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Rapid Pain Modulation with Nuclear Receptor Ligands

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Abstract

We discuss and present new data regarding the physiological and molecular mechanisms of nuclear receptor activation in pain control, with a particular emphasis on non-genomic effects of ligands at peroxisome proliferator-activated receptor (PPAR), GPR30, and classical estrogen receptors. PPAR α agonists rapidly reduce both acute and chronic pain in a number of pain assays. These effects precede transcriptional anti-inflammatory actions, and are mediated in part by IK_{ca} and BK_{ca} channels on DRG neurons. In contrast to the peripheral site of action of PPAR α ligands, the dorsal horn supports the expression of PPAR γ . Intrathecal administration of PPAR γ ligands rapidly (5 minutes) attenuated mechanical and thermal hypersensitivity associated with nerve injury in a dose-dependent manner that could be blocked with PPAR γ antagonists. By contrast, a PPAR γ antagonist itself rapidly increased the mechanical allodynia associated with nerve injury. These data suggest that ligand-dependent, non-genomic activation of spinal PPAR γ decreases behavioral signs of inflammatory and neuropathic pain. We also report that the GPR30 is expressed on cultured sensory neurons, that activation of the receptor elicits signaling to increase calcium accumulation and PKC ϵ translocation, and that this signaling may contribute to increased neuronal sensitivity as treatment with the GPR30 agonist induces hyperalgesia. Finally, application of the *membrane-impermeable* 17 β -E₂-BSA rapidly (within 15 min) enhanced BK-stimulated inositol phosphate (IP) accumulation and PGE₂-mediated cAMP accumulation in trigeminal ganglion cultures. We conclude that nuclear receptor ligands may operate through rapid, non-genomic mechanisms to modulate inflammatory and neuropathic pain.

1. INTRODUCTION

The nuclear receptor superfamily includes retinoid, thyroid hormone, steroid, and peroxisome proliferator-activated (PPAR) receptors. Unlike plasma membrane receptors that signal through second messengers, nuclear receptors can function directly as transcription

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factors that control gene transcription. The regulation of gene transcription by nuclear receptor ligands is commonly referred to as the “classical” or “genomic” pathway. Responses mediated by the genomic pathway typically have latencies of at least 30 to 60 minutes (and up to days) and are associated with changes in protein synthesis. All 75+ members of this superfamily share certain structural features, including a C-terminal ligand-dependent activation domain, a DNA-binding domain, and an N-terminal ligand-independent activation domain. The physiological actions of nuclear receptors are quite numerous, and extensive research in the past 20 years has led to the development of important pharmacotherapeutic agents for the treatment of a variety of medical problems. However, with the notable exception of steroidal anti-inflammatory drugs, only until recently has appreciation developed for the great potential of this superfamily as a reservoir of targets for the pharmacotherapy of chronic pain. We discuss and present new data regarding the physiological and molecular mechanisms of nuclear receptor activation in pain control, with a particular emphasis on non-genomic (very rapid) effects.

1.1 Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are transcription factors belonging to the nuclear receptor superfamily (Kota BP, 2005). PPARs are activated by fatty acids, eicosanoids, and synthetic ligands. Three PPAR isoforms have been identified – α , β/δ , and γ (Berger JP, 2005; Michalik L, 2006). Activated PPARs form functional heterodimers with retinoic acid receptors (RXR) (Berger and Moller, 2002; Willson et al., 2000). This complex interacts with various co-activators and a specific peroxisome proliferator response element (PPRE) on the promoter region of target genes to alter transcription (Tan NS, 2005). PPARs produces pleiotropic actions that are mediated not only through these slow-response genomic (transcription-dependent) (Berger and Moller, 2002; Willson et al., 2000), but also by rapid non-genomic (transcription-independent) mechanisms (Fu et al., 2003).

PPAR α —Genomic actions of PPAR α are well described in the literature (Berger and Moller, 2002; Willson et al., 2000). In metabolically active tissues, such as the liver, heart and skeletal muscle, activation of PPAR α induces expression of genes involved in mitochondrial and peroxisomal fatty-acid β -oxidation, lipoprotein and cholesterol metabolism, gluconeogenesis, triglyceride clearance and ketogenesis (Berger and Moller, 2002; Willson et al., 2000). A growing body of evidence has also implicated PPAR α in the control of inflammatory and immune responses. PPAR α is expressed in various immune cells that regulate these processes [Daynes,2002], mice lacking the gene encoding for this receptor display prolonged inflammatory responses [Devchand,1996] and synthetic PPAR α agonists exert profound anti-inflammatory effects (LoVerme et al., 2005a), including reductions in the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), interleukin-1 β (IL-1 β), prostaglandin E₂ (PGE₂), vascular cell adhesion molecule-1 (VCAM-1) (Jackson et al., 1999) and tumor necrosis factor alpha (TNF- α). PPAR α anti-inflammation has been linked to the inhibition of the pro-inflammatory signaling pathways mediated by the transcription-dependent nuclear factor κ B (NF- κ B) and activated protein-1 (AP-1) (Vanden Berghe et al., 2003).

More recent studies have identified a number of PPAR α -dependent rapid non-genomic actions. In the small intestine, PPAR α agonists rapidly engage peripheral vagal sensory fibers to reduce food intake (Fu et al., 2003). In liver and white adipose tissue these drugs rapidly induce lipolysis and fatty-acid oxidation, reducing tissue triacylglycerol levels (Guzmán et al., 2004). Both of these effects occur in a PPAR α -dependent manner on the order of minutes (Fu et al., 2003; Guzmán et al., 2004) —effects that are too rapid to occur through classic transcription-dependent mechanisms. Taylor et al. (in 2002 and 2005) were the first to report rapid antihyperalgesic actions of PPAR α agonists in the carrageenan

model of inflammatory pain (Taylor et al., 2002; Taylor et al., 2005). As reviewed below, LoVerme et al. (in 2006) extended this finding to a number of animal pain models and to a peripheral mechanism of action on sensory neurons (LoVerme et al., 2006).

PPAR γ —The PPAR γ isoform mediates numerous physiological functions, and of particular clinical significance is its role as a lipid sensor. PPAR γ activation leads to adipocyte differentiation and drives the gene expression of enzymes that facilitate lipid uptake and synthesis (Lehrke M, 2005). Abnormal PPAR γ function is associated with numerous diseases such as type 2 diabetes. Indeed, synthetic PPAR γ agonists of the thiazolidinedione (TZD) class act as insulin sensitizers. Although our understanding of the glucose lowering properties of PPAR γ ligands remains an intense area of investigation (Murphy GJ, 2000; Staels B, 2005), TZDs such as rosiglitazone (Avandia®) and pioglitazone (Actos®) represent an important pharmacotherapy for the treatment of glucose intolerance (Martens FM, 2002).

Beyond its peripheral actions, recent studies indicate that PPAR γ agonists regulate CNS inflammation and that PPAR γ is a powerful pharmacological target for counteracting neurodegeneration, ischemic stroke, and spinal cord injury (Culman et al., 2007). The dramatic pace of discovery is exemplified by recent clinical studies indicating that the PPAR γ ligand pioglitazone, an FDA-approved TZD that can cross the blood brain barrier to exert CNS actions (Heneka MT, 2005), reduced the incidence of stroke in patients with type 2 diabetes (Culman et al., 2007), and by current Phase 2 and 2b clinical trials on the benefits of pioglitazone in a subset of AD patients expressing the ApoE4 gene (GlaxoSmithKline). Recent reports describe the potential of PPAR γ agonists as anti-inflammatory agents (Taylor et al., 2002). As reviewed below, the latest data extends this therapeutic potential to neuropathic pain syndromes (Churi et al., 2008).

1.2 Estrogen Receptors

Estrogens regulate a wide range of cellular functions. These actions are mediated by a variety of receptor subtypes that signal via an assortment of pathways in a cell-type specific manner. The two best studied subtypes, ER α and ER β , are separate gene products (Beato et al., 1995; Kumar and Thompson, 1999). Recently it has become clear that estrogen can elicit rapid (within seconds to minutes) signaling events that are not mediated by the classical genomic pathway (for reviews see Cato et al., 2002; Ho and Liao, 2002; Levin, 2002; Beyer et al., 2003; Bjornstrom and Sjoberg, 2005; Song et al., 2005).

ER α and ER β —In some cases, the rapid actions of estrogen appear to be mediated by classical estrogen receptors (ER α , ER β , or their splice variants) in the plasma membrane or cytosol (Razandi et al., 1999; Clarke et al., 2000; Norfleet et al., 2000; Watson et al., 2002; Pawlak et al., 2005). Post-translational changes (e.g., palmitoylation) have been proposed to traffic the ER α and ER β subtypes to plasma membrane microdomains enriched in cholesterol, receptors and signaling molecules (Chambliss et al., 2000; Acconcia et al., 2003; Razandi et al., 2003a). Several signal transduction pathways have been implicated in rapid ER signaling, including mitogen-activated protein kinase (MAPK), adenylyl cyclase (cAMP), PLC (calcium, PKC), nitric oxide (cGMP) and phosphatidylinositol 3-kinase (PI3K and Akt) (for reviews, see Hall et al., 2001; Kelly and Levin, 2001; Levin, 2002).

GPR30—The GPR30 is a GPCR which was cloned in the late 1990's (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997); however, it was ~3 years before estradiol (17 β -E₂) was shown to bind with high affinity to the receptor and to induce activation of MAP kinase through transactivation of the epidermal growth factor (EGF) receptor in breast cancer cell lines which express the GPR30 but do not express the classical

ERs (Filardo et al., 2000). Expression of the GPR30 has been documented in the heart, skeletal muscle, bone, kidney, pancreas, placenta, lung, liver, prostate, ovary and discrete areas of the brain (Carmeci et al., 1997; Heino et al., 2008; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). There are some reports that the receptor is expressed in a sexually dimorphic manner (Canonaco et al., 2008); however, the mechanisms for regulation of GPR30 expression have largely been unexplored. Several laboratories have demonstrated that the GPR30 is upregulated by progesterone (Ahola et al., 2002; Thomas et al., 2005), but the identity of its promoters and other transcriptional activators remain unknown. It is possible that estradiol also regulates the expression of GPR30, since the loss of functional ER α results in the upregulation of mRNA for the receptor in the thymus (Wang et al., 2008).

The binding affinity profile of the GPR30 for traditional estrogen agonists and antagonists differs from that of the classical estrogen receptors. In binding assays, estradiol (Ki: 3-6 nM; (Revankar et al., 2005; Thomas et al., 2005)) and G-1 (Ki: 11 nM; (Bologa et al., 2006), a recently synthesized GPR30-selective agonist, were able to displace binding to the GPR30, whereas estrone and estriol did not demonstrate a high affinity for, and were unable to activate the receptor. Likewise, progesterone, testosterone, and cortisol were also unable to displace binding of estradiol from GPR30 (Thomas et al., 2005). Interestingly, the classic "antagonists" tamoxifen and ICI 182,780 both bind with high affinity and activate the GPR30 (Filardo et al., 2002; Revankar et al., 2005; Thomas et al., 2005), in contrast to their partial or complete antagonism of transcriptional effects of classical ERs (Aronica et al., 1994; Kelly et al., 1999; Kuiper et al., 1997; Lagrange et al., 1997). Thus, the pharmacology of the GPR30 distinguishes it from the other estrogen receptors and may provide an opportunity for the development of therapeutics which can selectively reverse GPR30 activity without affecting the actions of estrogens at the classical receptors.

Since the identification of estradiol as the ligand for the GPR30, multiple signaling mediators for the receptor have been proposed. In cell expression systems, estradiol activation of GPR30 has elicited increases in intracellular calcium (Revankar et al., 2005), stimulation of adenylyl cyclase through dissociation of G α_s and G $\beta\gamma$ (Filardo et al., 2002), dissociation of G $\alpha_{i/o}$ and G $\beta\gamma$ (Revankar et al., 2005), translocation of PI3K to the nucleus (Revankar et al., 2005) and ERK phosphorylation (Filardo et al., 2000; Filardo et al., 2002; Thomas et al., 2005). In cancer cell lines, GPR30 activates ERK, G $\alpha_{i/o}$, and src tyrosine kinase either directly or indirectly through transactivation of the EGF receptor (Maggiolini et al., 2004; Vivacqua et al., 2006a; Vivacqua et al., 2006b). In keratinocytes, estradiol induces increases in CREB phosphorylation which results in cyclin D2 upregulation and cell growth. This estradiol-induced response is blocked by the protein kinase A (PKA) inhibitor H89, but not by PKC or PI3K inhibitors, is mimicked by dibutyryl cAMP, and reversed by treatment with antisense to the GPR30 (Kanda and Watanabe, 2004). In macrophages, the application of estradiol induces a rapid increase in cAMP accumulation and c-fos expression which results in the increased release of NGF from the cells and this effect is also reversed by antisense knockdown of the GPR30 receptor (Kanda and Watanabe, 2003). Thus, the precise GPR30 signaling pathways appear to be cell-type specific. It is yet unknown what signaling cascades are engaged by the activation of the GPR30 in sensory neurons. Although sensory neurons do not express the EGF receptor, they do express other tyrosine kinase receptors, such as the high affinity receptor for nerve growth factor (trkA) which can be activated secondary to ligand binding to GPCRs (Lee et al., 2002).

Although numerous studies have investigated the genomic effects of estrogen on nociceptive responses (Coyle et al., 1996; Cruz et al., 1996; Drury and Gold, 1978; Flake et al., 2005; Frye et al., 1992; Frye et al., 1993; Martinez-Gomez et al., 1994; Ratka and Simpkins, 1991), the discovery that estrogen can rapidly activate intracellular signaling pathways through non-genomic mechanisms suggests the possibility that the steroid hormone might

also induce posttranslational changes of ion channels or receptors in trigeminal neurons to alter the sensitivity of neurons to thermal, mechanical, or chemical stimuli.

2. RESULTS

2.1 Rapid analgesia through peripheral PPAR α receptors

Drs. LoVerme, Piomelli, and colleagues used the formalin test to show that intraplantar injection of high affinity PPAR α agonists, including Wy-14643, GW747 and the natural PPAR α agonist palmitoylethanolamide (reported in vitro human receptor transactivation EC₅₀ values: 0.0065 μ M, 5 μ M and 3 μ M, respectively, (Brown et al., 2001; LoVerme et al., 2005b) reduced phase I pain (pain mediated by c- and A δ -fibers occurring 0-15 min after formalin injection) and phase II pain (pain mediated by ongoing activity of c-fibers and inflammation occurring 15-45 minutes after formalin injection) (LoVerme et al., 2006). Low-affinity PPAR α ligands, such as fenofibric acid (reported in vitro human receptor activation EC₅₀ value: 30 μ M) (Brown et al., 2001; Willson et al., 2000), however, were ineffective in this assay (LoVerme et al., 2006). In this model all three drugs reduced pain in a PPAR α -dependent manner as these compounds only reduce pain in wildtype, but not in PPAR α -null mice (LoVerme et al., 2006). Moreover, when administered peripherally (intraperitoneally), the analgesic effects of PPAR ligands were selective for PPAR α , since low doses of PPAR β and PPAR γ agonists (GW501516 and ciglitazone, respectively), failed to reduce formalin-evoked pain behaviors (LoVerme et al., 2006). Finally, GW7647 and palmitoylethanolamide (PEA) reduced magnesium sulfate writhing behaviors within 15 minutes in wild-type, but not in PPAR α -null animals (LoVerme et al., 2006).

The PPAR α agonists GW7647 or PEA reduced both thermal and mechanical hyperalgesia in wild-type, but not in PPAR α -null neuropathic mice (LoVerme et al., 2006). In addition, PPAR α agonists reduce mechanical and thermal hyperalgesia in two models of chronic inflammation: (i) experimental arthritis elicited by intradermal injection of complete Freund's adjuvant (CFA) into the base of the tail; and (ii) paw edema induced by i.pl. injection of carrageenan (LoVerme et al., 2006). In all three chronic pain models, PPAR α agonists are effective when administered 30 minutes prior to pain testing, indicating they reduce pain behaviors through a rapid-response mechanism (LoVerme et al., 2006).

The exact non-genomic mechanism by which PPAR α agonists reduces pain remains unclear. However, rapid inhibition of peripheral wide dynamic range (WDR) sensory neurons through an engagement of large-conductance K_{ca} (BK_{ca}, K_{ca}1.1) or intermediate-conductance (IK_{ca}, K_{ca}3.1), calcium-activated potassium channels is likely to occur for the following reasons. First, intraplantarly administered PEA in mice, results in markedly elevated tissue levels of the drug in the injected paw, but remains unchanged in the brain and lumbar spinal cord, indicating that PPAR α agonists may inhibit pain behavior through a peripheral mechanism (LoVerme et al., 2006). Second, mouse dorsal root ganglia (DRG) (L4-L8) contain both PPAR α mRNA and PPAR α immunoreactivity in both small and large neurons within L5-L6 DRG (LoVerme et al., 2006). Third, administration of PPAR α agonists strongly attenuates the electrophysiological response of spinal nociceptive neurons to peripheral noxious stimuli (LoVerme et al., 2006). In rats, in which recordings were made from spinal wide dynamic range (WDR) neurons, whose receptive fields were localized on the plantar surface of the hind-paw, GW7647 and PEA rapidly reduce action potential firing within 2 min following their administration (LoVerme et al., 2006). Fourth, charybdotoxin and clotrimazole, two inhibitors of large-conductance K_{ca} channels (BK_{ca}, K_{ca}1.1) and intermediate-conductance K_{ca} channels (IK_{ca}, K_{ca}3.1) (Faber and Sah, 2003), prevented the antinociceptive actions of GW7647 and PEA in the formalin test (LoVerme et al., 2006). These data indicate that IK_{ca} and BK_{ca} cooperate in mediating the rapid antinociceptive response to PPAR α agonists.

2.2. Rapid analgesia through central PPAR γ receptors

Taylor and colleagues recently discovered that PPAR γ is transcribed, translated, and bound to DNA in the spinal cord (Churi et al., 2008). To test the hypothesis that activation of spinal PPAR γ decreases nerve injury-induced allodynia, they next tested the effects of 15-deoxy- $\Delta^{12,14}$ -PGJ2 (15d-PGJ2, a cyclopentanone prostaglandin), a naturally-occurring PPAR γ agonist (Soares AF), and the commercially-available synthetic ligand, rosiglitazone, on behavioral signs of peripheral neuropathic pain in the spared nerve injury (SNI) model (Decosterd and Woolf, 2000; Taylor et al., 2007). Both PPAR γ agonists dose-dependently decreased mechanical and cold hypersensitivity (Churi et al., 2008). These effects occurred within 5 min of injection (unpublished observations), were maximal at a dose of 100 μ g, and peaked at ~60 min. This rapid time course suggested a transcription-independent mechanism of action. Anti-allodynia resolved within 4 hr, arguing against a toxic action. To test whether the anti-allodynic effects of 15d-PGJ2 were PPAR γ receptor mediated, a PPAR γ antagonist, bisphenol A diglycidyl ether (BADGE) (Wright et al., 2000), was concurrently administered. BADGE significantly and dose-dependently reduced the anti-allodynic effect of 15d-PGJ2 (Churi et al., 2008). No adverse effects of BADGE or 15d-PGJ2/BADGE combinations were discernable. In animals without nerve injury, rosiglitazone did not alter motor coordination, von Frey threshold, or withdrawal response to a cool stimulus (Churi et al., 2008). Intraperitoneal and intracerebroventricular administration of PPAR γ agonists (only tested at the 100 μ g that was used in the i.t. studies) did not decrease mechanical and cold hypersensitivity, arguing against effects subsequent to diffusion from the intrathecal space (Churi et al., 2008). Very recent unpublished data from the Taylor laboratory indicates that the BBB-permeant PPAR γ agonist, pioglitazone, reduced neuropathic pain not only when administered by the intrathecal route, but also by a systemic (intraperitoneal) route of administration.

These results indicate that exogenous administration of PPAR γ agonists act in a dose- and PPAR γ -dependent manner to reduce allodynia and hyperalgesia. Does this mimic an intrinsic pain inhibitory, spinal PPAR γ system? To begin to address this question, Dr. Taylor's laboratory further analyzed the effect of PPAR γ receptor antagonist administration on SNI-induced mechanical allodynia in the rat. As illustrated in Figure 1, SNI reduced VF threshold, from 15 gm to approximately 2 gm. Importantly, intrathecal administration of BADGE (200 μ g) decreased VF threshold even further, to approximately 1 gm. BADGE did not decrease VF threshold at the contralateral paw or in sham animals (data not shown). These data suggest that PPAR γ antagonism increases allodynia, and that endogenous PPAR γ systems tonically inhibit neuropathic pain.

2.3. The role of GPR30 on sensory neurons

Various signaling pathways previously implicated to increase nociceptor sensitivity are activated by GPR30 in both cell expression systems and native cells, yet only one study has characterized the effects of GPR30 activation on mechanical hyperalgesia (Kuhn et al., 2008). Thus, in contrast to the considerable effort focused on ER α and ER β in pain research, limited studies have evaluated the expression pattern or role of GPR30 in modulating nociceptors.

The expression of GPR30 on sensory neurons has not been carefully examined in sensory tissues. Here, Drs. Fehrenbacher and Hargreaves examined the presence of GPR30 immunoreactivity in cultures derived from trigeminal ganglia prepared from female rats. Figure 2 illustrates GPR30 expression (green) in trigeminal cultures derived from female rats and localization of the receptor within sensory neurons (N52; blue), specifically within the nociceptive subpopulation of neurons, indicated by TRPV1 (red).

To evaluate a functional role for the GPR30 in modulating sensory neurons, Drs. Fehrenbacher and Hargreaves examined the effects of a newly developed selective agonist for the receptor, G-1 (Bologa et al., 2006), on calcium accumulation in TG cultures. Figure 3A illustrates that application of G-1 rapidly and substantially triggers increases in $[Ca^{2+}]_i$ in TG neurons cultured from female rats. The time-response curves are depicted separately for neurons which were responsive (red) or insensitive (blue) to G-1 application. The columns in the right panel demonstrate the net increase in $[Ca^{2+}]_i$ elicited by administration of G-1 (Net = Peak – Basal; induced by 5-minute treatment with 100 nM G-1). The application of G-1 evoked a rapid accumulation of intracellular calcium in 60% (54 of 90) of the studied neurons, with a mean net increase of ~70 nM $[Ca^{2+}]_i$ (Figure 3B).

2.4. Rapid actions of estrogens regulate inflammatory mediator signaling in trigeminal ganglion cultures

Both ER α and ER β subtypes are expressed in sensory DRG neurons and the trigeminal ganglion (TG) (Lorke et al., 2000). (Yang et al., 1998; Taleghany et al., 1999; Cui and Goldstein, 2000; Papka et al., 2001; Papka and Storey-Workley, 2002). More than 80% of peptidergic sensory neurons in the DRG of female rats express the ER α receptor, whereas ER β is expressed in only 46% of male DRG neurons (Yang et al., 1998). In addition, there is abundant evidence that estrogen modulates the responsiveness of a peptidergic subpopulation of nociceptors (Lee et al., 2002; Bennett et al., 2003; Evrard et al., 2003; Flores et al., 2003) and thereby would be expected to influence pain neurotransmission at the level of the primary sensory neuron.

Dr. Clarke's laboratory examined the actions of estrogen on primary cultures of trigeminal ganglion neurons obtained from ovariectomized female rats. The new results of Figure 4 indicate that a brief (15 min) pre-treatment with 17 β -estradiol (17 β -E $_2$) enhanced the cellular responses to the inflammatory mediators, bradykinin (BK) and prostaglandin E $_2$ (PGE $_2$). Figure 4A shows that 17 β -E $_2$ enhanced BK-stimulated inositol phosphate (IP) accumulation and PGE $_2$ -mediated cAMP accumulation in cultures derived from female rats. Importantly, pretreatment with the membrane-impermeable 17 β -E $_2$ conjugated to bovine serum albumin (17 β -E $_2$ -BSA) also enhanced this inflammatory mediator action.

To assess the physiological relevance of estrogen action, Dr. Clarke's laboratory measured the effect of local injection of 17 β -E $_2$ on bradykinin-induced heat allodynia at the rat hindpaw. Figure 4B shows that intraplantar pretreatment with 1 ng but not 100 ng of 17 β -E $_2$ prevented the heat allodynia normally associated with paw injection of BK (1 μ g, intraplantar). The effect of estrogen was restricted to the ipsilateral paw, suggesting a local site of action. Estrogen injection itself had no effect on paw withdrawal latency during the 15 min pretreatment period.

To address the possibility that ovariectomy-induced changes in physiological estrogen levels confounded the rapid effects of estrogen, TG cultures were exposed to a continuous (5 days) physiological concentration of 17 β -E $_2$ (within the range of concentrations found in rat plasma during the estrus cycle). Acute exposure to 17 β -E $_2$ (50 nM, for 15 min) enhanced BK-induced increases in intracellular calcium (data not shown). When TG cells were grown with 100 pM 17 β -E $_2$ for 5 days, the effect of BK itself was increased (suggesting a possible genomic action of estrogen) and acute treatment with 17 β -E $_2$ further enhanced the BK-induced calcium influx. Acute 17 β -E $_2$ did not alter calcium levels on its own. These results indicate that the acute effects of estrogen occur in the background of physiological concentrations of estrogen.

3. Discussion

3.1. PPAR α

As summarized above, PPAR α agonists reduce both acute and chronic pain in a number of pain assays in mice and rats. These effects are rapid and occur independently of and precede the transcriptional anti-inflammatory actions exerted by this receptor (LoVerme et al., 2005a; LoVerme et al., 2006). Moreover, GW7647 and PEA fail to reduce inflammatory edema during the 30 min incubation period following their administration to carrageenan-inflamed paws (LoVerme et al., 2006). These results further support the conclusion that ligand-activated PPAR α rapidly suppresses hyperalgesia prior to alleviating inflammation. The ability of PPAR α to simultaneously reduce inflammation through a genomic mechanism, while rapidly reducing nociception through a non-genomic mechanism, offers a unique opportunity to simultaneously address both the inflammatory and nociceptive components of pain. Moreover, these drugs do not appear to induce tolerance in animal pain studies (LoVerme et al., 2006) and the safe use of low-affinity PPAR α agonists in the clinic for unrelated disorders (Gross and Staels, 2007) highlights their potential therapeutic use.

3.2. PPAR γ

Substantial evidence has rapidly accumulated in the past 5 years indicating that PPAR γ activation effectively attenuates neurodegenerative and inflammatory processes in the central nervous system. Taylor and colleagues suggest that PPAR γ agonists may ultimately be useful for chronic pain as well, without blocking transient pain or producing major adverse effects. With the detection of PPAR γ mRNA, protein, and DNA binding in spinal cord extracts (Churi et al., 2008), and unpublished data demonstrating PPAR γ immunoreactivity in thin sections, Dr. Taylor and colleagues suggest that the dorsal horn supports the expression of PPAR γ signaling elements. Furthermore, they reported that intrathecal administration of rosiglitazone and 15d-PGJ2 (an endogenous PPAR γ ligand) rapidly reduce the mechanical and thermal hypersensitivity associated with nerve injury in a dose- and PPAR γ -dependent manner (Churi et al., 2008). More recent unpublished data extends these results to systemic administration of the BBB-permeant ligand, pioglitazone. Based on these results, the new analysis of Figure 1, and other unpublished data from their laboratory, Taylor and colleagues conclude that ligand-dependent activation of PPAR γ in the dorsal horn decreases behavioral signs of inflammatory and neuropathic pain, perhaps through disruption of injury-induced activation of spinal neurons and glia.

3.3. GPR30

Despite a plethora of potential mechanisms ascertained by studies in other tissues and cell expression systems, there is a lack of knowledge on the role of GPR30 in modulating nociceptors. Dr. Fehrenbacher and Hargreaves' studies provide strong support for the hypothesis that GPR30 can significantly increase the activity of peripheral nociceptors. The new data of Figures 2-3 concurs with the findings of Kuhn and colleagues, who demonstrated that activation of GPR30 by acute treatment with G-1 induces translocation of PKC ϵ toward the plasma membrane in cultures of dorsal root ganglia neurons derived from male rats (2008). In addition to these *in vitro* studies, Kuhn and colleagues also demonstrated that injection of G-1 or ICI 182,780 into the hindpaw elicits mechanical hyperalgesia. This sensitization was reversed by pretreatment with PKC ϵ -inhibitory peptide, ϵ V1-2, suggesting that the activation of PKC ϵ may be a component of both *in vitro* and behavioral actions of the GPR30 agonist (Kuhn et al., 2008). Collectively, these studies suggest that the GPR30 is expressed on sensory neurons, that activation of the receptor elicits signaling to increase calcium accumulation and PKC ϵ translocation in cultured neurons, and that this signaling may contribute to increased neuronal sensitivity as treatment with the GPR30 agonist induces hyperalgesia.

3.4. Estrogens and inflammatory mediators

Dr. Clarke's new data of Figure 4 indicate that estrogen can rapidly enhance the responsiveness of nociceptors to activation by inflammatory mediators. The mechanism of estrogen action is likely via a non-genomic pathway since the response occurred within 15 min and could be produced with a membrane-impermeable form of 17β -E₂. Functional RGD-binding integrins may be required for the rapid estrogen action in nociceptors, since soluble peptide inhibitors that block a particular subclass of integrins (those that bind a tri-amino acid sequence (Arg-Gly-Asp [RGD]; (Scarborough, 1999)), completely eliminate the effect of estrogen to enhance BK signaling in TG nociceptors (Clarke, unpublished data). Furthermore, integrin-blocking peptides injected locally in the rat hindpaw also blocked the effect of estrogen to enhance BK-induced thermal allodynia (Clarke, unpublished data).

4. Conclusions

Chronic pain management is a major scientific and health care challenge, as current analgesic drugs rarely provide sufficient efficacy in the absence of serious side effects. Our review of the literature, together with new data, leads us to conclude that activators of peroxisome proliferator-activated receptors and/or inhibitors of estrogen receptors may come to represent novel classes of analgesic drugs. For example, Dr. Taylor suggests that PPAR γ receptors in the spinal cord are potential therapeutic targets of BBB-permeant PPAR γ agonists for the treatment of inflammatory or neuropathic pain. Future experiments should be designed to further understand the general mechanisms underlying TZD- or PPAR- α -mediated inhibition of chronic pain, with the long-term goal of determining the therapeutic potential of these FDA-approved drugs or mixed PPAR α/γ agonist in humans.

Considerable evidence implicates estrogens as critical factors in sex-dependent differences in pain (Fillingim, 2000). An understanding of the effects of classical estrogen receptor activation via rapid signaling mechanisms on inflammatory mediator signaling in primary sensory neurons may provide a first step toward a basis for understanding the enhanced sensitivity of women to painful stimuli. Alternatively, the pharmacology of the GPR30 distinguishes it from the other estrogen receptors and thus may provide an opportunity for the development of therapeutics which can selectively reverse GPR30 activity without affecting the diverse actions of estrogens at the classical receptors.

5. Experimental Procedures

The PPAR α studies were performed and analyzed as previously described (LoVerme et al., 2005a; LoVerme et al., 2006), as were the PPAR γ studies (Churi et al., 2008).

Animals of the Estrogen Receptor studies

Adult intact female Sprague Dawley rats (200–250 g; Charles River Laboratories, Wilmington, MA) were used in this study. All animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at San Antonio and conformed to the International Association for the Study of Pain and federal guidelines. Animals were housed for 1 week before the experiments with food and water available *ad libitum*. OVX rats received injections of 17β -E₂ (1 or 100 ng, 50 μ l) into the hindpaw followed 15 min later with BK (25 μ g, i.pl., 50 μ l). Paw withdrawal latencies (PWL) were measured using the Hargreaves method (Hargreaves et al., 1988) at 5 min intervals for 20 min post-BK injection.

Rat TG primary cultures

Fehrenbacher and Hargreaves: Trigeminal ganglia were quickly removed after decapitation, and neuronal cultures were prepared as described previously (Fehrenbacher et al., 2005; Patwardhan et al., 2005). Cells were plated on poly-D-lysine/laminin-coated coverslips at a density of 2 ganglia per 6 coverslips and maintained at 37°C and 5% CO₂ for 5 days in modified DMEM containing 10% FBS, penicillin (50 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), 5-fluoro-2-deoxyuridine (3 µg/ml), uridine (7 µg/ml), and 100 ng/ml NGF (Harlan, Indianapolis, IN).

Clarke—As previously described (Berg et al., 2007a; Berg et al., 2007b), neurons from trigeminal ganglion were harvested from OVX female rats and cultured for 5 days. 24 hrs before experiments, cells were placed in NGF- and FBS-free medium. For BK experiments, cells were pre-labeled with ³H-myoinositol for 24 h prior to experiment. Cells were washed three times in HBSS containing 10 mM HEPES and 20 mM LiCl followed by a 15 min incubation in the absence (DMSO, 0.01%) or presence of 17β-estradiol (50 nM). Total inositol phosphate (IP) accumulation in response to BK (1 µM) was measured in the presence of 20 mM LiCl for 30 min. For PGE₂ experiments, cells were treated with 17β-estradiol (50 nM, 15 min) or vehicle (DMSO, 0.01%) followed by PGE₂ (1 µM) for 30 min. cAMP accumulation was measured with RIA.

Immunohistochemistry

Cultured TG cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and then blocked with 10% normal goat serum (30 min each step). Cells were then incubated overnight at 4°C with a rabbit polyclonal antibody directed against GPR30 (Abcam, Cambridge, MA), a guinea pig anti-TRPV1 antisera (NeuroMics, Bloomington, MN), and a mouse anti-N52 antibody (Sigma, St. Louis, MO). Immunoreactivity was detected using appropriate secondary antibodies conjugated to Alexa-488, Alexa-594, or Alexa-633 (Invitrogen, Carlsbad, CA). Images were acquired using a Nikon (Melville, NY) E600 microscope. Images were analyzed using MetaMorph software (version 4.5 r6; Universal Imaging Corporation, West Chester, PA).

Calcium imaging

TG cells cultured on coverslips were loaded with the cell-permeable calcium-sensitive dye fura-2 AM (1 µg/ml) (Invitrogen) for 30 min at 37°C in SES of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH 7.4. Coverslips containing cells were placed in a chamber with constant infusion of SES. Images were detected by a Nikon Eclipse TE-2000 microscope fitted with a 20×/numerical aperture 0.75 Fluor objective. Fluorescence images were collected in 5 s intervals throughout the experiment and analyzed, and the F_{340}/F_{380} ratio was calculated by the Metafluor software (MetaMorph; Universal Imaging Corporation). The GPR30 agonist, G-1, was delivered locally to the cells. The magnitude of calcium influx was determined by subtracting the averaged baseline 30 s before G-1 application from the peak achieved by the G-1 stimulation for each cell (ratiometric method, $\Delta F_{340}/F_{380}$).

Data analysis

Calcium influx data are presented as percentage of basal levels (mean ± SEM). Data were analyzed using Prism software version 4 (GraphPad Software, San Diego, CA). The results were analyzed using two tailed *t* test. The statistical significance was tested at *p* < 0.05.

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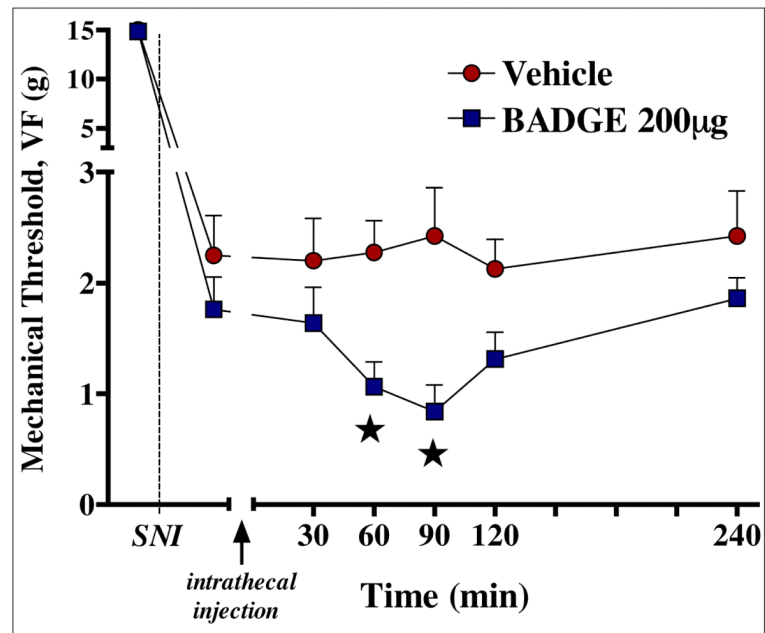


Figure 1. PPAR γ tonically inhibits neuropathic pain

Intrathecal administration of BADGE (200 μ g, n=8) but not DMSO vehicle (n=4) enhanced the drop in VF threshold associated with spared nerve injury. $F(1,70)=24$, $p<0.001$ by 2-way ANOVA. * $p<0.05$ by post-hoc tests. Error bars represent SEM. *Contributed by B. Taylor.*

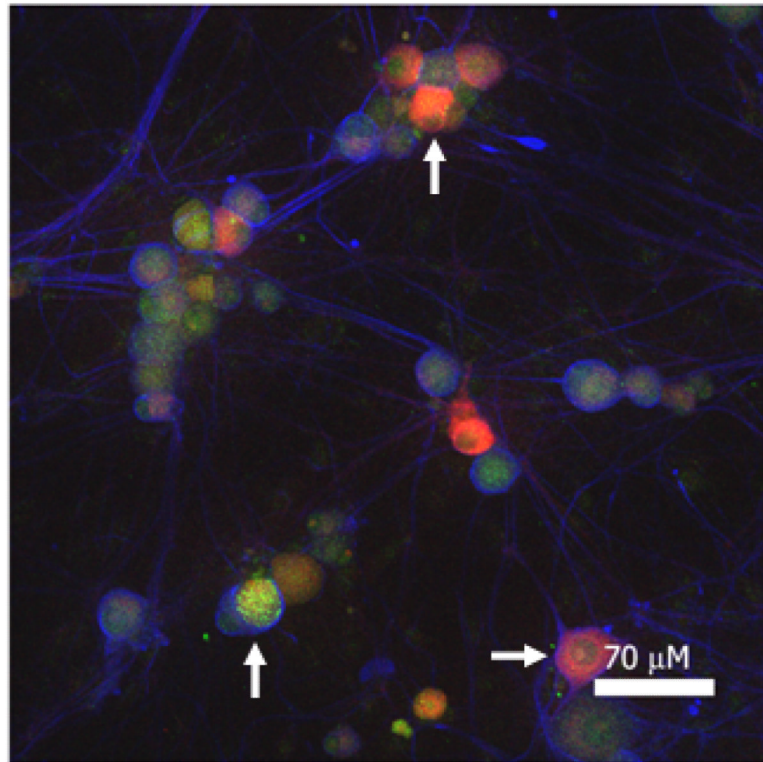


Figure 2. Neuronal expression (indicated by N52 staining, blue) of the GPR30 receptor (green) in trigeminal ganglia cultures derived from female rats
Arrows highlight GPR30-positive neurons which colocalize with TRPV1 (red). *Contributed by Fehrenbacher and Hargreaves.*

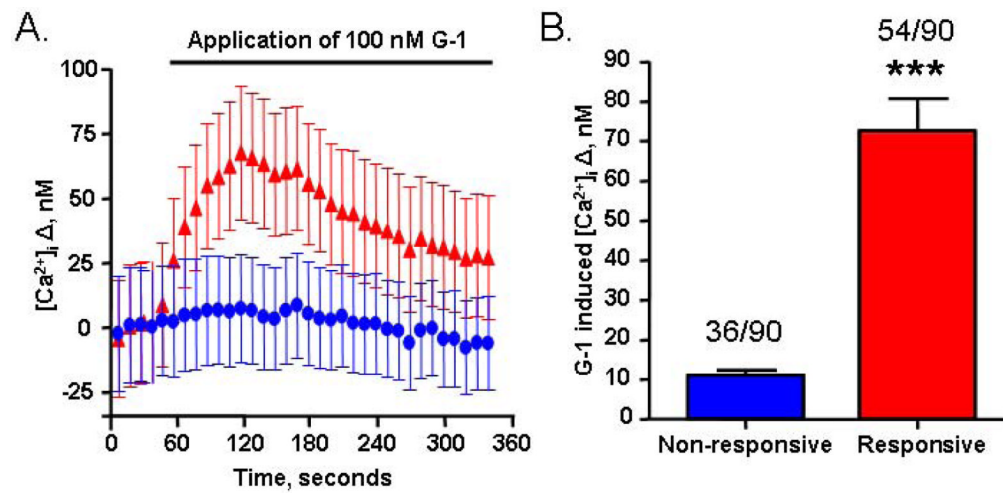


Figure 3. G-1 (100nM) increases the intracellular concentration of calcium in female TG neurons in culture

A. Time course of G-1-induced increases in calcium. All cells received the same treatment, but only those indicated by red ($n=54$) were responsive to G-1. In B, the average basal Ca^{2+}_i was subtracted from the peak Ca^{2+}_i to determine the extent of calcium mobilization by G-1 in both responsive and non-responsive cells. An asterisk indicates significant differences between responsive and non-responsive cells; $p<0.0001$ using t-test. *Contributed by Fehrenbacher and Hargreaves.*

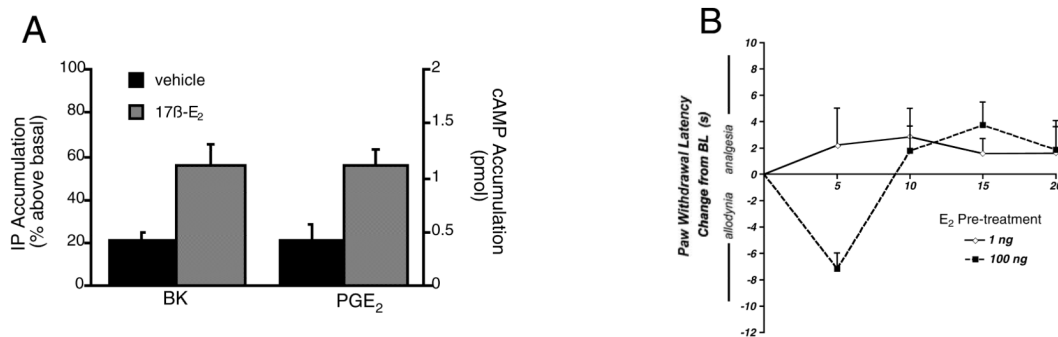


Figure 4. The effect of 17β-E₂ on cellular responses to BK and PGE₂ in TG nociceptors in culture (A) and on the time-course of BK-induced thermal allodynia *in vivo* (B)

Data shown in (A) represent the mean ± SD of 2 experiments measuring total inositol phosphate (IP) accumulation in response to BK (1 μM) in the absence (DMSO vehicle) or presence of 17β-estradiol. Data shown in (B) represents paw withdrawal latency to heat in response to bradykinin when tested 15 min after the injection of 17β-estradiol (1 or 100 ng).

Contributed by W. Clarke.