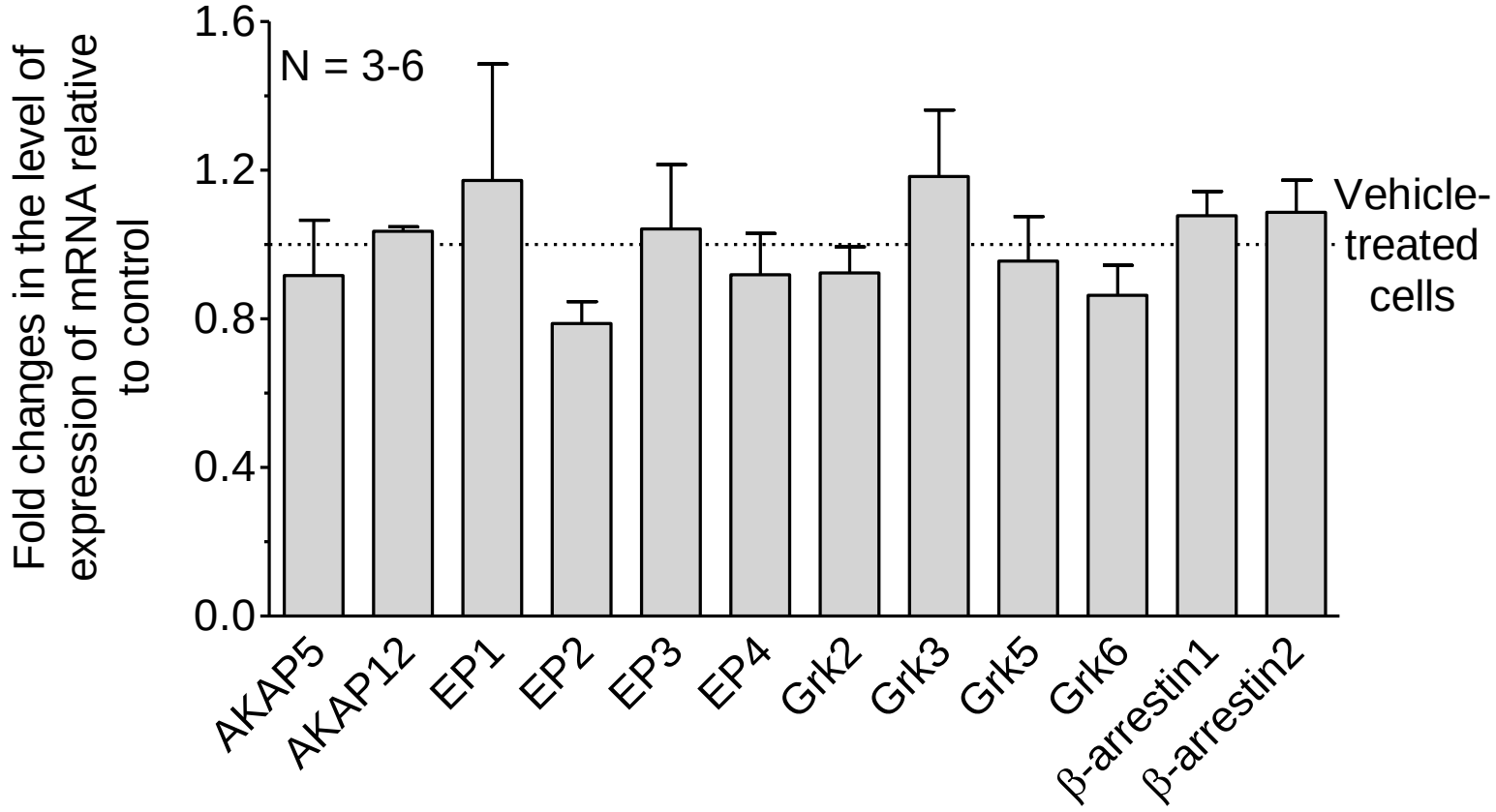

Figure 31. Five-day exposure to PGE_2 does not alter the relative mRNA level of AKAPs, EPs or Grks in adult rat sensory neuronal cultures. Each column represents the mean ± SEM of the relative level of mRNA normalized to that of control cultures. Grey columns represent cultures exposed to 1 μM PGE_2 for 5 days, while the dotted line represents control cultures exposed to vehicle for 5 days. There was no statistical significance using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Cholera toxin-induced PKA activation is maintained after long-term exposure to PGE_2

Classical GPCR desensitization is mediated by receptor uncoupling from the cognate heterotrimeric G-protein and the downstream signaling pathway (Davies and Lefkowitz, 1983; Leeb-Lundberg et al., 1985; Sibley et al., 1986). In sensory neurons exposed to PGE_2 for 5 days, it is possible that EP4 and EP3C receptors are no longer coupled to $G_{\alpha s}$/adenylyl cyclase/PKA pathway. If this is true, a tool that is able to bypass the receptor and directly activate $G_{\alpha s}$ or adenylyl cyclase should be able to activate PKA even after long-term exposure to PGE_2. Fortunately cholera toxin and forskolin can bypass the receptor and activate $G_{\alpha s}$ and adenylyl cyclase respectively (Gilman, 1984). Cholera toxin ADP-ribosylates $G_{\alpha s}$ and locks it in the activated state (Noel et al., 1993). Previously, Vasko and coworkers showed that 16-hour incubation with 1.5 µg/ml of cholera toxin ADP-ribosylates in excess of 95 percent of $G_{\alpha s}$ (Hingtgen et al., 1995). Overnight incubation with 1.5 µg/ml cholera toxin caused sensitization (twofold increase in iCGRP release from 10 ± 0.9 to 20.3 ± 0.8 percent of total content with or without cholera toxin, respectively). In neuronal cultures that were exposed to 1 µM PGE_2 for 5 days, cholera toxin also caused similar augmentation of capsaicin-evoked iCGRP release (97 percent increase from 9.8 ± 0.6 to 19.3 ± 0.7 percent of total content with or without cholera toxin, respectively) (Fig. 32). This finding supports the notion that activation of cAMP/PKA pathway, by cholera toxin, can still sensitize sensory neurons after long-term exposure to PGE_2.

I observed that PKA is similarly activated by the toxin even after long-term exposure to PGE_2 (Fig. 33). In cultures exposed to vehicle for 5 days, cholera toxin increased PKA activation from 0.06 ± 0.007 (vehicle) to 0.46 ± 0.01, while in cultures exposed to PGE_2 for 5 days cholera toxin increased PKA activity from 0.05 ± 0.003 (vehicle) to 0.46 ±0.02. In total these results corroborate the idea that desensitization occurs at the receptor level because activation of the effector directly downstream from the receptor, $G_{\alpha s}$, induced PKA activation to the same level as control cultures.

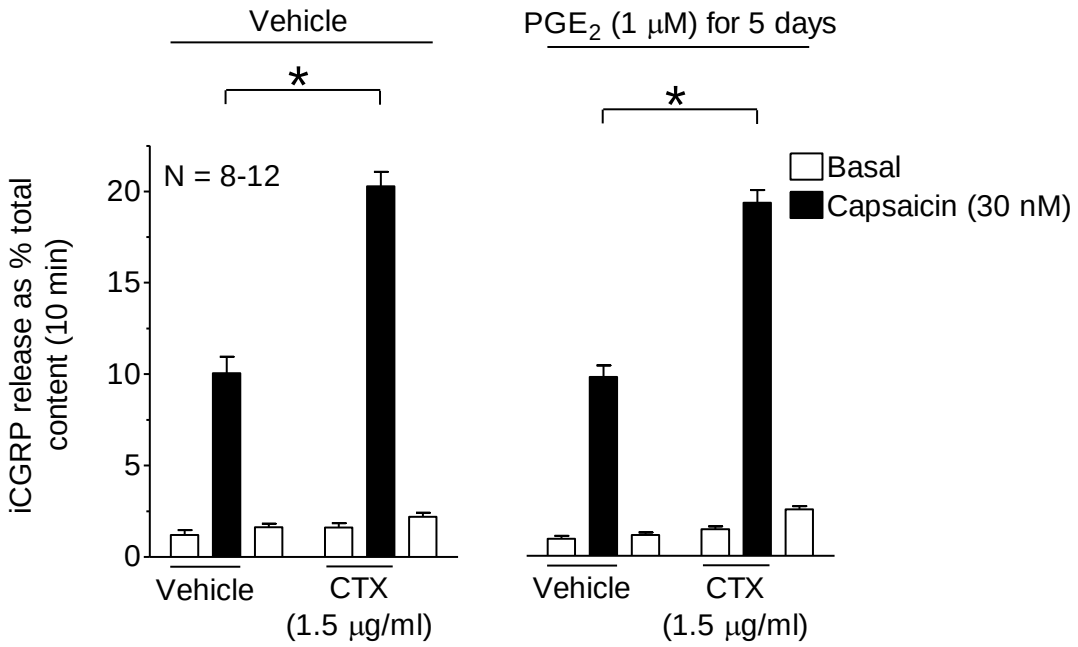

Figure 32. CTX-induced augmentation of capsaicin-evoked iCGRP release is not attenuated after five-day pre-exposure to PGE_2. Each column represents the mean ± SEM of iCGRP release as percent of total content from sensory neuronal cultures. Left panel represents release from cells exposed to vehicle for 5 days, while right panel represents release from cells exposed to PGE_2 (1μM) for 5 days. Open columns indicate basal release whereas closed columns represent capsaicin-evoked iCGRP release. Asterisks indicate statistically significant difference as indicated between iCGRP release from vehicle-treated versus that from CTX-treated cells (1.5 μg/ml). Statistical analysis was done using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

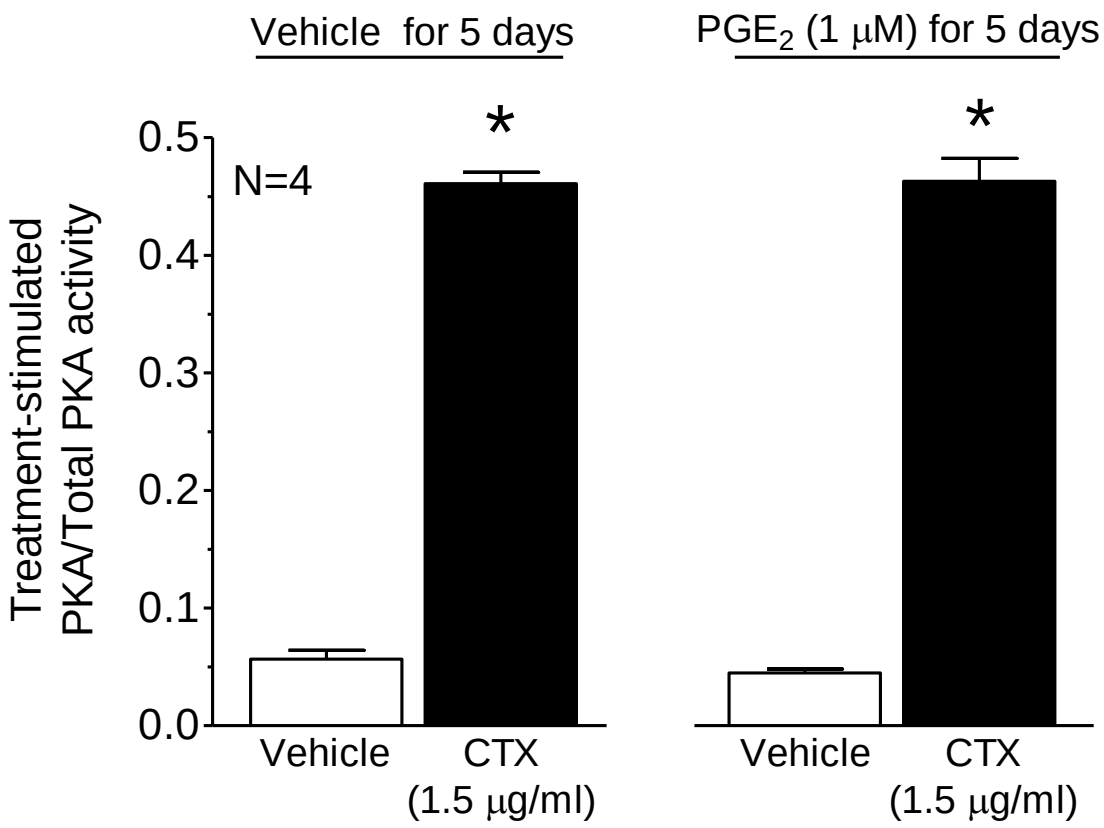

Figure 33. Five-day exposure to PGE_2 does not inhibit PKA activation by CTX. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to CTX (1.5 μg/ml). An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Long-term exposure to PGE_2 does not alter the extent of forskolin-activated PKA

As mentioned above, downregulation of PKA activation after long-term exposure to PGE_2 may be caused by uncoupling of the receptors EP3C and EP4 from the downstream signaling pathway. Forskolin can bypass the receptor and directly activate adenylyl cyclase (Gilman, 1984). Another possibility for the PKA desensitization after long-term exposure to PGE_2 is the loss of adenylyl cyclase activity. To address both possibilities, I measured the indirect activation of PKA by forskolin after long-term exposure to PGE_2. As shown in figure 34, long-term exposure to PGE_2 does not reduce PKA activation after exposure to forskolin to increase cAMP. PKA activity after exposure to forskolin was 0.34 ± 0.03 in cultures exposed to vehicle for 5 days and 0.34 ± 0.02 in cultures exposed to 1 µM PGE_2 for 5 days. This observation supports the previous findings that the uncoupling between PGE_2 and the PKA signaling pathway occurs at the receptor level leading to downregulation of PGE_2-activated PKA despite that the PKA signaling pathway itself remains functional.

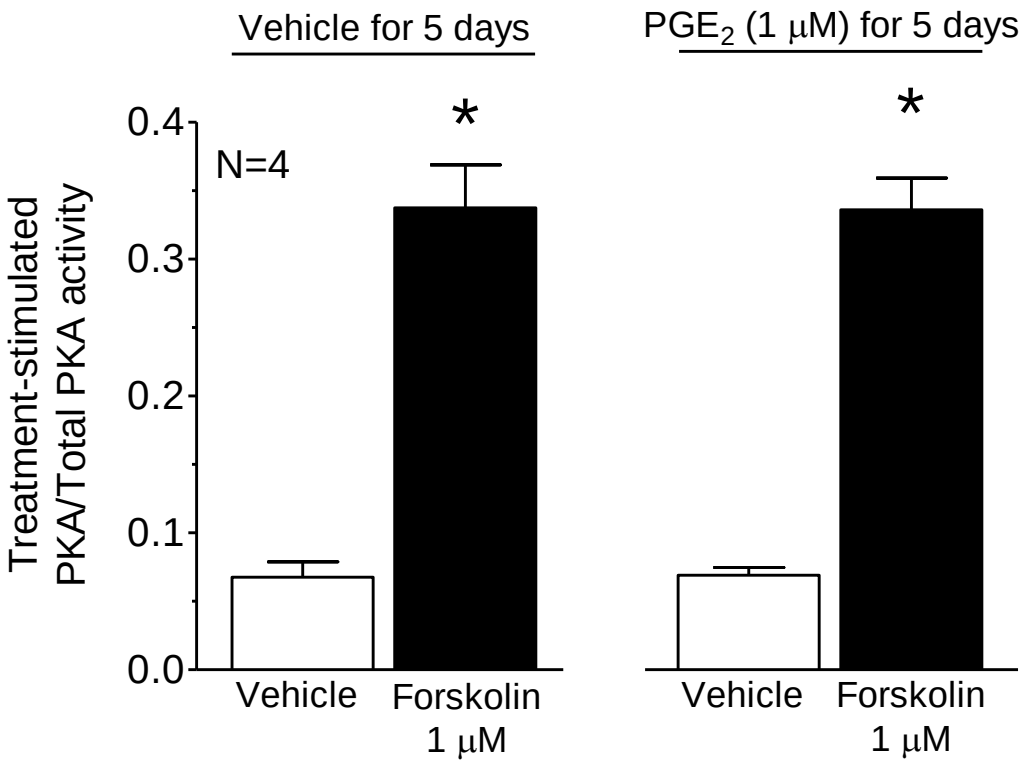

Figure 34. Five-day exposure to PGE_2 does not inhibit PKA activation by forskolin. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to forskolin (1 μM). An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

PKA and PKC do not mediate long-term induced desensitization of PKA activation by PGE_2

It is possible that second messenger-activated kinases, such as PKA or PKC, phosphorylate and uncouple GPCR from its downstream effectors (Hausdorff et al., 1989; Hausdorff et al., 1990; Premont, 2005). For example, it was previously shown that βAR can be phosphorylated and desensitized by PKA *in vitro* (Benovic et al., 1985) and *in vivo* (Wang et al., 2009). In analogous manner, PKA or PKC can phosphorylate and uncouple EP3C and EP4 from downstream signaling pathways in sensory neurons after long-term exposure to PGE_2. To investigate whether PKA or PKC mediate the observed downregulation of PGE_2-activated PKA, I used the kinase inhibitors, H-89 (10 µM) and BIM-I (1 µM), respectively. As shown in figure 21, 10 µM H-89 inhibits purified catalytic subunit of PKA ≥ 90 percent. Previously, Vasko and co-workers showed that 100 nM BIM-I was sufficient to attenuate sensitization by ATP through the P2Y/$G_{\alpha q/11}$/PKC pathway (Huang et al., 2003). It was also previously shown that classical, novel and atypical PKC isoforms are inhibited by BIM-I, however, novel and atypical PKC isoforms were 10-20 fold and up to 100 fold more resistant to inhibition by BIM-I than the classical ones (IC_{50} ≈ 8-18, 100-200 and 5800 nM for classical, novel and atypical PKC isoforms respectively) (Martiny-Baron et al., 1993). Since some of the novel PKC isoforms are expressed in DRGs (e.g. PKCε), I chose to use BIM-I at 10-fold higher concentration to ensure total blockade of classical and atypical PKC isoforms (Khasar et al., 1999a; Zhang et al., 2012). I began by exposing sensory neuronal cultures to 10 µM H-89 or 1 µM BIM-I for 5 days, but I found that exposure to these drugs in the manner described was toxic to sensory neuronal cultures (evidenced by the severe abnormal morphology of cell cultures and cell loss). Therefore, I exposed the cultures to the kinase inhibitors at the concentrations described along with PGE_2 or vehicle for only 12 hours. I observed that neither H-89 nor BIM-I applied in this manner show observable toxicity in our cultures. Moreover, at the time of stimulating the cultures with PGE_2, the media was replaced containing the kinase inhibitors was replaced with fresh media for 20 minutes, followed by exposure to vehicle or PGE_2. As seen in figure 35, pre-exposure to the kinase inhibitors for 12 hours did not interfere with PKA activation after acute re-exposure to PGE_2, indicating that presence of the kinase inhibitors for long period does not interfere with acute PKA activation. Under these conditions, long-term exposure to PGE_2 with or without the kinase inhibitors at the concentrations described above caused

downregulation of PKA activation by the re-exposure to the prostanoid. In naïve cultures exposed to vehicle, 10 µM H-89 or 1 µM BIM-I for 12 hours, acute 1 µM PGE_2-activated PKA was 0.49 ± 0.04, 0.59 ± 0.04 and 0.53 ± 0.04, respectively. They were not significantly different from each other. In cultures exposed to 1 µM PGE_2 alone, with 10 µM H-89 or with 1 µM BIM-I, acute re-exposure to the eicosanoid caused PKA activity values of 0.14 ± 0.02, 0.19 ± 0.02 and 0.16 ± 0.2, respectively. These values were not significantly different from each other or from baseline PKA activity (acute vehicle 0.06 ± 0.01) (Fig. 35). Atypical PKC isoforms, such as PKCζ, are known to be expressed in peripheral sensory neurons and also known to mediate sensitization of sensory neurons by NGF (Zhang et al., 2012). Despite the fact that no NGF was added in these experiments, possible downregulation of PGE_2-activated PKA by atypical PKCs in sensory neurons after long-term exposure cannot be excluded with certainty at the concentration of BIM-I that I used. Other tools, such as reduction of expression of atypical PKC isoforms using RNAi, can be used.

Subsequent to this finding, I employed an alternative approach to examine the potential involvement of PKA in the detected desensitization. Instead of inhibiting PKA, I asked whether long-term activation of PKA would cause PKA desensitization, in a manner analogous to its desensitization by long-term exposure to 1 µM PGE_2. I used forskolin to elevate cAMP concentration which in turn would activate PKA. Long-term exposure to forskolin did not alter acute activation of PKA by PGE_2 (0.46 ± 0.11 and 0.5 ± 0.1 in cultures exposed to vehicle of forskolin, respectively) which supports the conclusion that PKA does not mediate the observed desensitization (Fig. 36). These experiments demonstrate that in rat sensory neurons downregulation of PKA activation by EP receptors is mediated by PKA or PKC activity.

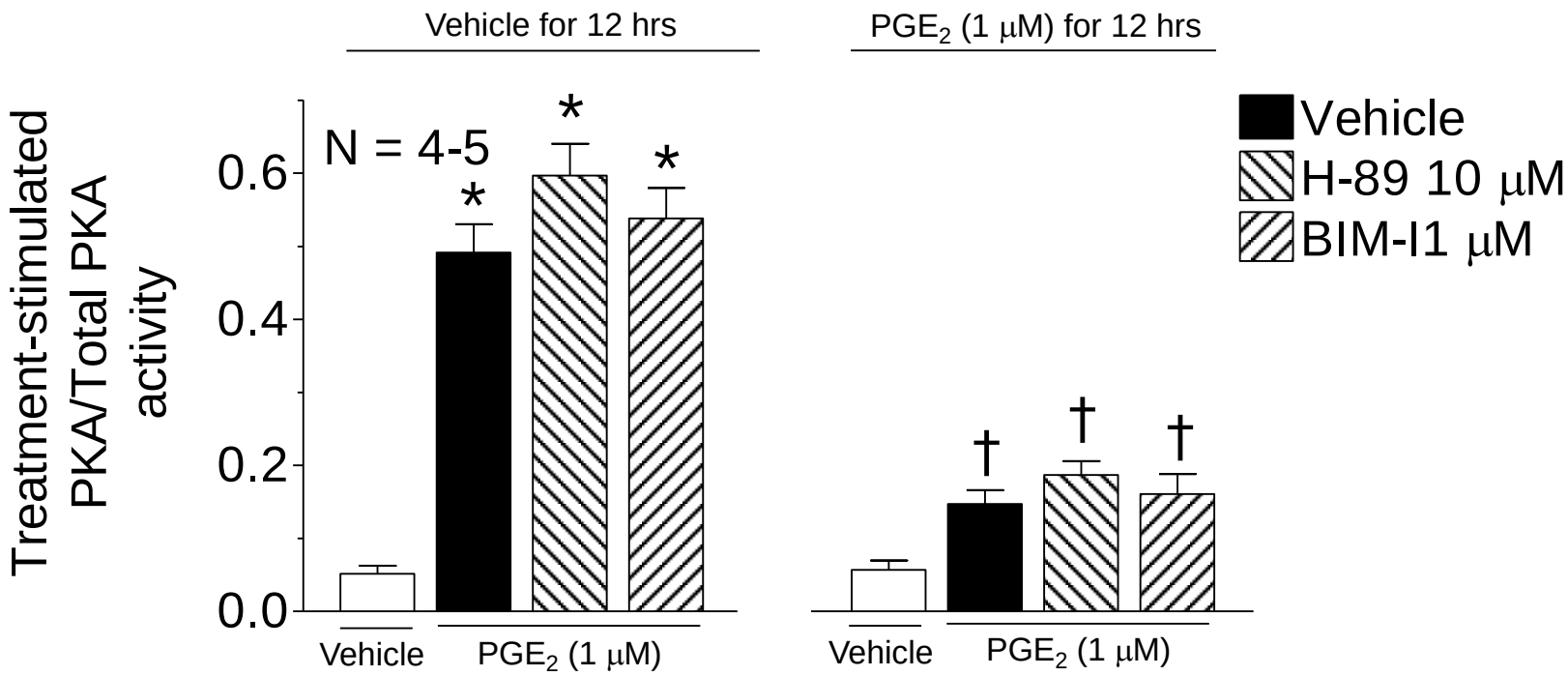

Figure 35. H-89 or BIM-I do not reverse desensitization of PGE_2-induced activation of PKA caused by 12-hours pre-exposure to the eicosanoid. Each column represents the mean ± SEM of treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 12 hours, while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 12 hours and both groups were pre-treated at the same time with vehicle, H-89 or BIM-I as indicated. Open columns represent cells treated acutely with vehicle, while all other columns represent cells acutely exposed to PGE_2 (1 μM). Asterisks indicate statistically significant difference in cells acutely-exposed to vehicle versus cells exposed to PGE_2 (1 μM) for 10 minutes. A dagger represents statistically significant difference in cells pre-exposed for 12 hours to vehicle and kinase inhibitors as indicated then exposed acutely to PGE_2 versus the corresponding groups in cells treated with PGE_2 for 12 hours. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

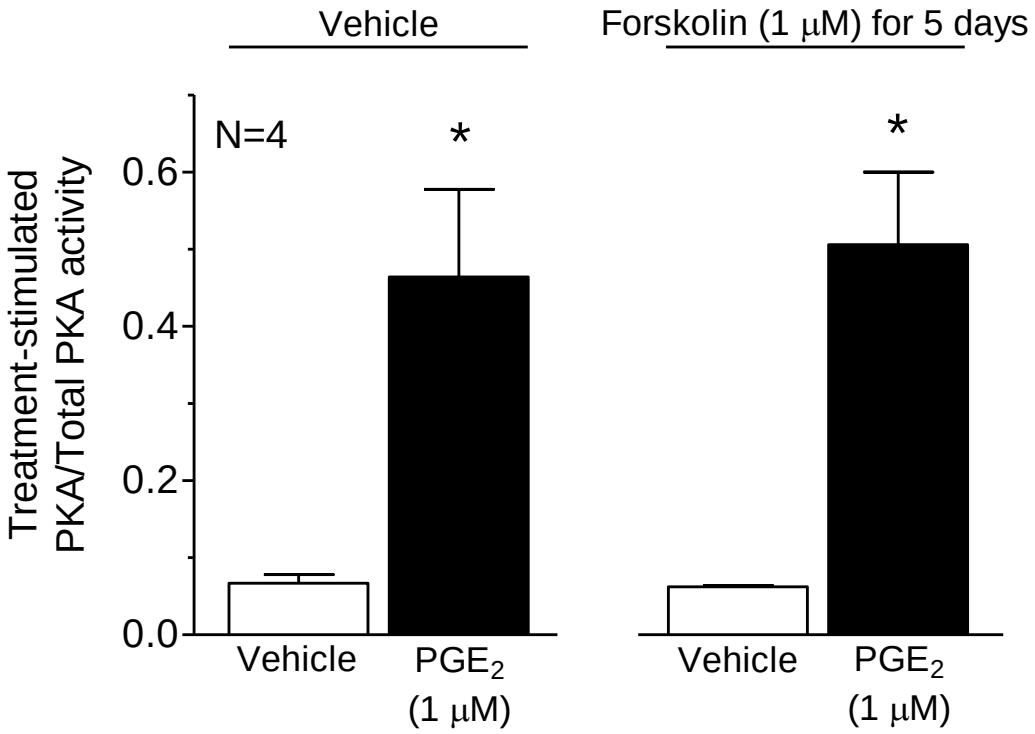

Figure 36. Five-day exposure to forskolin does not inhibit PKA activation by PGE_2. Each column represents the mean ± SEM of the treatment-stimulated PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to forskolin (1 μM) for 5 days. Open columns represent cells treated acutely with vehicle, while closed columns represent cells acutely exposed to PGE_2. An asterisk indicates statistically significant difference from vehicle using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

Grk2 mediates loss of PKA activation after long-term exposure to PGE_2

An important mechanism of termination of GPCRs activation is mediated by Grks which are Ser/Thr protein kinases that phosphorylate ligand-bound GPCRs (Ferguson, 2001; Pierce and Lefkowitz, 2001; Shenoy and Lefkowitz, 2011a; Sibley and Lefkowitz, 1985). Alteration of the level of expression of Grks is an important mechanism for long-term modulation of its activity (Penn et al., 2000). I asked whether long-term exposure to 1 µM PGE_2 alters the level of expression of Grk2, 3, 5 or 6. Using real time PCR, I observed no change of the mRNA levels of any of Grks 2, 3, 5 and 6 (Fig. 31).

Grks were shown to mediate desensitization of several GPCRs even in the absence of a change of the level of expression of Grks (Penn et al., 2000). As mentioned in the introduction of this dissertation, there is evidence that Grk2 modulates signaling pathway mediating PGE_2-induced sensitization in sensory neurons in models of chronic sensitization (Eijkelkamp et al., 2010a; Eijkelkamp et al., 2010b). Therefore, I asked whether inhibition of Grk2 function can attenuate loss of PKA activation by PGE_2 after long-term exposure to the eicosanoid. Since there are no selective small-molecules that inhibit Grks I used RNAi to reduce the expression of Grk2. Real time PCR was used to confirm that the siRNA against Grk2 effectively and selectively reduced the mRNA expression of Grk2. Grk2 mRNA was significantly reduced by approximately 65 percent. Grk5 mRNA level also reduced by a modest reduction (≈ 25 percent) (Fig. 37). It is unlikely that such a small reduction of expression of Grk5 mRNA would have an effect on signaling. However, it is important to confirm this finding by using a different method such as a different siRNA that is more selective, or by performing an add-back experiment. Another interesting observation is that the level of expression of Grk6 mRNA increased by approximately 36 percent after treatment with siRNA against Grk2. This could be a negative-feedback response by cells to compensate for the reduction of Grk2 expression.

Importantly, inhibition of PKA activation after long-term exposure to PGE_2 was partially prevented by the siRNA directed against Grk2 but not scramble siRNA (from 0.06 ± 0.007 to 0.20 ± 0.02 in ScRNA and Grk2-siRNA treated cultures, respectively) (Fig. 38). This indicates that Grk2, at least in part, mediates desensitization of PKA signaling after long-term exposure to PGE_2. Since level of expression of Grk5 mRNA was also reduced, albeit to a lesser extent (Fig. 37, and the discussion above), the

observed partial attenuation of downregulation of PGE_2-induced PKA activation after long-term exposure to the eicosanoid can be also mediated by Grk5. Interestingly, I also found that PKA activation after acute exposure to PGE_2 was significantly increased from 0.28 ± 0.04 in untreated cultures to 0.49 ± 0.05 in Grk2-siRNA treated ones. This indicates that even under conditions where peripheral sensory neurons are exposed to PGE_2 acutely, Grk2-mediated desensitization of receptors ensues and reduces the level of activated PKA. Therefore, Grk2 is not only responsible for partial downregulation of PGE_2-mediated PKA activation after long-term exposure to the prostanoid, but also after acute exposure of naïve DRG cultures to the eicosanoid.

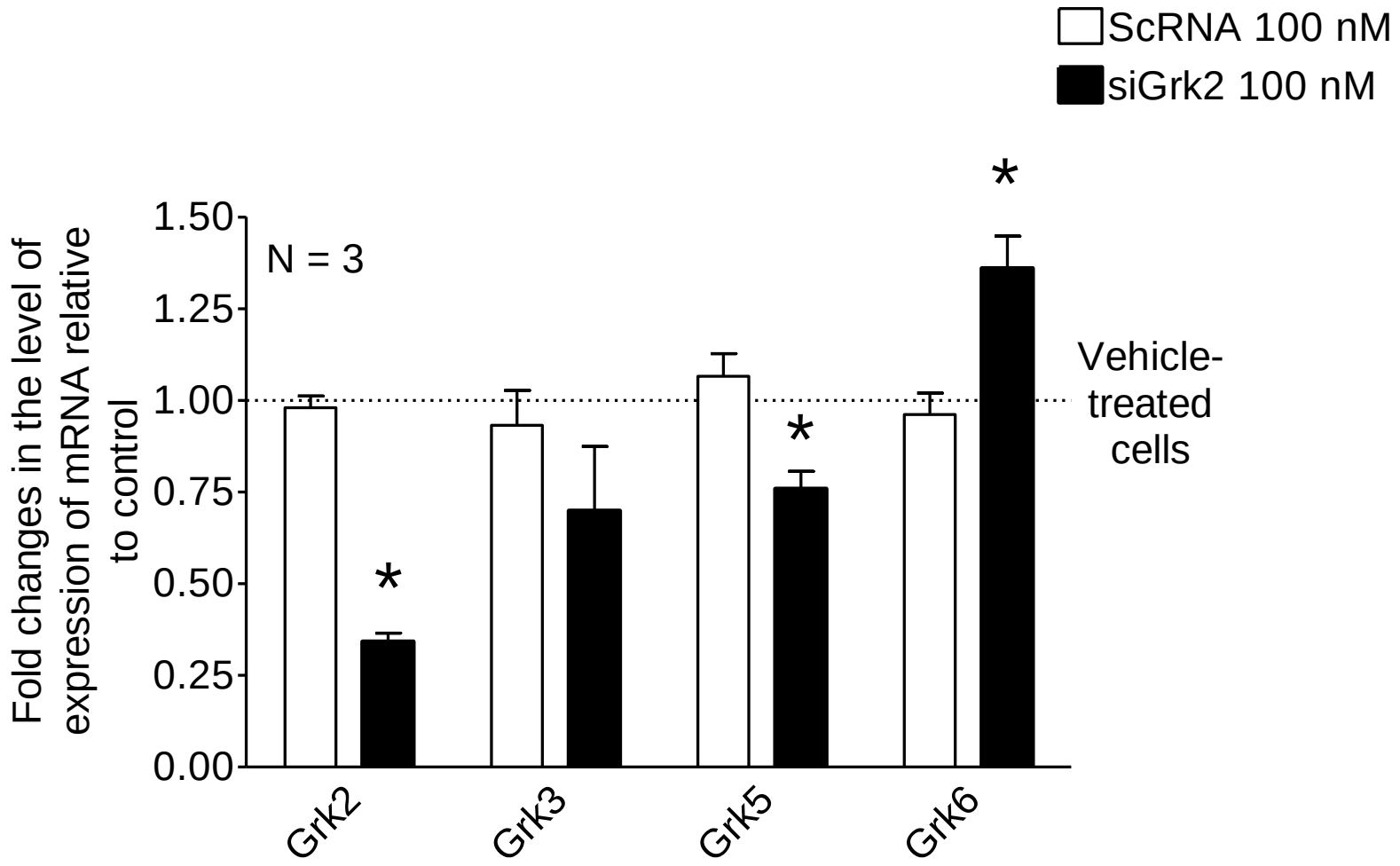

Figure 37. siRNA for Grk2 selectively reduces the level of expression of Grk2. Each column represents the mean ± SEM of the relative level of mRNA of different targets normalized to that obtained in vehicle-treated cultures. Open columns represent cells treated with control RNA (ScRNA), while closed columns represent cells treated with siRNA directed against Grk2 (siGrk2). The dotted line indicates vehicle only-treated cells. Real time PCR was performed for various Grk isoforms as indicated. Asterisks indicate statistically significant difference from ScRNA-treated cells using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

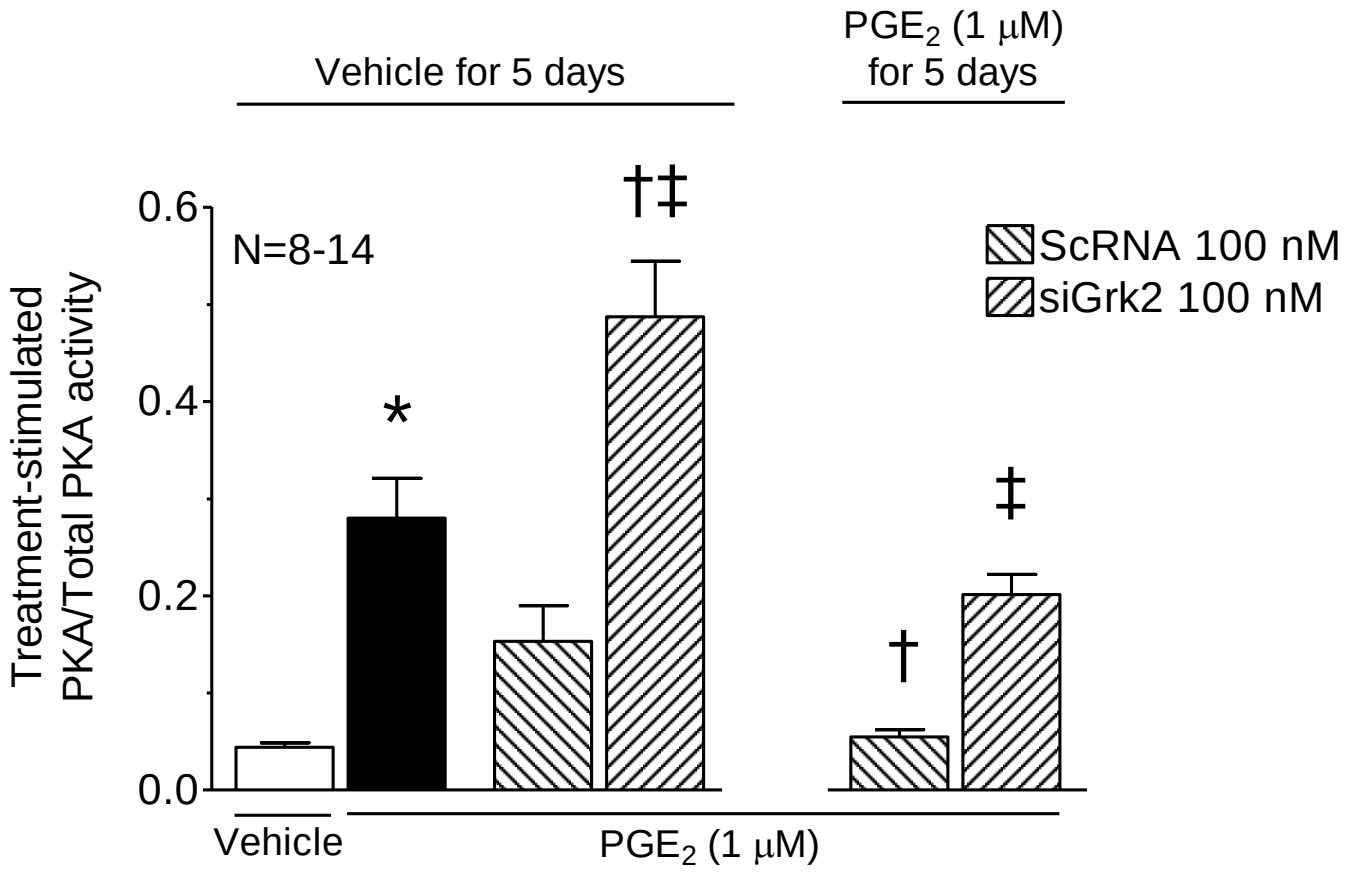

Figure 38. Inhibition of PGE_2-induced activation of PKA by five-day pre-exposure to the eicosanoid is attenuated by reduction of expression of Grk2 using siRNA. Each column represents the mean ± SEM of treatment-induced PKA activity normalized to total PKA activity measured after exposure to 10 μM cAMP. The left panel represents PKA activity from cells exposed to vehicle for 5 days while the right panel represents PKA activity from cells exposed to PGE_2 (1 μM) for 5 days. Open column represents cells treated acutely with vehicle, while all other columns represent cells acutely exposed to PGE_2 (1 μM) for 10 minutes after various chronic treatments. Closed column denotes cells chronically exposed to vehicle then acute PGE_2, hatched column denotes control siRNA-treated cells (ScRNA), or Grk2-siRNA treated cells (siGrk2) as indicated. An asterisk indicates statistically significant difference from vehicle, a dagger represents statistically significant difference from cells exposed only to acute PGE_2 (denoted by the closed column), while a double dagger represents significant difference from cells treated with ScRNA within the same panel. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-test, $p < 0.05$.

DISCUSSION

Extensive evidence suggests that sensitization of sensory neurons is maintained in chronic inflammatory states. In addition, there are numerous studies that show that signaling pathways change under these pathological persistent inflammatory diseases. In this dissertation, data are presented to provide evidence that sensitization is maintained in sensory neuronal culture after long-term exposure to NGF or PGE_2 in an analogous manner to what is observed in experimental animals and in humans. Evidence is also provided for the clear presence of a signaling switch from the canonical cAMP signaling pathway which mediates acute PGE_2-induced sensitization to another pathway. Indeed, work presented here shows that PKA no longer mediates sensitization induced by PGE_2 after long-term exposure to NGF or the prostanoid.

Investigating the mechanisms of persistence of PGE_2-induced sensitization is crucial since chronic pain is a substantial clinical problem (see introduction). Patients with chronic inflammatory painful conditions achieve only temporary relief by using classic NSAIDs or COX-2 selective ones (Holmes, 2012). Moreover, it is established that these drugs are not always effective, and suffer from severe gastrointestinal and renal toxicities (Buchanan, 1990; Long et al., 2001; Shah and Mehta, 2012a; Wolfe et al., 1999). This prompted researchers to investigate the mechanisms by which chronic sensitization is maintained in hope to discover new specific and more effective drug targets.

Use of isolated adult rat DRG cultures

Cell cultures offer a number of advantages over use of *in vivo* animal models, and suffer from a number of shortcomings. Using isolated neurons in culture reduces interference from other unrelated cells that are present in the same or near-by tissue in the animal. This offers two important advantages; 1) studying signaling pathways in a cell-specific manner and 2) studying the effects of defined inflammatory mediators. These two crucial advantages are not realized with whole animal experiments, since several types of immune cells, glial cells and even fibroblast cells are involved in the process of inflammation. Furthermore signaling pathways coupled to a particular receptor may differ in different cell types (Popper, 1984; Yao et al., 2009) and thus traditional whole animal studies do not allow cell-specific examination of signaling

pathways (Merighi et al., 2008; Oliveira et al., 2007; Pezet and McMahon, 2006; Ren and Dubner, 2010). Methods that are commonly used for the induction of inflammation in animal models, such as intraplantar injection of carrageenan or CFA, cause the release of a many inflammatory mediators (Barnes et al., 1992; Cunha et al., 2005; Popper, 1984; Woolf et al., 1997; Woolf et al., 1994). Therefore it is difficult to distinguish the effect of an individual inflammatory mediator on nociceptive neurons in classic animal models.

Elucidating signaling pathways in sensory neurons is difficult in experiments involving animal models. For example, intraplantar injection of small molecular weight inhibitors of PKA attenuates sensitization caused by subsequent injection of PGE_2, but the interpretation that the inhibition specifically affects neurons is difficult (Aley and Levine, 1999). At the site of injection, there are many different types of cells, including epithelial, adipose and connective tissues, smooth and skeletal muscles and endothelial, immune and glial cells. Because of the small molecular weight and the favorable hydrophilic-lipophilic balance of non-peptide PKA kinase inhibitors, they diffuse and cross cell membranes relatively freely, and thus could inhibit PKA in all of these cell types that are close to the site of injection. Therefore, it is difficult to conclude that PKA in a particular cell type mediates an observed effect. In contrast, adult rat DRG cell cultures contain few cell types (neurons, glial cells and fibroblasts). This offers a means to reduce the number of possible cell types involved in PGE_2-induced sensitization, and thus allows demonstrating that PGE_2 directly sensitizes sensory neurons (Hingtgen et al., 1995). Other techniques such as electrophysiology and cell imaging offer an additional level of specificity in determining the exact cell type in which inhibition of PKA attenuates PGE_2-induced sensitization (Evans et al., 1999).

As mentioned earlier, methods used to induce chronic inflammation in animal models cause the release of inflammatory mediators, the quantity or identity of which cannot be controlled (Loram et al., 2007). Even when a single inflammatory mediator is injected, it results in the subsequent release of multiple other inflammatory mediators as evidenced by several studies (Cunha et al., 2005; Safieh-Garabedian et al., 1995; Woolf et al., 1997). Cell culture allows for a more precise control of the inflammatory mediators present and the quantity at which they are added.

Augmentation of stimulus-evoked neuropeptide release from DRG cultures by inflammatory mediators is used as an endpoint to model sensitization. For example, PGE_2 and $cPGI_2$ (stable analogue of PGI_2), were shown to enhance capsaicin or

bradykinin-evoked neuropeptide (iCGRP or iSP) release from adult and embryonic DRG neurons grown in culture (Hingtgen and Vasko, 1994b; Hingtgen et al., 1995; Vasko et al., 1994). Purinergic receptor agonists, such as the inflammatory mediator ATP, also cause sensitization of sensory neurons (Huang et al., 2003). Activation of the PKC signaling pathway, known to enhance sensitization, augments capsaicin-evoked peptide release from cultures as well (Barber and Vasko, 1996). Thus DRG cultures offer a controllable and faithful model of sensitization of DRG neurons.

Using neurons in culture also has a number of limitations. For example, the effect of removal of sensory neurons and growing them in culture on signaling, excitability and response to inflammatory mediators is not precisely understood. Technically, harvested DRG neurons are axotomized and this has known pathological effects from studies in neuropathic pain models (Colleoni and Sacerdote, 2010). However, several studies showed that axotomy alone is not sufficient to maintain hypersensitivity in animal models beyond 6 days without the contribution of descending pain pathways from the rostral ventromedial medulla (Burgess et al., 2002; Gardell et al., 2003; Porreca et al., 2001). Therefore it is possible that in isolated DRG sensory neurons, axotomy enhances their sensitization only temporarily, and that this enhanced sensitization subsequently subsides due to the lack of the rostral ventromedial medulla descending pathways. Another aspect that changes the behavior of cells once removed from the animal and cultured, is the fact that cells interact with surrounding cells as well as with various components of the extracellular matrix (ECM) such as proteins or glycosaminoglycans (Abbott, 2003). Harvesting a particular tissue and preparing it for cell culture deconstructs (dismantles) its three-dimensional architecture and hence alters cells-ECM interactions.

In the fields of cancer, stem cell and endothelial cell research it is clear that isolated cells behave differently when cultured in a three-dimensional system using matrigel, compared to cultures prepared in the traditional “two-dimensional” way (Bissell and Hines, 2011; Brafman et al., 2012; Discher et al., 2009; Kraehenbuehl et al., 2011; Kshitiz et al., 2012). There also is indirect evidence that ECM interactions with neurons in general affect multiple aspects of their physiology and properties (Dityatev et al., 2010) and with peripheral sensory neurons in particular. ECM surrounding DRG neurons is modulated by these neurons. It was shown that a constrictive peripheral nerve injury altered immunostaining of the laminin ECMs surrounding DRG neurons (Dubovy et al., 2006). In animal models, it was also shown that blocking integrins with monocloncal

antibodies [integrins are cell surface receptors that interact with the ECM (Cox et al., 2010)] can attenuate persistent hyperalgesia (Dina et al., 2005; Dina et al., 2004). Integrin also binds NGF and may serve as a putative receptor for the neurotrophin (Staniszewska et al., 2008). Fibronectin, an ECM protein, was found to enhance tyrosine phosphorylation of TRPV1 channel in adult rat sensory neurons in culture and showed an increase of its translocation to the cell membrane. This leads to augmentation of capsaicin-evoked iCGRP release, TRPV1-mediated calcium accumulation and increase in the number of capsaicin responsive neurons (Jeske et al., 2009).

DRG neurons are surrounded not only by ECM, but also by satellite glial cells and in very close proximity to other neurons. There is clear evidence that cell-cell interactions between sensory neurons and satellite glial cells in the whole ganglion (*in situ*) occur and affect their sensitization. For example, Huang and co-workers demonstrated that sensory neurons stimulate satellite glial cells by secreting ATP, which in turn causes these satellite glial cells to release TNF-α, and hence enhance neuronal excitability (Zhang et al., 2007). In addition, studies demonstrated that satellite glial cells also communicate with each other through gap junctions as indicated by diffusion of a fluorescent dye from one satellite glial cell to another (Huang et al., 2005). Induction of inflammation by intraplantar injection of CFA in the hind paw, enhances this communication, as evidenced by increased diffusion of a fluorescent dye (Dublin and Hanani, 2007). Reduction of expression of connexin 43, the protein that makes up the gap junction, results in reduction of nociception in animal models of neuropathic injury, and paradoxically enhances nociceptive behavior in control animals (Ohara et al., 2008).

In the trigeminal ganglion, silencing of an inwardly-rectifying potassium channel Kir4.1 in satellite glial cells, to mimic its reduction of expression after nerve injury, results in pain like behavior in the absence of an actual nerve injury (Vit et al., 2008). These studies suggest that there is cross-talk resulting in reciprocal effects between neurons and the surrounding satellite glial cells. Therefore these findings must be always kept in mind and that dissociating DRGs may have unpredictable and unknown effects on findings.

Another limitation of studying sensory neurons in culture is that they do not represent the rest of the nociceptive pathway in the whole organism. Therefore, care must be always taken when interpreting results. As mentioned earlier, central axons of DRG neurons relay their signals in the dorsal horn of the spinal cord. In particular nociceptive neurons synapse on spinal neurons in laminae I in primates and human [in

rodents, lamina II appears also to receive input from peripheral C-fibers (Craig, 2003)]. It is also noteworthy that spinal neurons in laminae IV, V, VII and VIII receive input from peripheral C fibers as well as A-β fibers, although the latter is not known to transduce or communicate nociceptive information under normal conditions (Dostrovsky and Craig, 2006). Axons from these spinal neurons in laminae I, IV, V, VII and VIII ascend in the spinal cord in groups known as lateral and anterior spinothalamic tracts, spinobulbar tract and spinohypothalamic tract which terminate in the thalamus, brain stem and hypothalamus, respectively (Craig et al., 2002; Dostrovsky and Craig, 2006). In addition to these ascending pathways, there are descending pathways by which various higher brain centers modulate the processing of pain signals along the ascending pain pathway (Fields et al., 2006).

Detailed discussion of central nervous system mediation and modulation of nociception is beyond the scope of this dissertation. However, it is clear that as important as the peripheral nociceptive neurons are to nociception in the whole organism, they are but one player of a complex, integrated and interconnected system. Therefore conclusions drawn from studies using cultures of peripheral neurons must be interpreted with care and restricted only to these peripheral neurons. An example is the case of EP receptors necessary for mediation of PGE_2-induced pathological hypersensitivity of nociceptive neurons. It was shown by multiple groups that receptors EP4 and EP3c are essential for sensitization of peripheral sensory neurons (Lin et al., 2006; Southall and Vasko, 2001). On the other hand, in spinal neurons EP2 receptors are essential for their sensitization (Ahmadi et al., 2002; Harvey et al., 2004; McCoy et al., 2002; Reinold et al., 2005). Also other groups showed that EP1 receptors mediate certain kinds of hypersensitivity such as acetic acid-0069nduced writhing (Sarkar et al., 2003; Stock et al., 2001). The exact cell type mediating expressing EP1 and mediating effects of PGE_2 in the latter study were not determined. It is thus clear that different findings can be obtained depending on what component of the nociceptive pathway is being studied. This affirms the need to cautious interpretation of findings from experiments done using DRG cultures.

Collectively, DRG cultures are invaluable means to study sensitization of peripheral sensory neurons and the signaling pathways mediating this sensitization. However, great care must be taken when interpreting findings obtained from such studies.

Choice of PGE_2 concentration

Higgs and Salmon reported that after subcutaneous implantation of carrageenan-impregnated sponges, the inflammatory exudate contained approximately 180 nM PGE_2 (Higgs and Salmon, 1979). In patients with untreated rheumatoid or psoriatic arthritis, synovial fluid collected from the knee joint contained approximately 4-25 nM PGE_2 (Bombardieri et al., 1981; Trang et al., 1977). Concentrations as small as 100 nM of PGE_2 were able to augment bradykinin-evoked iCGRP release from rat sensory neurons in culture (Vasko et al., 1994). At 1 µM, PGE_2 causes a 33 percent increase of the capsaicin-evoked iCGRP release without affecting the basal one (Vasko et al., 1994). It was later discovered that PGE_2 causes a significant increase in cAMP synthesis at both 100 nM and 1 µM (Hingtgen et al., 1995).

The PGE_2 concentration that is considered effective to increase cAMP production to 50 percent of its maximal value (EC_{50}) in adult rat DRG cultures is approximately 1.2 µM (Smith et al., 1998). In the experiments described in this dissertation, a concentration response curve showed that the EC_{50} is approximately 0.8 µM. Thus, EC_{50} obtained from the work described in this thesis, is close to previous literature (Smith et al., 1998).

The EC_{50} value from the current work is approximately 10-fold higher than the values measured from arthritic or psoriatic patients and from experimental animals with inflammation (see above). There are multiple possibilities to explain the difference. It is possible that extracellular first messengers (inflammatory mediators and hormones) are not uniformly dissolved and distributed in the extracellular compartment, but rather exist in highly localized compartmentalized fashion similar to intracellular compartmentalization of second messengers. Precedent for this comes from a recent finding that an extracellular enzyme that synthesizes lysophosphatatidic acid (LPA) is bound to the GPCR that the ligand is activated by the product of the enzymatic reaction (Fulkerson et al., 2011; Hausmann et al., 2011; Tabchy et al., 2011). Moreover, inhibition of a metabolic enzyme that catabolizes LPA, lipid phosphate phosphatase, resulted in localized increase in LPA concentration and hence amplification of its signaling through LPA1 receptor (Aaltonen et al., 2012). Thus, it is possible that COX enzymes and organic anion transporting protein [OATP, the exporter that is thought to pump PGE_2 outside cells (Kanai et al., 1995)] are localized to the same microdomains as the EP receptors and thus creating a localized compartment of high concentration of PGE_2 right next the receptor. It is known that PGE_2 is released in a “polarized” fashion. Expression

of PGE_2 transporter only on the apical membranes of canine kidney cell line and the transport of PGE_2 to the basolateral side was increased by 100 fold (Endo et al., 2002). Endo and co-workers, the authors of this paper, suggested that this polarized transport of PGE_2 may aim at driving PGE_2 to stimulate a particular population of EP receptors on the basolateral membrane (Schuster, 2002).

Another potential explanation is that PGE_2 acts on some of the effectors of sensitization on their intracellular domains rather than through binding to an extracellular binding site on their cognate specific GPCR. Again, the precedent for this comes from a study on LPA. LPA acts as a sensitizing agent via activation of LPA receptors. However, recently, LPA was found to directly activate TRPV1 channel via its intracellular C-terminal domain (Nieto-Posadas et al., 2012). This discovery indicates that LPA can sensitize sensory neurons by activating its receptor and the coupled signaling pathway or it can directly alter the activity of its effector. It is possible to speculate that PGE_2 acts in a similar manner. If this was the case, PGE_2 in the cytosol would be the deciding factor, the concentration of which is not frequently measured, but is expected to be much higher than the plasma one. Despite the controversy, it is thought that prostaglandins activate peroxisome proliferator-activated receptors, which are intracellular targets, similar to the direct activation of TRPV1 by LPA binding to its intracellular C-terminus. For example it was found that PGI_2 can activate PPARδ at physiologically relevant concentrations (Gupta et al., 2000).

A third explanation that may underlie the low values of PGE_2 in biological samples from clinical or animal experiments is its degradation. It must be remembered that PGE_2 is a short-lived inflammatory mediator that is synthesized and broken down relatively rapidly. Lysed cells and broken tissues contain large activity of prostaglandin dehydrogenase, a main PGE_2-metabolizing enzyme. This means that the reported values of PGE_2 from clinical and animal studies may underestimate the amount of PGE_2 present due to its rapid breakdown by the enzyme released from lysed cells and tissues (Bito and Baroody, 1975; Bito et al., 1977).

Collectively, the EC_{50} for PKA activation by PGE_2 in sensory neurons in the current study is similar to some findings in literature from clinical and animal experiments, but higher than others. It is possible that these differences are due to yet undiscovered properties of how PGE_2 is released and its different sites of action. Further work is needed to uncover the mechanism by which PGE_2 is released and whether the

synthesis/release mechanisms are localized to EP receptors, and the site of action of PGE_2.

Choice of NGF concentration

Studies involving NGF employed it at the concentration of 30 ng/ml. This concentration was selected based on the following literature. NGF increases TRPV1-mediated $^{45}Ca^{2+}$ uptake in isolated adult DRG neurons after 6 days of exposure to the growth factor in a concentration dependent manner (Winter et al., 1988). The EC_{50} reported by the authors of that work was 20 ng/ml. In a different study by Pasricha and coworkers, NGF increases the expression and release of iCGRP in a concentration dependent manner and although they did not perform non-linear regression and determine the EC_{50}, it seems that 30 ng/ml is significantly higher than 1 ng/ml and significantly less than 300 ng/ml which indicates concentration-dependency (Winston et al., 2001). It is noteworthy that both of the studies by Lindsay and coworkers and by Pasricha and coworkers used 2.5S NGF while in all the work presented in this dissertation 7S NGF was used. Generally, it is considered that the 2.5S and 7S forms of NGF to be biologically equivalent, especially in sensory neurons (Pezet and McMahon, 2006). Nevertheless, it has to be recognized that a difference might exist between the 2.5S and the 7S forms of NGF that is still undiscovered. Currently, there is a single report in literature that actually showed a difference in the biological activity between the 2.5S and the 7S forms of NGF. Neonatal cerebellar, hippocampal and cortical astrocytes proliferate in response to 2.5S but not 7S NGF (Shao et al., 1993).

As mentioned in the introduction, production of NGF is increased in inflammatory conditions. Various studies attempted to measure concentration of NGF in clinical or animal experimental setting. It was reported that NGF concentration was increased approximately 50 percent from 8 to 12 ng per hind paw (Safieh-Garabedian et al., 1995; Woolf et al., 1997). In another study NGF was increased more than 2 fold to 0.9 ng per gram wet weight in mouse knee joint injected with carrageenan (Manni and Aloe, 1998). In patients of various inflammatory disorders, it was reported that NGF levels increase between 2.4 fold as in urticaria and up to 30 fold as in vernal keratoconjunctivitis. In acute myelogenous leukemia patients who recently developed the disease, NGF in plasma peaked at approximately 50 pg/ml (Simone et al., 1999).

It is noticeable from the brief discussion of NGF concentrations reported from clinical studies in humans and those actually used in *in vitro* experiments using isolated sensory neurons that there is large concentration difference (compare 50 pg/ml in the study by Simone et al versus 30 ng/ml in the work by Winter et al and Winston et al (Simone et al., 1999; Winston et al., 2001; Winter et al., 1988). Simone and co-workers measured NGF in samples from patients with acute myelogenous leukemia and therefore it is possible that NGF concentration reaches higher levels in inflammatory conditions such as rheumatoid arthritis or osteoarthritis. Another possibility is that the concentration of NGF detected in plasma or in wet tissue or skin might not represent the concentration of NGF at the receptor. NGF concentration might be higher at or close to the receptor site than in plasma or whole tissue; i.e. NGF concentration is compartmentalized in the extracellular compartment. A precedent comes from the case of BDNF, another inflammatory mediator and neurotrophin. It was shown that when the glycosaminoglycan chondroitin sulfate, is sulfated in a particular pattern it becomes able to bind the growth factor and thus create a microdomain by definition. Since chondroitin sulfate exists extracellularly as a component of the ECM, it binds to and concentrates BDNF close to its receptor on the cell surface and thus enhances neurite outgrowth (Gama et al., 2006). Thus an ECM component effectively creates a compartment of higher BDNF concentration close to the cell membrane than the total extracellular fluid. This gradient or compartmentation effect is undetectable using conventional assay techniques since the tissue gets homogenized and all BDNF is extracted. The study by Gama and co-workers described above raised the possibility that other growth factors and mediators, including NGF, might also be compartmented in a manner that results in their much higher concentration at their cognate receptors than previously thought. This could explain the difference between NGF concentrations that are sufficient for producing biological effects experimentally from concentrations measured in the whole organism.

Spare receptors

In the introduction to this dissertation, spare (or reserve) receptors were suggested as a potential mechanism explaining persistence of PGE_2-induced sensitization after long-term exposure despite the fact that there is a decrease in receptors available on the cell membrane both *in vivo* and *in vitro*. Spare receptors

enable biological effects of their cognate ligands to persist even after receptor desensitization. As mentioned earlier, it was shown before that the K_D of EP receptors in DRG cultures does not change after 24 hours exposure to 1 µM PGE_2 despite the reduction of the maximal receptor binding (B_{max}) by approximately 40 percent (Southall et al., 2002). The concept of spare receptors describes the presence of a larger number of receptors than are needed to evoke the maximal effect. With an overabundance of receptors, those that get activated and downregulated are replaced by "naïve" receptors that initially were not activated by the ligand. Spare receptors have two consequences on the manner in which they propagate signaling; 1) responses to the ligand still activate the same signaling pathway that is coupled to the receptor after acute exposure and 2) the EC_{50} is significantly smaller than the actual K_D (i.e. the amount if drug required to reach half maximal full effect is much lower than the amount of drug required to bind half the receptors available, since most receptors are spare). Multiple eicosanoid receptors act in accordance with spare receptors (see introduction). Thus spare receptors seem a plausible explanation for the persistence of PGE_2-induced sensitization either in animal models (Aley and Levine, 1999; Aley et al., 2000; Southall et al., 2002; Southall et al., 1998) or in isolated sensory neurons (Bolyard et al., 2000). However a closer look reveals a significant difference between the manner in which PGE_2-induced sensitization persists and the typical spare receptors behavior.

Much evidence presented in this dissertation as well as in literature suggest that signaling is switched after prolonged exposure to PGE_2. This switch in signaling is uncharacteristic of spare receptors, because spare receptors maintain the same signaling pathway associated with them available for subsequent activation after prolonged exposure to the ligand. Moreover, as mentioned earlier, EC_{50} changes when spare receptors are removed from a population of receptors and shifts to the right (Brodde, 1993). Therefore further experiments are needed to determine the EC_{50} of PGE_2-incuded sensitization of capsaicin-evoked iCGRP before and after long-term exposure to the eicosanoid or NGF. A rightward shift of EC_{50} would suggest the presence of spare receptors. These two arguments make the concept of spare receptors an unlikely mechanism for explanation of persistence of sensitization by PGE_2 after long-term exposure.

mRNA versus protein

In multiple experiments in this dissertation real-time PCR was used to quantify relative expression level of different mRNAs encoding variety of targets essential for mediating PGE_2-induced sensitization. There are several advantages of using real-time PCR including accuracy, the ability to quantify mRNA and specificity of the assay. There are also inherent disadvantages of analyzing mRNA using real-time PCR, namely that level of expression of mRNA does not always reflect level of expression of the encoded protein. Real-time PCR is able to detect transcripts levels over a dynamic range that is 7-8 orders of magnitude wide (Morrison et al., 1998). Real-time PCR also is thousands of times more sensitive than other RNA quantification techniques such as RNase protection assay and dot blot hybridization (Malinen et al., 2003; Wang and Brown, 1999). In some instances, real-time PCR can be optimized to detect a single copy of mRNA (Palmer et al., 2003). Real-time PCR is sensitive enough to discriminate changes in the expression of mRNA that are as small as approximately 25 percent (Gentle et al., 2001).

Specificity becomes of prime importance when studying one particular isoform of multiple highly homologous members of family of signaling molecules. For example, studying the change of the level of expression such a molecule as a result of exposure to a mediator or when attempting to reduce its level of expression using RNAi, it is important to use a tool that offers high level of specificity. Specificities of antibodies that are commercially available are seldom verified by the manufacturer since it is an extensive and laborious process and thus it is up to the researchers to validate the antibody properly (DeSilva et al., 2003). Therefore, it is clear that real-time PCR offers a highly specific and relatively feasible tool to conduct these studies.

Despite the great advantages of real-time PCR discussed above, there is increasing evidence that mRNA levels may not reflect the corresponding protein levels (Maier et al., 2009). This was recently quantitatively demonstrated at genome-wide level in the study by Selbach and coworkers. In this work, the copy number of more than 5000 unique mRNA and protein in NIH3T3 cells was accurately assayed and compared to each other (Schwanhausser et al., 2011). The correlation was found to be poor (R^2 = 0.41), indicating that the level of expression of mRNA does not accurately indicate the level of expression of the protein.

An important example of the lack of correlation between the level of mRNA and the corresponding protein is the change of expression of Grk2 caused by inflammation. As mentioned earlier, Grk2 is an important mediator of GPCR desensitization. Expression of Grk2 was reported to be reduced at the protein but not at the mRNA level previously (Lombardi et al., 1999). The authors of this study used northern blot to demonstrate that there is no change in the level of mRNA of Grk2. On the other hand, using western blot they showed that the level of iGrk2 is reduced. Clearly, these findings are represent an important example that warrants further experiments examining the protein level of Grk2 in rat DRG sensory neurons after long-term exposure to NGF or PGE_2. However, these findings must be interpreted with care. Northern blot is not a highly sensitive or quantitative tool to measure mRNA levels. Also in northern blot analysis RNA samples are exposed to a greater risk of degradation during the procedure which may result in even more inaccurate quantification (Streit et al., 2009; Valasek and Repa, 2005; Wittwer et al., 2004).

Historically, similar incorrect conclusions were made regarding effects of NGF on expression of TRPV1 channel mRNA and protein. Due to the usage of non-quantitative mRNA assay techniques it was concluded that NGF does not alter the level of expression of TRPV1 mRNA (Ji et al., 2002; Puntambekar et al., 2005). Subsequently, using real-time PCR other groups showed that expression of both TRPV1 protein and mRNA are increased by NGF (Kim et al., 2004; Obata et al., 2004; Simonetti et al., 2006; Yang et al., 2007).

As shown in figure 9, 30 ng/ml NGF significantly reduced the expression of AKAP5 protein despite the lack of clear statistically significant effect on the level of the mRNA. This illustrates the need to investigate effects of various experimental manipulations on the amount of protein present as well as its biological function in order to reach scientifically more accurate conclusions.

Although real-time PCR offers a quantitative and highly selective tool to study the level of expression of mRNA of different targets, but further experiments to investigate the changes of expression on the level of protein are warranted.

PKA activity assay

A significant portion of the work presented in this dissertation utilized an assay of PKA activity. There are multiple advantages for assaying PKA activity over cAMP synthesis, which is frequently used as a surrogate for activation of $G_{\alpha s}$/adenylyl cyclase/PKA pathway. These advantages include the following; 1) the specificity of signaling downstream from cAMP (since cAMP can also activate Epacs and HCN), 2) more sensitivity due to signal amplification, 3) assaying PKA activity is a more selective way of implicating PKA than the use of kinase inhibitors (H-89 is of limited selectivity), 4) PKA activity represents the integrated activity of cAMP generation machinery which is highly regulated by complex interaction of PDEs, adenylyl cyclases, AKAPs and PKA itself, which makes interpretation of cAMP concentration data more difficult, 5) there are well developed tools to measure PKA activity in subcellular compartments using microscopy.

Kinase inhibitors were frequently used to infer that PKA mediates PGE_2-induced sensitization (Taiwo et al., 1989; Taiwo and Levine, 1991). H-89 is marketed as a selective inhibitor of PKA, while in fact its selectivity was largely unknown until relatively recently. For example, H-89, a commonly used inhibitor of PKA, is also able to inhibit ribosomal protein S6 kinase β-1 (S6K1), S6K2, PKBα (protein kinase B α), PKBβ, Rho-associated coiled-coil-containing protein kinase 2 (ROCK2), protein kinase N2 (PRK2), PKCζ, protein kinase D1 (PKD1), mouse homologue of SNF-like kinase (MSK1), and maternal embryonic leucine-zipper kinase (MELK) (Bain et al., 2007; Davies et al., 2000). In another high-throughput study, inhibition of 300 protein kinases by an array of 178 known kinase inhibitors was tested (Anastassiadis et al., 2011). Up to 27 kinases were inhibited 40 percent or more by 10 μM H-89. Of these 27 kinases, 7 were more sensitive than PKA to inhibition by H-89. Some of these kinases are known to mediate sensitization such as several of the MAPK pathway (Hudmon et al., 2008; Ji et al., 2002; Obata and Noguchi, 2004; Stamboulian et al., 2010). PKCζ (and its N-terminus truncated isoform) was recently shown to mediate sensitization of sensory neurons by NGF (Zhang et al., 2012). ROCK is also thought to mediate LPA-induced sensitization in sensory neurons (Ahn et al., 2009; Inoue et al., 2004). This brief account demonstrates that H-89, like many small molecule drugs, has likely effects on signaling proteins other than PKA. The contribution of most of these signaling molecules to sensitization and to

the effect of PGE_2 is unknown, thus necessitating experiments to demonstrate that PKA gets activated by PGE_2, directly in sensory neuronal cultures.

Another important advantage of the usage of PKA activity assay rather than cAMP, is that an increase in cAMP synthesis not only activates PKA, but other effectors, such as Epac and HCN channels. For over 40 years, the cAMP pathway has been implicated in mediating PGE_2-induced sensitization (Ferreira and Nakamura, 1979). Several studies confirmed that interference with this signaling pathway inhibits PGE_2-induced sensitization both *in vitro* and *in vivo* (England et al., 1996b; Evans et al., 1999; Hingtgen et al., 1995; Taiwo et al., 1989; Taiwo et al., 1992). Studies also provided evidence that cAMP generation is induced by application of PGE_2 to DRG sensory neuronal cultures (Hingtgen et al., 1995; Nakao et al., 2007; Smith et al., 2000a; Wise, 2006). Historically, PKA was assumed to be activated when cAMP concentrations increase in response to a particular ligand. However, with the discovery of multiple other effectors of cAMP such as Epacs and cyclic nucleotide-gated channels, it became clear that there are several effectors through which cAMP may act to propagate the signal (Kopperud et al., 2003; Seino and Shibasaki, 2005). Distinguishing which cAMP effector is mediating sensitization has acquired special importance since the discovery that Epacs, in addition to PKA, mediate response to PGE_2 and isoproterenol in conditions of chronic inflammation (Hucho et al., 2005; Wang et al., 2007). HCN channels, which also are activated by cAMP, mediate sensitization in conditions of chronic hyperalgesia (Chaplan et al., 2003; Emery et al., 2011a; Sun et al., 2005; Takasu et al., 2010; Weng et al., 2012). In embryonic DRG neurons, CREB (cAMP-response element binding protein) mediates sensory neuronal survival (Cox et al., 2008). In adult rat DRG neurons, PGE_2 causes increase of expression of BDNF in a CREB-dependent pathway (Cruz Duarte et al., 2012). Inhibition of CREB phosphorylation by cannabinoid receptors was also showed to be analgesic, pointing to its potential role in mediating hyperalgesia (da Silva et al., 2011). These studies point to the fact that cAMP has multiple effectors that mediate different aspects of sensitization of sensory neurons. Therefore elevation of cAMP concentration must be supplemented by further investigation of the downstream effector that gets activated.

Signal amplification may allow undetectable increases in cAMP concentration to be able to activate PKA and propagate signaling. It is now recognized that cAMP signaling is highly compartmentalized. Therefore it is possible that cAMP concentration increases in specific and localized compartments, which are below the detection limit of

commonly used cAMP assay methods, yet still enough to activate PKA in that compartment. Due to signal amplification, PKA activity might be detectable while increase in cAMP might not.

Despite these clear advantages there are some disadvantages for measuring PKA activity in the manner described in this dissertation. A classic and reliable assay method used a peptide substrate, called kemptide, which is selectively phosphorylated by PKA (Demaille et al., 1979; Kemp et al., 1977). This assay is feasible, inexpensive and reliable. However one of its most important shortcomings is the lack of a good method to identify the cell in which PGE_2 activates PKA. DRG cultures, as described earlier in the discussion do not contain one type of cells. Therefore assaying PKA activity in cell lysates does not eliminate the contribution of non-neuronal glial cells present in cultures. This is of particular importance since it was shown that EP and IP receptors activation increase cAMP concentration in whole DRG cultures and non-neuronal (glia-only) cell cultures prepared from DRGs (Ng et al., 2011). It is noteworthy that the protocol for preparing DRG cultures in that study is different from the one I employed in the work presented in this dissertation. In addition to different type of media and mitotic inhibitors used to prevent non-neuronal cell growth, an important and noticeable difference is that cells were grown at 20-fold lower density than the cultures used in this dissertation. In the work by Ng and coworkers cells were seeded at a density of approximately 300 cells/ cm^2, while throughout this dissertation cells were seeded at approximately 6,000 cells/cm^2. The density at which DRG cultures are grown affects cAMP production greatly, the lower the density of cells in culture, the higher the maximal response to IP and EP agonists (Rowlands et al., 2001). Therefore the much-lower cell culture density in the study by Ng and coworkers might explain the significant cAMP levels obtained in non-neuronal cultures. Also in the study by Ng and coworkers, IBMX was used, which is a pan-PDE inhibitor, while in both the lysis or PKA activity assay buffers used in this thesis, no PDE inhibitors were used.

Further work to measure PKA activity in a cell-specific manner is needed. Cell-specific PKA activity assays would enable measurement of PKA in neurons only, and even in compartments of interest, such as the neurites. PKA fluorescence resonance energy transfer (FRET) reporters has been developed and even and multiple versions with enhanced dynamic response to PKA are available (Allen and Zhang, 2006; Zhang et al., 2005a; Zhang et al., 2001). Under normal conditions DRG neurons transduce stimuli into an orthodromic electric signal of action potentials generated in the peripheral

nerve endings, propagated along the axon to the central nerve endings. Electric signals in the central terminals stimulate the release of neurotransmitters and relay this signal to the second order neuron in the spinal cord. Antidromic electric signals can also occur in peripheral sensory neurons in some pathological conditions as in neurogenic inflammation, however its discussion is not within the focus of this dissertation (Willis, 1999). Therefore it would be interesting to measure PKA activity in these compartments specifically as it was shown before that cAMP waves (and hence PKA activation) may not occur uniformly throughout the neuron (Bacskai et al., 1993; Hempel et al., 1996b). Multiple peptide signal sequences capable to trafficking proteins to axonal compartments in neurons are known (Francesconi and Duvoisin, 2002; Tiao et al., 2008). Some of the discovered targeting sequences were successfully used in DRG neurons (Babetto et al., 2010). Furthermore, it is worth mentioning that Epac-based FRET reporters for measuring cAMP are also available (DiPilato et al., 2004; Nikolaev et al., 2004; Ponsioen et al., 2004). Thus, although cAMP was not the focus of the current work, it would be useful to monitor cAMP concentration in neurites to determine if EP receptor interaction with adenylyl cyclase is altered in specifically in these compartments. In summary, the above discussion considers the disadvantages of cAMP assays in the light of advantages of measuring PKA activity in order to elucidate the signaling pathways activated by PGE_2 to mediate sensitization in sensory neurons.

Design of PKA activity assay

Endpoint PKA activity was measured after duration of exposure of the culture to various ligands for 10 minutes. The duration of exposure was chosen based on the fact that in experiments involving neuropeptide release, 10-minute exposures to an evoking stimulus (capsaicin or potassium) with or without PGE_2 are used. Like all reversible reactions, if we considered phosphorylation by kinases to be the forward reaction, then it is counterbalanced by a reverse reaction by dephosphorylation by phosphatases. This serves the function of maintaining homeostasis and resetting the system after the cell responds appropriately to the stimulus initiating the signaling process. Traditionally, when measuring kinase activity, phosphatase inhibitors are used to prevent the reverse reaction from proceeding. In the PKA assay used in this dissertation, phosphatase inhibitors are included in our assay and lysis buffers, except when otherwise mentioned. However it is important to note that no phosphatase inhibitors were added to the media

during treatment; i.e. live cells were never exposed to phosphatase inhibitors. The rational for this is that kinases and phosphatases are themselves modified by phosphorylation and these modifications affect their activity, and hence the outcome. For example, enzymes that synthesize or breakdown cAMP, and hence affect PKA activation, are modulated by phosphorylation; PDE4D is phosphorylated by PKA leading to enhancement of its activity (Sette and Conti, 1996), while phosphorylation of the phosphodiesterase by Erk on a different residue inhibits its phosphodiesterase activity (Hoffmann et al., 1999). Also studies showed that adenylyl cyclases are phosphorylated and their activity is modulated by PKA in HEK293 cells (Bauman et al., 2006), although this observation was found to play a minimal role in adult rat cardiac myocytes (Rochais et al., 2004). Therefore, interfering with addition or removal of these posttranslational modifications would influence cAMP level and subsequent PKA activation.

It is well known that the activation of the MAPK pathway involves stepwise phosphorylation of a cascade of kinases and its inactivation involves the reverse; i.e. dephosphorylation of various kinases to reset the system. The catalytic subunit of PKA, on the other hand, is constitutively phosphorylated during “maturation” of the enzyme (Cheng et al., 1998; Shoji et al., 1979; Steinberg et al., 1993; Toner-Webb et al., 1992). This phosphate group is exceptionally resistant to dephosphorylation and thus phosphorylation and dephosphorylation are not believed to be mechanisms for regulation of PKA activity (Bechtel et al., 1977; Humphries et al., 2005; Mei et al., 2002; Shoji et al., 1979; Toner-Webb et al., 1992). The catalytic subunit can still be dephosphorylated under some conditions, however, these conditions are drastic and difficult to encounter in experiments where living cells are used (Liauw and Steinberg, 1996; Zakany et al., 2002). The regulatory subunit of PKA isoform II (abbreviated PKA-RII) is phosphorylated, and this phosphorylation is responsible for its high-affinity binding to a cardiac anchor protein known as AKAP15 (Manni et al., 2008). Upon stimulation, mutants of PKA-RII that cannot be phosphorylated are not able to phosphorylate its effectors upon stimulation, such as ryanodine receptor and phospholamban. This could be due to improper localization leading to its inability to receive the signal from its upstream activators (adenylyl cyclase and the GPCR activating it) or due to mis-localization in relation to its targets.

Phosphorylation also is important for the regulation of phosphatases. Membrane bound PTPs activity was also increased in a PKA-dependent manner in African green monkey kidney CV-1 cells and inhibition of PSPs increased PTPs activity (Brautigan and

Pinault, 1991a). The PP2A regulatory subunit B56δ is also phosphorylated by PKA leading to activation of PP2A in Sf9 cells (Ahn et al., 2007). β-adrenergic receptor stimulation activates PP2A in human keratinocytes affecting their migration and consequently wound healing (Pullar et al., 2003). Regulation of phosphatases by phosphorylation and dephosphorylation is not only limited to serine/threonine residues, but posttranslational modification of tyrosine residues by phosphorylation can serve as a means of regulation as well. In mouse fibroblast cell line 10T/2, tyrosine phosphorylation of PP2A in response to growth stimulation regulates its activity (Chen et al., 1994). On the other hand, in mouse B-lymphocytes, phosphorylation of tyrosine phosphatases on serine/threonine residues by PKA (activated by β2-adrenergic receptor stimulation) increased PTP activity (McAlees and Sanders, 2009).

It is noteworthy that culture media (which contained stimulating drugs), lysis buffer and assay buffer of the PKA activity assay used in this dissertation did not contain PDEs inhibitors. As mentioned above, PDEs (and adenylyl cyclases) can be phosphorylated as a way to modulate their activity. Once PKA becomes activated various positive and negative feedback loops are executed which modulate activities of PDEs, adenylyl cyclases, AKAPs and even PKA itself, eventually sculpting the cAMP compartment, its concentration and consequently PKA activation (Violin et al., 2008). Since the goal of PKA activity assay in this thesis is to have a reliable and faithful picture of its status in DRG cultures upon activation by PGE_2, it was decided that it is best to avoid interference with the assay, by not adding inhibitors of various PDEs.

Collectively, this discussion provides strong evidence for the presence of an extensive regulatory network between kinases, phosphatases, PDEs and adenylyl cyclases. Since assaying PKA activity is the most frequently used endpoint in this dissertation, it was judged that minimal interference with the processes underlying the activation of PKA would best reflect its status of activation within DRG cultures. Therefore, during the process of stimulating DRG cultures with PGE_2, phosphatase inhibitors and PDE inhibitors were not used.

Isoproterenol and sensitization of sensory neurons

One interesting observation in the current work is that 10 µM isoproterenol only increases PKA activity modestly compared to 1 µM PGE_2, $cPGI_2$, forskolin, 1.5 µg/ml cholera toxin or 300 nM L902688 (Fig. 4) Moreover, in pilot experiments that are not

shown in this thesis, isoproterenol concentrations from 1-10 μM did not cause an appreciable difference between PKA activation, suggesting a lack of concentration-response relationship.. This was a surprising finding since isoproterenol is used as a sensitizing agent (Hucho et al., 2005; Khasar et al., 1999b; Levine et al., 1988; Ouseph and Levine, 1995), presumably, acting through the cAMP pathway. Previous studies showed that activation of β-adrenergic receptors can lead to analgesia in some studies as well as hypersensitivity in others. Much of the work published about the effect of adrenergic stimulation and sensitization comes from whole animal experiments. Therefore, whether activation of β-adrenergic receptors causes hyperalgesia or analgesia may depend on the site of drug administration, the prior state of the animal (naïve or inflamed) and the concentration of the agonist.

Adrenergic receptors are ubiquitously expressed on many different cell types, notably the immune cells, which contribute significantly to release of inflammatory mediators and thus to sensitization. There are two major subtypes of adrenergic receptors, α and β receptors. There are α1A, α1B and α1D, α2A, α2B, and α2C, and β1, β2 and β3 adrenergic receptors and selective agonists and antagonists are clinically used for several of them to treat a variety of diseases in humans (Pertovaara, 2006). Nicholson *et. al* published a study that provides evidence for the expression of mRNA of α1A, α1B and α2C, but none of the other receptors, including β-adrenergic receptors, in adult rat DRGs and in superficial dorsal horn of the spinal cord *in situ* (Nicholson et al., 2005). Mizukami demonstrated using immunohistochemistry that β2-adrenergic receptors are expressed on nociceptive termini as well as spinal cord neurons in the dorsal horn of the spinal cord of rats (Mizukami, 2004). The conflicting results of these two studies may stem from the difference in techniques used to show receptor expression. It is possible that the immunoreactivity indicative of β2-adrenergic receptors in the study by Mizukami, was due to non-specific or off-target labeling due to antibody selectivity. RNA hybridization probes used for in-situ hybridization by Nicholson and co-workers to investigate mRNA expression are much more specific.

Functionally, 10 μM isoproterenol failed to augment capsaicin-evoked TRPV1 currents in DRG neurons (Moriyama et al., 2005). In whole-cell and perforated-patch recordings from DRG neurons, activation of α1-adrenergic receptors caused depolarization of the membrane potential and increase in excitability. On the other hand activation of β-adrenergic receptors caused hyperpolarization, and thus rendered DRG neurons more resistant to depolarization (Pluteanu et al., 2002). In female adult rats,

hyperpolarization-activated current was inhibited in medium and large diameter neurons but a lot less frequently in small diameter neurons by application of clonidine, a selective α2 adrenergic receptor agonist (approximately 37 percent of small, 96 percent of medium and 100 percent of large diameter neurons). It is thought that inhibition of hyperpolarization-activated currents in DRG neurons enhances their sensitivity (Yagi and Sumino, 1998). Therefore, it seems that there is evidence to support that activation of α-adrenergic receptors, but not β-adrenergic receptors, sensitizes DRG neurons. It is not clear from literature how compounds acting on the β-adrenergic receptors produce hyperalgesia. However, from PKA activity assay data presented in this thesis as well as from evidence reviewed from literature it seems that isoproterenol is not a reliable activator of PKA in DRG neurons and hence it is not a useful positive control.

PGE_2 sensitizes sensory neurons

As shown in figures 5, 6, 19, 20 and 32, acute exposure to PGE_2 sensitizes sensory neurons in culture and that activation of the cAMP/PKA pathway is the likely pathway mediating this sensitizing effect of the prostanoid (Hingtgen and Vasko, 1994b; Hingtgen et al., 1995; Southall et al., 2002; Vasko et al., 1994). Furthermore, it has long been appreciated that PGE_2 mediates inflammation-induced hypersensitivity in human (Collier and Schneider, 1972; Ferreira, 1972; Ferreira et al., 1973) and in animal models (Ferreira et al., 1978). One potential mechanism through which PGE_2 augments capsaicin-evoked iCGRP release, is through enhancement of excitability by inhibition of potassium-currents in sensory neurons (Evans et al., 1999).

PGE_2-induced sensitization is persistent

Persistent hyperalgesia is a significant clinical problem that necessitates investigating the mechanisms underlying its development. DRG neuronal cultures allow studying the effect of defined inflammatory mediator(s) on a more defined cell population. The question remains as to how to model persistent sensitization in cell cultures. One method to investigate mechanisms of chronic sensitization is by inducing it in animal models (such as by injection of carrageenan or CFA), and subsequently harvesting peripheral sensory neurons from these animals and studying them *in vitro*. A concern with this method is that once the DRG neurons are removed from the animal,

they likely revert back to their quiescent or baseline state and the pathological changes associated with persistent hyperalgesia might be abolished. For example, Bolyard and co-workers found that 24 hours - 7 days of continuous exposure of DRG neuronal cultures to forskolin or inflammatory cocktail caused sensitization (i.e. augmentation of potassium-evoked neuropeptide release). This sensitization does not desensitize and persists even after prolonged exposure to forskolin or inflammatory cocktail. However, one hour after removal of either agent, augmentation of potassium-evoked neuropeptide release was abolished. This indicates that DRG neurons can be sensitized in animal after creation of experimental inflammation. However, once the neurons are excised from the animal and maintained in culture they could revert to their baseline state presumably due to loss of inflammatory mediators in the extracellular milieu. An alternative method is to use a cocktail of inflammatory mediators (inflammatory soup) applied to the culture. For example, Vasko and co-workers showed that in DRG neuronal cultures grown in the presence or absence of NGF (Southall and Vasko, 2000), or after long-term exposure to “inflammatory cocktail” (Bolyard et al., 2000), or PGE_2 (Southall et al., 2002), the ability of the prostanoid to induce sensitization persists.

PGE_2-induced sensitization in animal models also persists (Ferreira et al., 1990). In the case of using animal models of sensitization, it is difficult to rule out the involvement of inflammatory mediators other than PGE_2 as it is known that PGE_2 can cause the release of other inflammatory mediators. For example, PGE_2 increases the expression of basic fibroblast growth factor mRNA in human fibroblasts (Sakai et al., 2001), which in turn plays a role in maintenance of persistent sensitization (Ji et al., 2006). PGE_2 also increases the expression and release of matrix metalloprotease 9, an enzyme that is released in (Pavlovic et al., 2006) and contributes to inflammatory and neuropathic hyperalgesia (Kawasaki et al., 2008; Liu et al., 2010c; Liu et al., 2012). More importantly PGE_2 increases BDNF synthesis and secretion from DRG cultures and explants (Cruz Duarte et al., 2012) and release of both BDNF and NGF from astrocytic cultures (Toyomoto et al., 2004). These released growth factors, enzymes and neurotrophins are known to contribute to persistence of sensitization (see introduction). Therefore, unlike the usage of cultured peripheral sensory neurons to study sensitization, when using *in vivo* models it is difficult to define and correlate persistence of sensitization to any particular inflammatory mediator(s).

In another animal model of persistent hyperalgesia (hyperalgesic priming), prior injection of an inflammagen, such as carrageenan, results in more intense and

prolonged reaction to sensitizing agents such as PGE_2 (Reichling and Levine, 2009). Similar to Ferreira's model, intraplantar injection of carrageenan (and similar inducers of inflammation) causes the release of several inflammatory mediators (see introduction). Since intraplantar injection of carrageenan, or repeated PGE_2 injection, cause the release of several cytokines, the augmented hyperalgesia could be replicated by injection of cytokines without having to inject the original agent. For example Ferreira's group showed that TNF-α, IL-1β and IL-8 induce persistent hyperalgesia in an identical manner to the repeated injection of PGE_2 (Sachs et al., 2002). Similarly, Levine and his group showed that IL-6 in skeletal muscle, produced hyperalgesic priming in adult rats (Dina et al., 2008). They also showed that TNF-α is capable of induction of hyperalgesic priming through its receptor 1 (Dina et al., 2008). Collectively, PGE_2-induced sensitization persists both *in vitro* and *in vivo* and cytokines are capable of producing a persistent state of hyperalgesia even without prolonged exposure to PGE_2 *in vivo*. The mechanism of action of these cytokines (TNF-α, IL-1β, IL-8) in producing persistent hypersensitivity is not understood. However, it was shown that these cytokines initiate cascades of release of various inflammatory mediators that culminate in increase of COX expression and consequently PGE_2 synthesis and release (Cunha et al., 2005). Nevertheless, none of the above mentioned studies attempted investigating the mechanism by which PGE_2 directly induces persistent hyperalgesia without the use of cytokines of agents that cause their release (e.g. carrageenan).

Based on the above discussion, prolonged application of either NGF or PGE_2 to adult rat DRG cultures was investigated as an *in vitro* model of persistent sensitization. NGF or PGE_2 were applied individually, rather than a mixture of several inflammatory mediators (as opposed to inflammatory soup). As detailed in the introduction, these two inflammatory mediators were selected because of the extensive body of evidence supporting their immutable role in persistent hyperalgesia (see introduction). Data shown in this dissertation provide evidence that when adult rat DRG cultures are grown in the presence of NGF or after long-term-exposure to PGE_2, sensitization induced by the eicosanoid is maintained (Fig. 6, 19 and 20). These data suggest that the use of adult rat DRG cultures as a model system to study persistent PGE_2-induced sensitization is valid and that it faithfully reproduces maintained sensitization, the hallmark of *in vivo* models.

PKA inhibition does not attenuate PGE_2-induced sensitization in DRG neurons grown in the presence of NGF or after long-term-exposed to PGE_2

As mentioned earlier, acute PGE_2-induced sensitization is mediated via EP3C and EP4, which are coupled to $G_{\alpha s}$ that activates adenylyl cyclase, which in turn increases cAMP synthesis and hence activates PKA which causes sensitization (Fig. 39 and Fig.40). The data presented in this dissertation demonstrate for the first time that PKA does not mediate PGE_2-induced sensitization in adult rat sensory neurons grown in the presence of 30 ng/ml NGF or after 5 days of exposure to 1 µM PGE_2. H-89 is a well-known PKA inhibitor and at the concentration of 10 µM it is able to completely inhibit PKA activity (this was confirmed as shown in Fig. 21). This is the first account of the effect of the interaction between NGF and PGE_2 or the effect of long-term exposure to PGE_2 on activation of PKA by the eicosanoid. This also suggests that the signaling pathway mediating PGE_2-induced sensitization clearly switches from being a PKA-dependent to a PKA-independent process after chronic exposure to NGF or PGE_2.

Possible mechanisms of signaling switch from PKA mediating the PGE_2-induced sensitization after long-term exposure to NGF or the eicosanoid

Multiple mechanisms could explain how PKA no longer mediates PGE_2-induced sensitization after chronic exposure to either NGF or PGE_2. These include; 1) alteration in EP receptors expression profile, 2) alteration of the level of expression of the heterotrimeric G protein subunit $G_{\alpha s}$, 3) alteration of the activity/expression of adenylyl cyclase, 4) alteration of the expression of AKAPs, 5) switch in the signaling by either increase of the activity of phosphatases or by receptor desensitization and engaging alternative signaling through Grk2. These various possibilities will be discussed in detail throughout the remainder of this discussion.

Possible signaling switch mechanism: 1) Alteration of EP receptors expression profile

One potential mechanism is that the expression profile of EP receptors changes such that a receptor that is not coupled to the PKA signaling pathway becomes responsible for mediating chronic PGE_2-induced sensitization (Fig. 30 and 40). It was shown previously that the only two receptor subtypes that are important for PGE_2-induced sensitization acutely are EP3C and EP4 by multiple groups, effectively ruling out receptors EP1 and most splice variants of receptor EP3 (Lin et al., 2006; Murase et al., 2008; Nakao et al., 2007; Southall and Vasko, 2001). Of particular importance is the study by McCoy and co-workers. In this study, it was clearly demonstrated that in genetically modified mice, only animals deficient in EP4 receptor were resistant to collagen-induced arthritis, indicating that this receptor is important for the development of this inflammatory disease (McCoy et al., 2002). However, the cell types in which EP4 is important were not determined in this study.

Few studies attempted to investigate changes in the expression profile of EP receptors in conditions that model chronic inflammation in isolated DRG cultures. Fehrenbacher and co-workers found that growing adult rat DRG cultures in the presence of NGF, TNF-α or IL-1β has no effect on the relative level of expression of mRNA of EP receptors (Fehrenbacher et al., 2005). In this dissertation, real time PCR data show that long-term exposure to PGE_2 also did not affect the level of EP receptors mRNA in DRG cultures (Fig. 31). This indicates that change of the expression profile of EP receptors at the transcriptional level is an unlikely mechanism to account for the ability of PGE_2 to activate PKA after chronic exposure to the eicosanoid. On the other hand, Ma found that in adult rat DRG explants exposure to dimethyl-PGE_2, a stable form of PGE_2, causes the modest increase in protein expression of EP1, EP3 and EP4, and reduction in expression of EP2 (Ma, 2010). Western blot detection of splice variants of the EP3 receptor was not done. Consequently, unlike the lack of change in the level of expression of mRNA in the study by Fehrenbacher and co-workers, the study by Ma provides some evidence for increased expression of the protein. However, whether a modest increase in expression of a receptor is functionally relevant is not clear. It is essential to recognize that receptors amplify signaling when activated and thus the added effect of modest increase of expression is not well understood. Therefore functional evidence is crucial, meriting more experiments studying which receptor

subtype is essential for mediating PGE_2-idnuced sensitization in DRG cultures grown in the presence of NGF or after long-term exposure to PGE_2.

Possible signaling switch mechanism: 2) Alteration of $G_{\alpha s}$ subunit activity/expression

Experiments described in this thesis show that in neurons grown in the presence of NGF, PKA was activated to a similar level by 1 µM PGE_2 (Fig. 9). This suggests that the pathway of activating PKA by PGE_2, $G_{\alpha s}$ and adenylyl cyclase, remains intact. Also in neurons exposed to PGE_2, over-night exposure to cholera toxin caused similar activation of PKA, suggesting that the pathway upstream from PKA also remains intact (Fig. 33). Previously, Southall and Vasko showed that expression of the heterotrimeric G-protein $G_{\alpha s}$ was not reduced in embryonic DRG cultures after 24 hours exposure to 1 µM PGE_2. However, it is important to measure not only the expression, but also the activity of $G_{\alpha s}$ in adult rat DRG neurons grown in the presence of NGF or after 5-day exposure to PGE_2. It is noteworthy that multiple receptors are coupled to $G_{\alpha s}$. Measuring the expression of all the $G_{\alpha s}$ in DRGs might confound specific changes in the pool that is coupled to EP receptors. Hence measurement of activation of $G_{\alpha s}$ after growing DRG cultures in the presence of NGF or after long-term exposure to PGE_2 would provide more functionally relevant answers.

Possible signaling switch mechanism: 3) Alteration of adenylyl cyclase activity/expression

As mentioned above, PKA activity was not different in neurons grown in the presence or absence of NGF, indicating that the signaling pathway upstream from PKA (including adenylyl cyclase) was not altered by growing neurons in NGF. However, exposing sensory neurons in the absence of NGF to PGE_2 for 5 days resulted in inhibition of cAMP synthesis as shown in figure 25, and inhibition of PKA activation (Fig. 22) suggesting that activation of adenylyl cyclase activity is impaired. However treatments that bypass EP receptors, such as cholera toxin or forskolin, activated PKA to the same degree, independent of pre-exposure to PGE_2. Similarly, forskolin increased cAMP production by 1 µM PGE_2 to the same extent even after long-term exposure to the eicosanoid. This can be interpreted that the loss of cAMP/PKA signaling occurs at the

receptor level rather than downstream signaling molecules. Previously, Fehrenbacher showed that when DRG cultures were grown in the absence of NGF and exposed to 1 μM PGE_2 for 24 hours then re-exposed to the eicosanoid acutely, cAMP synthesis was not different from vehicle; i.e. cAMP synthesis was inhibited. When the same paradigm was repeated, only this time with cultures grown the in the presence of 250 ng/ml NGF (but not 30 ng/ml NGF), cAMP synthesis significantly increased after exposure to 1 μM PGE_2 (Fehrenbacher, 2005). Thus the results presented in this dissertation confirm Fehrenbacher's findings. Fehrenbacher's work also indicates that at relatively higher concentrations of NGF adenylyl cyclase expression or activation by PGE_2 is altered, enabling it to overcome desensitization by long-term exposure to PGE_2. Further work is warranted to study the interaction between long-term exposure to NGF and long-term exposure to PGE_2 at the level of adenylyl cyclase activation.

Possible signaling switch mechanism: 4) Alteration of AKAPs expression

Alteration of expression of AKAPs by long-term exposure to NGF or PGE_2 could underlie the loss of mediation of sensitization by PKA. AKAP150/79 was previously shown to be necessary for acute PGE_2-induced sensitization of TRPV1 channel (see introduction for literature review). Also aberrant expression of AKAPs is known to underlie cancer, heart failure, arrhythmias and Alzheimer's diseases and dwarfism (Aye et al., 2012; Chen et al., 2007; Chiriva-Internati et al., 2012; Jin et al., 2008; Kammerer et al., 2003; Proctor et al., 2011; Rauch et al., 2008; Soderling et al., 2007; Wirtenberger et al., 2007). Changes in the mRNA levels of AKAPs are used to predict ovarian cancer prognosis (Sharma et al., 2005). After growing DRG cultures in 30 ng/ml NGF or exposing them to 1 μM PGE_2 for 5 days, no change in the level of expression of AKAP mRNA was detected using real-time PCR. As mentioned above, real-time PCR offers highly selective and quantitative means that is able to detect small changes in the level of expression of mRNA. However, this does not obviate the need to assay the protein level as changes in expression of protein may not be reflected in their cognate mRNA levels.

Indeed when the protein level of AKAP5 was semi-quantitatively measured using Western blotting, NGF significantly reduced its expression by more than 50 percent (Fig. 9), highlighting the necessity to measure the level of expression of the proteins of the mRNAs quantified in this dissertation using real-time PCR. However, it is not clear how

NGF-mediated reduction of expression of AKAP5 can contribute to loss of PKA-mediated PGE_2-induced sensitization. In the presence of phosphatase inhibitors, PGE_2 activated PKA similarly whether sensory neuronal cultures were grown in the presence or absence of NGF. This might be interpreted that reduction of AKAP5 protein expression occurs in compartments other than the ones that PGE_2 utilize for PKA activation. Therefore, as mentioned below, further studies are needed to measure AKAP5 expression in cell-specific as well as subcellular compartments-specific manner using live-cell microscopy.

Future experiments are also warranted to measure protein expression of the other AKAPs (AKAP12 as well as others) after chronic treatment with NGF or PGE_2. Since AKAPs exist in pools or compartments associated with various signaling complexes or signalosomes (see introduction), it is important to study its level of expression in these relevant compartments, such as TRPV1-associated signaling complexes (see introduction for detailed literature review of this topic). Another dimension of regulation may exist by controlling how AKAPs are trafficked to their specific compartments. It is possible that by altering the mechanisms for regulating the trafficking of AKAPs, their function as organizers for signaling, and hence the downstream PKA function, can be regulated. For example, the AKAP5 is anchored to cell membranes through a domain that recognizes and binds phospholipids (Dell'Acqua et al., 1998) while AKAP12 is targeted to the cell membrane through a similar domain in addition to a myristoylation on the N-terminus (Malbon et al., 2004). In theory modulation of the enzymes that myristoylate AKAP12, can regulate targeting of this anchor protein to the cell membrane and thus affect activation of the PKA pool that it anchors (Farazi et al., 2001).

Possible signaling switch mechanism: 5a) NGF inhibits PKA-mediated signaling by increasing phosphatase activity

Experiments described in results section show that, in the absence of phosphatase inhibition, activation of PKA by 1 μM PGE_2 is significantly lower in lysates obtained from DRG cultures grown in the presence of NGF (Fig. 10). This significant difference in PGE_2-activation of PKA was not due to alteration of the level of total PKA activity, as PKA activated by 10 μM cAMP was not significantly different whether the cultures were grown in the presence or absence of NGF (Fig. 7). In order to identify the

phosphatase mediating this effect an array of phosphatase inhibitors was used. Sodium vanadate, a selective PTPs inhibitor, did not reverse the observed reduction of PKA activation by PGE_2 in cultures grown in the presence of NGF (Fig. 12). Selective inhibition of PP1 and calcineurin also did not reverse the reduction of PGE_2-activated PKA in cultures grown in NGF (Fig. 17 and Fig. 14, respectively). The difference of PGE_2-activated PKA in lysates from DRG cultures grown in the presence or absence of NGF is totally abolished if lysates contain MCS-LR, a potent and nonselective PSPs inhibitor. MCS-LR is a toxic naturally occurring cyanobacterial cyclic heptapeptide. It is considered the most potent toxin in its family (Swingle et al., 2007). MCS-LR inhibits the PSPs PP1, PP2A, PP4, PP5, PP6 but not PP7 (Huang and Honkanen, 1998; Prickett and Brautigan, 2006; Swingle et al., 2007). Taken together, data presented in this thesis point to a MCS-LR-sensitive phosphatase that is responsible for the reduction of PGE_2-activated PKA in sensory neuronal cultures grown in the presence of NFG (Fig. 39). There are numerous examples of a kinase and a phosphatase having opposing effects on a given target. For example, a PTP antagonizes the effect of a tyrosine kinase in live cells derived from patients with chronic myelogenous leukemia (LaMontagne et al., 1998). An similar example of the PSPs family is PP2A which antagonizes the effect of PKA on L-type calcium channel in rat cortical neurons (Davare et al., 2000). Numerous examples are further discussed in specialized reviews (Dai et al., 2009; Herzig and Neumann, 2000).

Phosphatases were repeatedly shown to be involved in models designed to study sensitization of sensory neurons. For example, okadaic acid, but not its inactive structural analogue L-norokadaone, attenuated in a dose-dependent manner antinociception caused by clonidine and baclofen, but not by κ-opioid receptor agonists (Moncada et al., 2005). Also, as mentioned earlier, Hingtgen and Vasko showed that okadaic acid, by itself, can significantly potentiate capsaicin-evoked iCGRP release from embryonic rat sensory neurons (Hingtgen and Vasko, 1994a). Okadaic acid also attenuated prolongation of PGE_2-induced hyperalgesia produced by rolipram, which indicates involvement of phosphatases in regulating the hypernociception signaling in animal models (Ouseph et al., 1995). The data presented in this dissertation point to another role of PSPs in NGF-mediated switching of the signaling pathway mediating PGE_2-induced sensitization (Fig. 39).

Identity of the phosphatase mediating NGF-induced attenuation of PKA signaling

Because MCS-LR is a potent but relatively non-specific PSPs inhibitor that is capable of inhibiting PP2A/PP4/PP6 as well as PP1/PP5 (Swingle et al., 2007), use of this drug cannot distinguish which PSP is critical in reversing desensitization of PGE_2-activated PKA by NGF (Fig. 39). I-2, a selective inhibitor of PP1, had no effect on NGF-induced reduction of PGE_2-activated PKA, suggesting that PP1 does not mediate the effect by NGF. Unfortunately, there are no small-molecule inhibitors of PP2A/PP4/PP6 (the remaining MCS-LR-sensitive PSPs) that would allow discrimination among them (Swingle et al., 2007). Therefore alternative methods must be used to identify the critical phosphatases. Reduction of expression using RNAi can provide a means to conduct cause-effect studies to identify the possible phosphatase involved. One difficulty with knock-down experiments is that PSPs are composed of several subunits which exist in multiple isoforms, and thus redundancy is a problem. For example, PP1 is a heterodimer composed of catalytic and regulatory subunits. There are four isoforms of the catalytic subunit of PP1 (α, β, γ1 and γ2) and over 100 PP1 regulatory subunits (Shi, 2009; Virshup and Shenolikar, 2009). Consequently there are more than 400 possible PP1 holoenzymes alone. PP2A is a heterotrimer that is composed of catalytic, regulatory and scaffolding subunits. Similarly, there are between 30 and 160 possible holoenzymes of PP2A (Shi, 2009; Virshup and Shenolikar, 2009). These subunits assemble in a variety of assortments to give rise to large number of holoenzymes that differ in their substrate specificity, subcellular localization, and regulation (Virshup and Shenolikar, 2009). The lack of a means to distinguish these isoforms or the lack of selective inhibitors makes studying the function of each of them a daunting task. Therefore, future experiments need to proceed by initially narrowing down the potential phosphatases involved.

As mentioned before, ion channels are an important effector of the sensitizing actions of PGE_2 (and other inflammatory mediators). Therefore, it might be useful to further identify which PSPs associate with the ion channels mediating the sensitization caused by PGE_2. This can be done by immunoprecipitating these ion channels, then analyzing the associated PSPs subunits using mass spectrometry to determine which catalytic, regulatory and anchoring subunits are involved. Once the subunits are identified, additional tools could be employed to pinpoint the PSPs involved in the observed effect.

Although identifying whether NGF alters expression of PSPs subunits is useful, altering expression of any protein in a cell is only one way to modulate its effect. There are numerous examples showing that phosphatase activity is altered by post-translational modification rather than altering the level of expression. In fact Paul Greengard was awarded the Nobel Prize (along with Eric Kandel and Arvid Carlsson) for his pioneering work on the effects of posttranslational modifications of several phosphatase regulatory proteins on learning and memory, including the well-studied DARPP-32 (Greengard, 2001). Mass spectrometry would be beneficial in identifying post-translational modifications as well, since it is possible to identify not only the molecular fingerprint, but also its mass and thus whether a post-translational modification occurs (Thelen and Miernyk, 2012). It is important to identify which phosphatase is mediating NGF-induced attenuation of PGE_2-activated PKA, and the mechanism of modulating this phosphatase activity. This would help identify novel targets that might be useful in developing alternative safer and more effective therapies for alleviating chronic pain in patients.

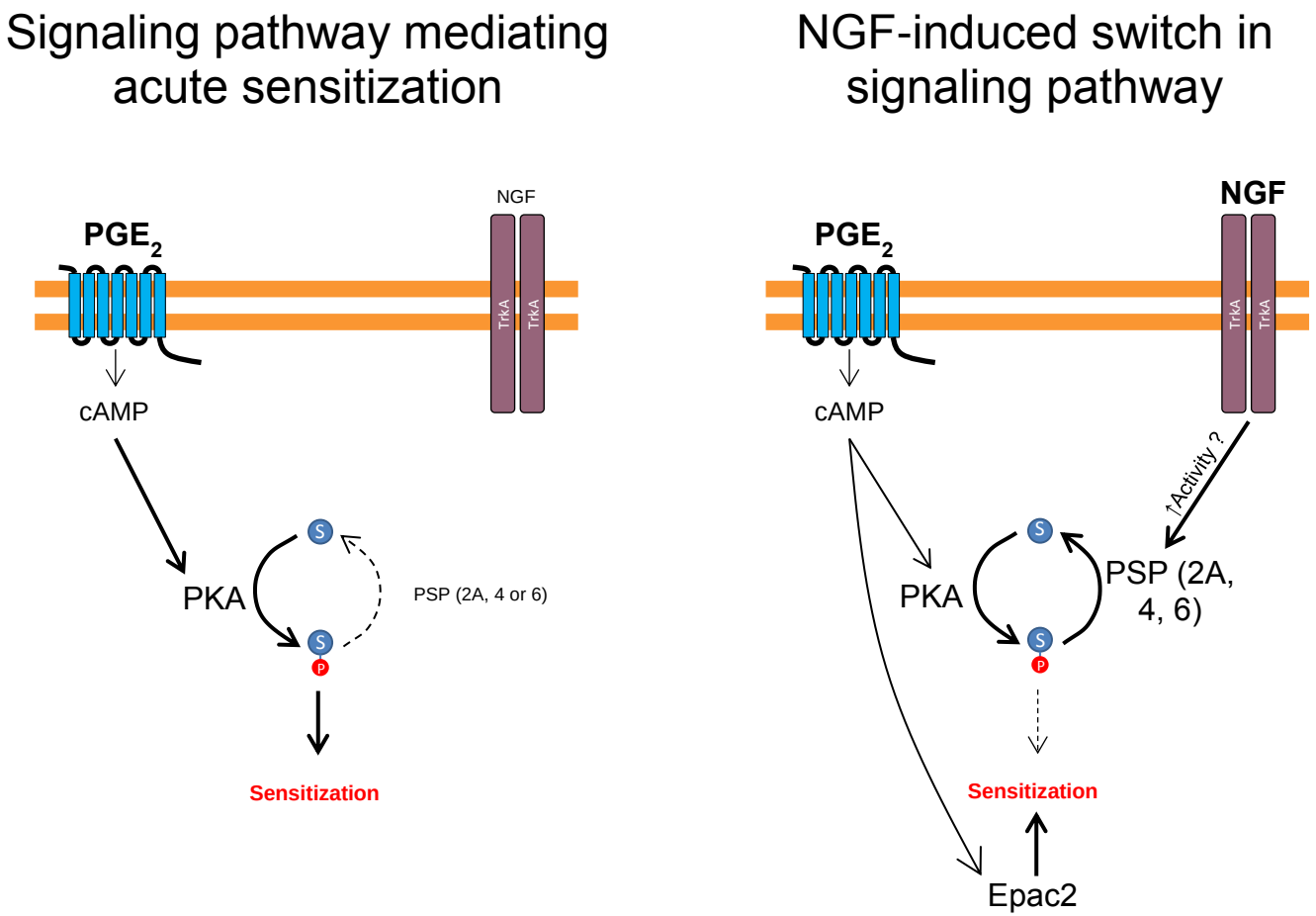

Figure 39. Mechanism of switching PGE_2-activated signaling from PKA to alternative signaling pathways by PSPs in adult rat sensory neurons grown in the presence of NGF

Possible signaling switch mechanism: 5b) Long-term exposure to PGE_2 causes Grk2-dependent homologous desensitization of PKA

It has been suggested that there is a switch in signaling that mediates PGE_2-induced sensitization under chronic inflammatory conditions (Hucho et al., 2005; Wang et al., 2007). Controversy exists whether PKA still partially mediates PGE_2-induced effects (Parada et al., 2005; Villarreal et al., 2009; Wang et al., 2007). For example using different behavioral and cellular models of sensitization, it was shown that in models of chronic hypersensitivity, dual inhibition of PKA and PKC is essential for total blockade of PGE_2-induced sensitization (Hucho et al., 2005; Sachs et al., 2009; Wang et al., 2007). My results clearly show that PKA does not get activated after prolonged exposure of isolated rat sensory neurons to PGE_2 and that H-89 lacks any inhibitory effect on PGE_2-induced sensitization after 5-day exposure to the eicosanoid.

The difference between various studies and my work could be explained by the fact that some of the previous studies were done *in vivo* and thus it is possible that signaling in some of the cells that participate in hypersensitivity continues to be PKA-dependent. As mentioned earlier in this discussion (see section “Use of isolated adult rat DRG cultures” in this discussion) different types of cells which have different roles contribute to hypernociceptive behavior in animal models. The behavioral response by the animal is integrated through the participation of different types of cells (such as immune cells, keratinocytes, fibroblasts, glial cells, endothelial cells, neurons and other cell types) and their respective signaling pathways. Therefore it is possible that *in vivo*, PKA-mediated signaling remains to be the conduit for processes important for hyperalgesia, in some of the non-neuronal cell subtypes but not in sensory neurons. The lack of cell specificity when studying signal transduction pathways in experimental animals highlights the importance of using either isolated cell cultures or animals that have cell-specific genetic modifications (such as by cell-specific overexpression or knockdown of different signaling molecules).

Wang *et al.* (2007) showed that PKA alone mediates PGE_2-induced sensitization of ATP-activated purinergic receptor currents in IB4+ sensory neurons harvested from naïve rats. However, in sensory neurons harvested from CFA-treated rats, both PKCε and PKA partially mediate PGE_2-induced augmentation of ATP purinergic currents. In the work by Wang and co-workers, purinergic receptor currents were measured from isolated single cells (Wang et al., 2007). Therefore, in Wang’s work it cannot be argued

that other cell types were involved in PKA-mediated signaling that contributes to PGE_2-induced sensitization. A potential reason that might explain why PKA does not mediate PGE_2-induced sensitization in my work while it partially does in the study by Wang *et al.* is that the signaling was studied in IB4+ sensory neurons, and that this signaling may be different from that in neuropeptide-containing neurons (Bennett et al., 1998a; Bradbury et al., 1998). Thus assaying PKA activity in cell lysates cannot detect a difference in different subpopulations of neurons that represent a fraction of all sensory neurons. Another explanation could be differences in the experimental model since Wang *et al.* used sensory neurons freshly dissociated from DRGs harvested from animals whose paws were inflamed by CFA injection, whereas I used sensory neurons harvested from naïve animals and kept in culture for 12 days (Wang et al., 2007). During the course of inflammation induced by CFA, multiple inflammatory mediators are released, including NGF.

The interaction between these inflammatory mediators and their effect on PKA activation by PGE_2 was not studied in detail in the current work. Of interest, however, is the observation that growing adult rat sensory neurons in 250 ng/ml NGF versus 30 ng/ml resulted in PGE_2-induced increase in cAMP in the former but not the latter even after 24 hours of pre-exposure to the eicosanoid (Fehrenbacher, 2005). This raises the possibility that in sensory neurons grown in the presence of NGF and PGE_2, re-exposure to the prostanoid may still activate PKA. Since it is known that CFA causes increased production of NGF (see introduction), it is possible that neurons harvested from CFA-injected animals have signaling pathways already altered and modulated by that exposure.

Long-term exposure to PGE_2 causes homologous desensitization

Findings presented in this dissertation show that there is cross desensitization of activation of PKA by PGE_2 and of that by L902688. There was a lack of similar interaction between desensitization of PGE_2 and $cPGI_2$-activated PKA. It was anticipated that PGE_2 would be able to desensitize PKA activation by L902688, because both ligands activate EP4 receptors which are expressed on DRG neurons (Fehrenbacher et al., 2005). On the other hand, it was somewhat surprising that long-term exposure to PGE_2 did not influence $cPGI_2$-activated PKA in sensory neurons. As mentioned earlier, EP and IP receptors are both expressed on sensory neurons, they both cause neuronal

sensitization of sensory neurons and they share the same signaling pathway that mediates this sensitization (Hingtgen and Vasko, 1994b; Hingtgen et al., 1995). The finding that the desensitization of PGE_2-activated PKA is homologous suggests the mechanisms mediating this desensitization because homologous desensitization of GPCRs is mediated by Grk and not by second messenger-activated kinases (Premont, 2005). This finding also shows that long-term exposure to one eicosanoid will not result in desensitization to other ones. One might speculate that subsequent (and even cyclical) production of eicosanoids would help maintain sensitization.

Second messenger kinases do not mediate loss of PKA-activation by long-term exposure to PGE_2

To study whether desensitization of PKA activation by PGE_2 was mediated by PKA or PKC (second messenger-activated kinases), manipulations aimed at both these kinases were used. Neither H-89 nor BIM-I attenuated the loss of PKA activation after long-term exposure to PGE_2. It was previously shown in HEK cells that PKA-mediated phosphorylation of the IP receptor is capable of desensitizing it (Lawler et al., 2001). There is no precedent, however, for an analogous phenomenon with the EP4 receptor. Furthermore, Ichikawa and co-workers showed that inhibition of PKA using PKI did not prevent EP4 receptor desensitization in CHO cells (Nishigaki et al., 1996). Although evidence of a role for EP1 (and the coupled PKC) in PGE_2-induced sensitization of DRG sensory neurons is sparse, there are 2 reasons to examine whether PKC could mediate desensitization of PGE_2-activated PKA. First, EP1 receptors are expressed in adult rat DRG cultures (Fehrenbacher et al., 2005) and some splice variants of the EP3 receptor are coupled to $G_{\alpha q/11}$ and thus can activate the PKC pathway (Zeilhofer, 2007). Second, several studies showed that an eicosanoid receptor that is coupled to PKC can desensitize another PKA-coupled eicosanoid receptor in the same cells. For example, IP receptors can become desensitized by PKC-dependent phosphorylation as a result of activation of EP1 in isolated rabbit lungs (Schermuly et al., 2007). Also, activation of EP1 receptors or $PGF_{2\alpha}$ receptors causes heterologous PKC-dependent phosphorylation and desensitization of thromboxane A_2 receptors α and β in HEK293 and renal mesangial cells (Kelley-Hickie and Kinsella, 2004). Thus it is possible that a similar mechanism occurs in sensory neuronal cultures after long-term exposure to PGE_2. However, using the non-selective PKC inhibitor BIM-I at 1 µM, no evidence was found of PKC-dependent

desensitization of PGE_2-activated PKA. BIM-I used at lower concentration blocks PKC-mediated sensitization of sensory neurons in culture by P2Y receptor agonists (Huang et al., 2003). Collectively, data shown in this dissertation demonstrate that second messenger-activated kinases such as PKA and PKC do not mediate desensitization of PGE_2-activated PKA after long-term exposure to the eicosanoid.

Indirect PKA activation through activating $G_{\alpha s}$ is not changed

Using cholera toxin to lock $G_{\alpha s}$ in the activated state shows that PKA activation via direct $G_{\alpha s}$ stimulation is not altered after long-term exposure to PGE_2. This supports the notion that the integrated effect of activating $G_{\alpha s}$ and the adenylyl cyclase are not altered by chronic exposure to PGE_2, and thus it is logical to infer that the changes that lead to loss of PKA activation are upstream from $G_{\alpha s}$, i.e. the EP receptors themselves. This observation was confirmed using long-term exposure to forskolin, which also bypasses the receptors and activates adenylyl cyclase directly and thus, activates PKA without receptor activation.

One of the limitations of using cholera toxin is that it will activate not only the $G_{\alpha s}$ that is coupled to EP receptors, but all the $G_{\alpha s}$ in DRG cultures. Therefore whether the PKA, activated indirectly by cholera toxin, belongs to the same pool activated by PGE_2 or not, remains unknown. This drawback of this experiment warrants further work to ask whether relevant EP receptors, i.e. EP3c and EP4 are able to activate $G_{\alpha s}$ when stimulated using selective agonists after long-term exposure to PGE_2. Using long-term exposure of sensory neurons in culture to PGE_2, it will be also possible to ask whether the coupling of EP3c and EP4 from $G_{\alpha s}$ to another heterotrimeric G protein is switched, akin to the switching of coupling of βARs from $G_{\alpha s}$ to $G_{\alpha i/o}$ (Daaka et al., 1997). It was previously shown that EP4 switches coupling from $G_{\alpha s}$ to $G_{\alpha i/o}$ in heterologous expression systems (Fujino and Regan, 2006; Neuschafer-Rube et al., 1997). It was also shown in HEK cells that prolonged activation of human EP4 receptors results in MAPK activation, a hallmark of switching signaling to $G_{\alpha i/o}$ (Desai and Ashby, 2001). Thus signaling mediating PGE_2-induced sensitization could be switched from being $G_{\alpha s}$/cAMP/PKA mediated to a different heterotrimeric G-protein, such as $G_{\alpha 11/q}$/PKC, $G_{\alpha 12/13}$/Rho/ROCK or $G_{\alpha i/o}$. It also is now recognized that $G_{\beta\gamma}$ subunits can mediate signaling (Albert and Robillard, 2002; Birnbaumer, 1992; Lin and Smrcka, 2011). Evidence for $G_{\beta\gamma}$-mediated signaling in neuronal tissues are also well described in

literature (Dev et al., 2001; Strock and Diverse-Pierluissi, 2004). Previously, it was observed that other GPCRs switch their coupling in sensory neurons. Levine and co-workers argued that μ-opioid receptors switch signaling from $G_{\alpha i/o}$ to $G_{\alpha s}$ and PGE_2 receptors switch signaling from $G_{\alpha s}$ to $G_{\alpha i/o}$ in behavioral experiments using experimental animals (Dina et al., 2009; Joseph et al., 2010; Khasar et al., 2008). As mentioned earlier, it is difficult to identify the cell type in which these signaling changes occur from the *in vivo* experiments performed in these studies. In addition, there is no evidence in literature that $G_{\beta\gamma}$ can mediate sensitization in sensory neurons.

Long-term exposure to PGE_2 does not alter the mRNA expression of β-arrestins or Grks

Previously it was shown that chronic Δ^9-tetrahydrocannabinol treatment causes upregulation of Grk2, Grk4, β-arrestins 1 and 2 in multiple brain regions (Rubino et al., 2006). In animal models of hypertension Grk2 activity and expression increase and this is described as one of the pathophysiological mechanisms underlying the disease (Gros et al., 1997b; Gros et al., 2000). In rheumatoid arthritis patients Grk2 activity and expression are reduced in leukocytes isolated from the systemic circulation (Lombardi et al., 1999). In DRG cultures IL-1β reduces the expression of Grk2 and thus reduces internalization of bradykinin receptors after prolonged exposure to the ligand (von Banchet et al., 2011). As mentioned earlier, these previous studies suggested the possibility that expression of the mRNA of the ubiquitously expressed forms of Grks (Grk2, 3, 5 and 6) or β-arrestins may change after long-term exposure to PGE_2. Real time PCR showed that the relative level of mRNA for these molecules did not change, suggesting that PGE_2 does not modulate their expression on the transcriptional level. Modulation of the level or activity of Grks or β-arrestins can happen on the level of translation and independent of transcription and mRNA levels. Therefore further studies on the level of protein expression of Grks and β-arrestins are warranted. Also activity of Grks can be modulated by post-translational modifications. So studies that examine the kinase activity of Grks might shed light on their modulation via signaling rather than level of protein expression.

Grk2-mediates desensitization of PKA activation

Accumulating literature is providing evidence that Grks can act as the main component in desensitizing certain receptors and that this can occur independent of β-arrestins. For example it was shown that histamine H2 receptors, which are $G_{\alpha s}$-coupled, are desensitized in a Grk2 dependent manner in HEK cells (Fernandez et al., 2011). Interestingly this desensitization was independent of the kinase activity of Grk2, but required its RGS domain (regulator of G-protein signaling, a group of negative regulators of GPCRs). In the study by Fernandez and co-workers, H2 receptors desensitized by a "kinase-dead" mutant of Grk2 were unable to become internalized and consequently did not re-sensitize. This means that the kinase function, for H2 receptors, is not needed for desensitization, but it is essential for internalization and resensitization. This was observed with other receptors as well, such as the receptor for follicle stimulating hormone in rat Sertoli cells (Reiter et al., 2001) and dopamine D2 receptors in HEK cells (Namkung et al., 2009), which are coupled to $G_{\alpha q/11}$ and $G_{\alpha i/o}$ respectively. In other examples, Grk was sufficient for both desensitization and internalization of the receptor due to presence of "clathrin-box" which allows for the binding of Grk and clathrin and subsequent internalization of the receptor (Mangmool et al., 2006; Shiina et al., 2001). Reduction of expression of Grk2 using RNA interference in experiments described herein will lead to reduction of the expression of the protein (Fig. 40). Consequently, whether Grk2 is mediating the observed desensitization through a phosphorylation-dependent or independent mechanism cannot be determined. Future experiments that involve methods to inhibit the kinase activity of Grk2 would help delineating whether desensitization of PKA activation by PGE_2 is dependent on phosphorylation. To date, no small molecules that are capable of selectively inhibiting Grk2 are available. Overexpression of "kinase-dead" mutants of Grk2 can be used. Alternatively, an aptamer that was recently developed that selectively inhibits Grk2 kinase activity can be also used (Mayer et al., 2008). Aptamers (Latin; to fit) are small nucleic acid or peptide molecules that are capable of selectively binding to and thus modifying the activity of variety of targets including receptors, enzymes and other molecules (Keefe et al., 2010). It is even possible to envision engineering a viral vector that is capable of cell-specific expression of the Grk2-selective inhibitor aptamer for *in vivo* use in animal models. Developing such tool would be relatively easy and highly selective. High-affinity peptide inhibitors known to stop Grk2 kinase activity are also available and similarly developing

viral vectors for cell-specific overexpression of these peptides is also relatively feasible (Winstel et al., 2005).

It is noteworthy that reduction of expression of Grk2 only partially reversed desensitization by PGE_2. This can be explained in terms of insufficient reduction of expression by the siRNA. Despite that siRNA against Grk2 used in this dissertation caused more than 40 percent reduction of expression of Grk2 mRNA, the remaining Grk2 might still be sufficient to desensitize EP receptors to some extent and hence prevent full reversal of desensitization. It is also important to note that expression of Grk6 was significantly increased in DRG cultures after treatment with Grk2 siRNA. The increase was small (approximately 35 percent), however it may be sufficient to prevent total reversal of PKA desensitization after long-term exposure to PGE_2. Finally, the lack of total reversal can be explained by the redundant nature by which Grks act; i.e. other Grk(s) might be involved as well. This was reported recently with β-adrenergic receptor (Nobles et al., 2011) where it was found that multiple Grks phosphorylate and desensitize βARs. It was named “bar-coding” since phosphorylation of βARs by different kinases resulted in activation of different alternative signaling pathways.

Interestingly, expression of Grk2 in DRGs was shown to be only partially reduced by IL-1β on the protein level (Kleibeuker et al., 2008a; von Banchet et al., 2011). Expression of IL-1β is controlled by several inflammatory mediators in different chronic pathological painful conditions (Marchand et al., 2005; Schafers and Sorkin, 2008). Reduction of expression of Grk2 may lead to only partial desensitization of the EP receptors, which allows them to continue generating cAMP, which then activates other cAMP effectors such as the Rap-GEF Epac. Accumulating evidence from multiple groups suggests that Epac mediates an alternative signaling pathway that is implicated in the switch to chronic inflammation (Eijkelkamp et al., 2010b; Hucho et al., 2005; Wang et al., 2007).

My work demonstrates for the first time in isolated DRG cultures that prolonged exposure to PGE_2 leads to loss of PKA activation by the ligand despite persistence of sensitization, and that this loss is mediated at least in part by Grk2 (Fig. 40).

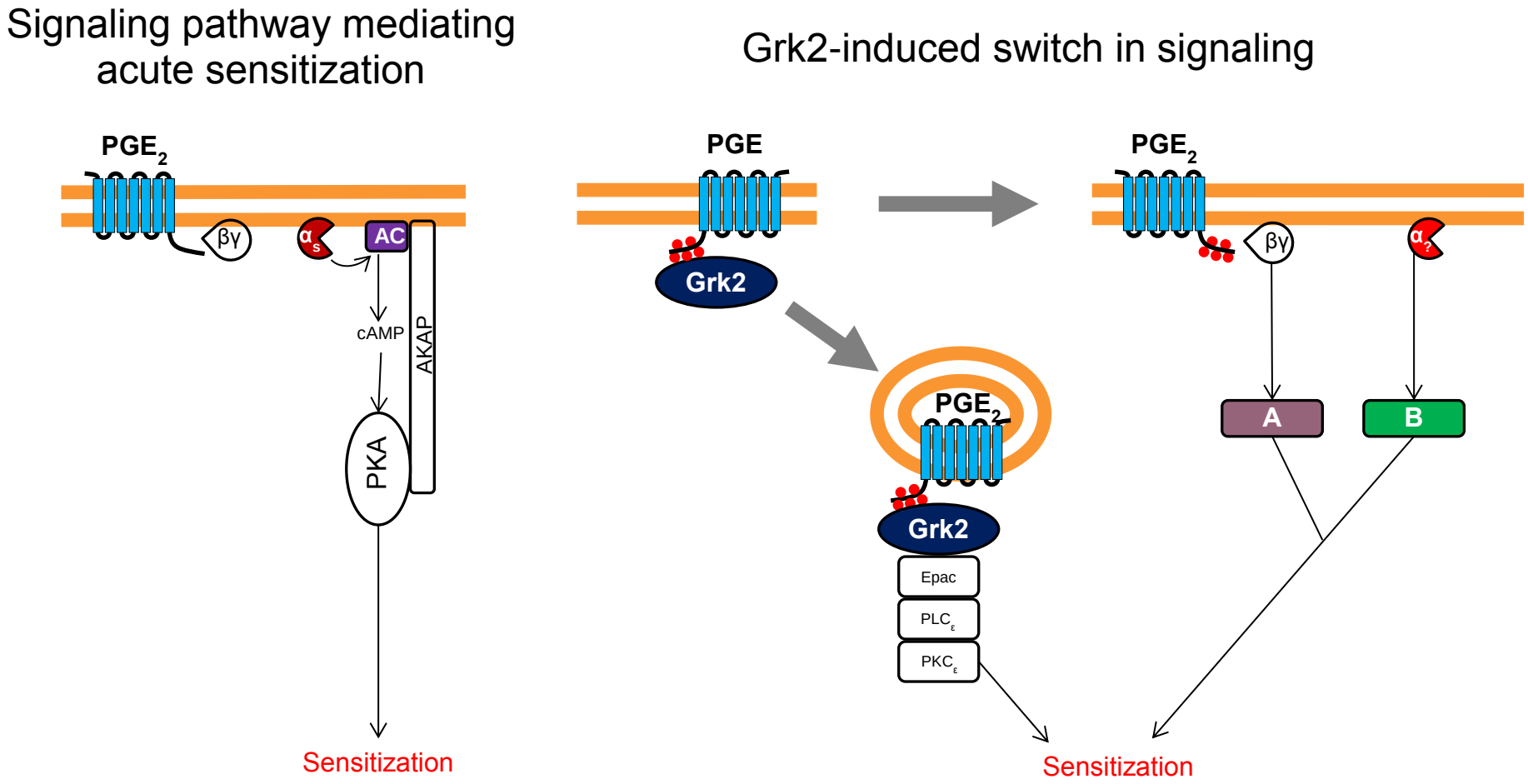

Figure 40. Mechanism of switching signaling from PKA to alternative signaling pathways by Grk2 in adult rat sensory neurons after long-term exposure to PGE_2

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Sigma Xi Graduate Research Competition 2010, co-First Place
Raymond Paradise Award for the 2006 Graduate Student Group

TEACHING EXPERIENCE

Assistant Lecturer
Ain Shams University-Cairo-Egypt-Department of Pharmacology and Toxicology, 2004-2006

Teaching Assistant
Ain Shams University-Cairo-Egypt-Department of Pharmacology and Toxicology, 2000-2004

RESEARCH EXPERIENCE

M.Sc. RELATED WORK EXPERIENCE

For my M.Sc. project, I answered the question whether a natural product, Jojoba liquid wax, possesses anti-inflammatory effects, as claimed in folk medicine. To this end, I used carrageenan-induced inflammation in paws as well as acrolein-induced inflammation in ear pinnae of rats as a model to study the anti-inflammatory effects of jojoba liquid wax. I used plethysmography, myeloperoxidase activity, tumor necrosis factor and interleukin1-β quantitation assays and immunohistochemistry. I showed that Jojoba liquid wax has anti-inflammatory properties applied topically or administered orally.

Ph.D. RELATED WORK EXPERIENCE

From 2006-2008, I worked on LPA as a putative novel inflammatory mediator. I used techniques such as real time PCR, western blotting, capsaicin-induced release of neuropeptides from sensory neuron cultures and immunohistochemistry. I also used FM1-43, an activity-dependent fluorescent reporter of synaptic vesicle recycling.

From 2008-2012, I worked on the switch in signaling pathways that are activated by prostaglandin E2. In addition to the techniques listed above, I am also using kinase and phosphatase activity assays.

PEER-REVIEWED PUBLICATIONS

Abstracts

R. R. Habashy, A. Hudmon, M. R. Vasko. Long-term exposure to PGE_2 causes homologous desensitization of receptor-mediated activation of protein kinase A. Society for Neuroscience Annual Meeting 2011.

R. R. Habashy, C. Guo, A. Hudmon, M. R. Vasko. Long-term exposure to NGF or PGE_2 reduces PGE_2-stimulated protein kinase A activity. Society for Neuroscience Annual Meeting 2010.

Papers

Accepted

Habashy, R. R., A. B. Abdel-Naim, *et al.* (2005). "Anti-inflammatory effects of jojoba liquid wax in experimental models." Pharmacol Res 51(2): 95-105.

In preparation

C. Guo, R. R. Habashy, D. B. Duarte, E. Thompson, M. R. Vasko. Epac2 mediates PGE_2-induced sensitization of adult rat DRG neurons cultured in the presence of nerve growth factor.

R. R. Habashy, A. Hudmon, M. R. Vasko. Homologous desensitization of receptor-mediated activation of PKA after long-term exposure to PGE_2 by Grk2.

MEMBERSHIPS

Society for Neuroscience 2010-2012