STIMULATING GLIAL G_q-COUPLED GPCR PATHWAYS BLOCKS ACUTE PAIN AND ITCH.

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ABSTRACT

SUZANNE KURTZER MINTON: Stimulating G_q GPCRs in GFAP-positive glia blocks acute pain and itch
(Under the direction of Ken D. McCarthy)

Glial G protein-coupled receptor (GPCR) pathways have been linked to synaptic modulation. However, their roles in pain and itch processing are unknown and have been difficult to study because glia and neurons often express overlapping GPCRs. The transgenic mouse model glial fibrillary acidic protein-Designer Receptor Exclusively Activated by a Designer Drug (GFAP-DREADD) is a useful model for selectively stimulating G_q-GPCR pathways on GFAP-positive cells. G_q-DREADD is expressed in astrocytes, but not neurons, in the spinal cord. Imaging studies demonstrated that G_q-DREADD stimulation causes calcium increases in astrocytes in lamina II and V of the spinal cord in slices from G_q-DREADD mice but not in those from wild-type mice. Stimulating G_q-DREADD caused a long-lasting antinociceptive phenotype for G_q-DREADD mice but not for wild-type mice as measured using the Hargreaves test. Selective stimulation of spinal cord and DRG G_q-DREADD was sufficient to mediate this effect. Surprisingly, removal of the IP_3R2 receptor did not affect G_q-DREADD-mediated antinociception, suggesting that stimulation of GFAP-positive G_q-GPCR pathways can inhibit pain transmission in an IP_3R2-independent manner. C-fos has been used as a marker of neuronal activity in response to noxious stimuli. G_q-DREADD stimulation did not alter the ability of noxious stimuli to induce c-fos in the dorsal horn. Nociceptors make their central projections in the dorsal horn, and a decrease in

excitatory neurotransmission at this synapse would be antinociceptive. We examined this hypothesis by performing dorsal root stimulation experiments and found that G_q-DREADD stimulation did not modulate evoked field potentials in lamina II. The neurocircuitry that is used in pain processing has a large degree of overlap with itch-sensitive neurocircuitry. G_q-DREADD stimulation was also shown to block histamine-dependent itch, and this phenotype appears to be peripherally mediated. Collectively, our experiments indicate that G_q-GPCR pathways in GFAP-positive glia can be antinociceptive in the setting of acute pain and can mediate antinociception in an IP_3R2-independent manner. In addition, G_q-GPCR pathways in astrocytes can block acute itch. These data provide insight into the roles that glial G_q-GPCR pathways play in the modulation of acute pain and itch, which may lead to the development of novel therapies for chronic pain and itch neuropathies.

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AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
Ach	Acetylcholine
ACM	Astrocyte conditioned media
ACSF	Artificial cerebral spinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
AD	Atopic dermatitis
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CIP	Congenital insensitivity to pain
CCD	Chronic compression of the DRG
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CNO	Clozapine N-oxide

CNS	Central nervous system
Cx	Connexin
DAG	Diacylglyercol
DHPG	(S)-3,5-Dihydroxyphenylglycine
DREADD	Designer receptor exclusively activated by a designer drug
DRG	Dorsal root ganglia
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
fEPSP	Field excitatory post synaptic potential
FMRF	Phe-Met-Arg-Phe peptide
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GAP	GTPase activating protein
GAT	GABA transporter
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein

GFP	Green fluorescent protein
GLAST	Glutamate Aspartate Transporter
GLT-1	Glutamate transporter-1
G protein	Heterotrimeric guanine nucleotide-binding protein
GPCR	G-protein coupled receptor
GRPR	Gastrin-releasing peptide receptor
GTP	Guanosine-5'-triphosphate
HA	Human influenza hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5-HT	5-hydroxytryptamine (serotonin)
HRP	Horseradish peroxidase
Hz	Hertz
IL-1β	Interleukin-1 beta
IL-10	Interleukin-10
I.P.	Intraperitoneal
IP_3	Inositol 1,4,5-trisphosphate
IP_3R2	Inositol 1,4,5-trisphosphate receptor type 2
I.T.	Intrathecal
JNK	c-Jun N-terminal kinase

kB	Kilobase
Kir	Inward rectifying potassium channel
KO	Knock out
KOR	Kappa opioid receptor
LPS	Lipopolysaccharide
LTP	Long term potentiation
M3 AchR	Muscarinic acetylcholine receptor M3
MCP-1	Monocyte chemoattractant protein-1
mGluR	Metabotropic glutamate receptor
mM	Millimolar
MrgA1	Mas-related genes receptor A1
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NMDA	*N*-methyl-$_D$-aspartic acid
NS	Nociceptive-specific
NSAIDs	Non-steroidal anti-inflammatory drugs
PAG	Periaquaductal gray
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PET	Positron emission tomography
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PHI	Postherpetic itch
PHN	Postherpetic neuralgia
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PMA	Premotor area
PNS	Peripheral nervous system
RASSL	Receptor activated solely by a synthetic ligand
RhoGEF	Ras homology guanine nucleotide exchange factors
RIPA	Radio immunoprecipitation assay
ROI	Region of interest
RVM	Rostroventral medulla
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SC	Schaffer collateral
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sEPSC	Spontaneous excitatory post-synaptic current

SFPN	Small-fiber polyneuropathy
sIPSC	Spontaneous inhibitory post-synaptic current
SG	Substantia gelatinosa
SGC	Satellite glial cells
SMA	Supplementary motor area
SMB	Scopolamine methyl bromide
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SR101	Sulfarhodamine 101
SSRI	Selective serotonin reuptake inhibitor
SV40	Simian virus 40
TBST	Tris-buffered saline with Tween 20
TNF-α	Tumor necrosis factor-alpha
TTX	Tetrodotoxin
VAS	Visual Analog Scale
WDR	Wide dynamic range
WT	Wild type

CHAPTER 1.

THE ROLE OF ASTROCYTES IN NEUROPHYSIOLOGY

1.1. A BRIEF HISTORY OF ASTROCYTE BIOLOGY

Our understanding of astrocytes and their role in neurophysiology has expanded tremendously over the last 175 years. Prior to the mid-19th century, conventional wisdom stated that the central nervous system contained no connective tissue. But when the pathologist Virchow detected inflammation in the brain— an early observation of what would later be called "reactive astrogliosis" — he recognized that this could not be accounted for by neurons alone (Schmidt and Weber, 2006). In 1856 he coined the term "neuroglia," writing that "this connective substance forms in the brain, in the spinal cord, and in the higher sensory nerves a sort of putty (neuroglia) in which the nervous elements are embedded" (Somjen, 1988). Astrocytes are a type of neuroglia, a category that also includes oligodendrocytes, NG2 glia, and microglia.

Astrocytes comprise nearly half the cells in the brain (Ullian et al., 2001), and they are more than "putty." Golgi started the modern study of glia by establishing that one may reliably distinguish glia from neurons by the lack of an axon. He also proposed that astrocytes provide nutritive support to neurons. Astrocytes are so named because their numerous fine bushy processes, which are distinct from the morphology of neurons or other glial cells, reminded early researchers of stars. In the early 20th century, Lugaro proposed that astrocyte processes contact both synapses and the vasculature (Somjen, 1988). The unique morphology of astrocytes plays a crucial role in their ability to perform many of their

functions in the central nervous system. This dissertation will further our understanding of the role and function of astrocytes.

1.2. ASTROCYTE MORPHOLOGY

1.2.1. Astrocytes ensheath synapses.

An astrocyte possesses a small soma and several thick processes from which arise numerous fine processes. Unlike neurons, astrocytes do not have axons or dendrites, nor do they generate action potentials. Astrocytes can be divided into two major categories: grey matter astrocytes (protoplasmic astrocytes) and white matter astrocytes (fibrous astrocytes) (Miller and Raff, 1984).

The classic astrocyte marker, GFAP, is a component of their thick processes; staining for GFAP highlights the classic "star-like" appearance of astrocytes (Bignami et al., 1972). However, GFAP immunostaining does not fully label the vast network of fine astrocyte processes. Astrocyte fine processes envelop both the pre-synaptic and post-synaptic elements of the synapse— a concept known as the tripartite synapse (Araque et al., 1999; Peters et al., 1976). A single astrocyte can envelope 100,000 synapses (Bushong et al., 2002). As such, these cells are well-positioned to modulate synaptic transmission.

Astrocyte processes show spontaneous motility at synaptic regions (Hirrlinger et al., 2004) as well as activity-dependent changes in synaptic ensheathment (Cornell-Bell et al., 1990a). The extent to which astrocytes ensheath synapses varies by region within the CNS. For example, astrocyte processes ensheath 57% of CA1 hippocampal synapses (Ventura and Harris, 1999). By contrast, only 29% of neocortical synapses have astrocyte ensheathment (Spacek, 1985). Haydon and coworkers have proposed a "synaptic island" model wherein a single astrocyte can coordinate the activity of groups of neurons through its contacts with

neighboring neuronal soma and dendrites (Halassa et al., 2007). In addition to making contacts with synapses, astrocytes also help compose and maintain the blood-brain barrier (BBB), which separates the circulatory system from the CNS.

1.2.2. Astrocytes contact the cerebral vasculature.

The German microbiologist and pharmacologist Paul Ehrlich discovered the BBB more than a century ago. He observed that when animals were injected with aniline dyes, staining would occur in all organs except for the brain. The BBB is composed of endothelial cells, which line the cerebral vasculature. The function of the BBB is to regulate ion gradients, facilitate nutrient transport, and shield the brain from potentially harmful substances (Hawkins and Davis, 2005). Endothelial cell permeability is restricted by the presence of high-resistance tight junctions (TJ) (Reese and Karnovsky, 1967). Astrocyte endfeet envelope CNS capillaries that compose the BBB (Risau and Wolburg, 1990; Davson and Oldendorf, 1967).

During development, astrocytes provide factors important to the induction of the BBB (Janzer and Raff, 1987). Stewart and Wiley showed that avascular brain tissue transplanted into the coelomic cavity developed vasculature with BBB characteristics whereas avascular somite grafts transplanted into the cerebral ventricles developed leaky vasculature (Stewart and Wiley, 1981). This suggested that neural tissue provides some factor that is crucial for BBB development, but it was still unclear which neural tissue cells provided the crucial factor. Further evidence that astrocytes help induce the BBB came from Janzer and Raff who showed that injecting astrocytes into the anterior chamber of rat eyes formed aggregates containing non-leaky vasculature (Janzer and Raff, 1987). Astrocytes release several factors to induce the BBB including SSeCKS (Lee et al., 2003), TGF-β (Tran et al., 1999), and

GDNF (Igarashi et al., 1999). Astrocytes are involved in functional hyperemia: the coordination of blood flow to meet neuronal metabolic demands. Astrocytes are well positioned to regulate functional hyperemia by virtue of their extensive contact with the BBB and with synapses.

1.2.3. Astrocytes couple to form cellular networks.

While neurons often have extensive overlap in their dendritic arbors, astrocytes occupy largely separate anatomical domains (Bushong et al., 2002). Astrocytes exchange information via an extensive network of gap junctions: specialized intercellular connections that directly connect the cytoplasm of two cells (Giaume and McCarthy, 1996). Dye-coupling experiments illustrate the extent of astrocyte gap junction coupling. When an individual astrocyte in a brain slice is loaded with a low-weight fluorescent dye, dye diffusion through gap junctions labels tens to hundreds of neighboring astrocytes (Duffy and MacVicar, 1995; Gutnick et al., 1981).

A gap junction is a cellular channel composed of two facing hemichannels, each of which contains a ring of six connexin (Cx) protein subunits (Caspar et al., 1977). Cx43 and Cx30, the major connexins in astrocytes (Dermietzel et al., 1991; Kunzelmann et al., 1999), are enriched at astrocytic perivascular endfeet (Simard et al., 2003). Gap junctions are permeable to ions and small (<1000 Da) signaling molecules such as ATP, inositol 1,4,5-trisphosphate (IP_3), calcium, glutamate, glucose, and lactate (Lawrence et al., 1978). Astrocyte gap junctions have also been implicated in pathological conditions such as mirror image pain, which is the spread of pain to the contralateral side from the neuropathic lesion (Milligan et al., 2003). Carbenoxolone, a gap junction decoupler, blocks neuropathy-induced

mirror image pain; this suggests that astrocytes mediate the spread of mirror image pain via gap junctions and are involved in pain processes (Spataro et al., 2004).

1.3. ASTROCYTE FUNCTIONS

1.3.1. Astrocytes support neurons metabolically.

One of their earliest proposed functions for astrocytes was supplying nutrients to neurons. Although the brain composes only 2% of the body by weight, it consumes 20% of the body's metabolic energy (Rolfe and Brown, 1997). Glucose is the major energy source for neurons in the central nervous system. Astrocytes provide metabolic support to neurons in an activity-dependent manner (Pellerin and Magistretti, 1994). During neuronal activity, astrocytes take up glucose from circulation (Kacem et al., 1998) and metabolize it to lactate or glycogen, which is then trafficked through astrocyte networks via gap junctions in order to sustain synaptic transmission and neuronal survival (Rouach et al., 2008; Pentreath and Kai-Kai, 1982; Tsacopoulos and Magistretti, 1996).

In addition to regulating glycogen, glucose, and lactate, astrocytes also regulate glutamate, which is the major excitatory neurotransmitter (Nicholls and Attwell, 1990). Excessive extracellular glutamate leads to excitotoxicity; astrocytes prevent excitotoxicty by controlling the concentration of extracellular glutamate (Brew and Attwell, 1987). In the glutamate-glutamine cycle, astrocytes take up extracellular glutamate via the glutamate transporter-1 (GLT-1), metabolize it to glutamine by glutamine synthetase, and then release glutamine to neurons as a precursor for glutamate synthesis (Schousboe et al., 1997). Meanwhile, GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter (Krnjevic and Schwartz, 1967). Since GABA is synthesized from glutamate, the glutamate-glutamine cycle in astrocytes is also important for supporting inhibitory neurotransmission (Kaufman et

al., 1986). In addition to supporting the metabolic activity of neurons, astrocytes are crucial in regulating the ionic and neurotransmitter content of the extracellular space.

1.3.2. Astrocyte buffering of potassium and neurotransmitters.

Astrocytes help maintain homeostasis by regulating potassium, glutamate, and GABA. Neurons release potassium into the extracellular space during normal neurotransmission. Excessive extracellular potassium leads to neuronal depolarization, hyperexcitability, and seizures (Wang and Bordey, 2008). Astrocytes import potassium released into the extracellular space during normal neuronal activity (Kuffler and Nicholls, 1966) and redistribute potassium through the astrocyte syncytium via gap junctions (Orkand et al., 1966). Due to their extensive contacts with the cerebral vasculature, astrocytes can also release excess potassium into the blood stream (Newman, 1986).

Potassium buffering by astrocytes is achieved using passive inward rectifying potassium (Kir) channels as well as electrogenic sodium-potassium ATPases (Ballanyi et al., 1987). In inwardly rectifying potassium channels, the inward flow of potassium ions is greater than the outward flow (Nichols and Lopatin, 1997). Kir4.1 is a glial-specific inwardly rectifying potassium channel (Takumi et al., 1995). The importance of astrocyte potassium buffering is illustrated by the conditional knockout of Kir4.1 in GFAP-positive glia, which results in severe ataxia, seizures, and early death (Djukic et al., 2007).

In addition to potassium buffering, astrocytes regulate extracellular levels of glutamate and GABA. Glutamate, GABA, and other neurotransmitters are released with potassium during neurotransmission; astrocytes express transporters that take up neurotransmitters during synaptic activity (Huang et al., 2004; Shigetomi et al., 2011). Glutamate transporters work by importing glutamate with sodium while extruding potassium (Storck et al., 1992).

Astrocytes express the glutamate transporters GLT-1 and GLAST (Glutamate Aspartate Transporter) (Storck et al., 1992; Pines et al., 1992). GLT-1 and GLAST are differentially expressed in the CNS. GLT-1 is primarily restricted to astrocytes in adults. By contrast, GLAST is expressed by radial glia and astrocytes during early development but is downregulated in adults (Regan et al., 2007).

Astrocytes regulate GABA concentrations via GABA transporters (GATs); they have been shown to express GAT-1 and GAT-3 (Brecha and Weigmann, 1994; Yan and Ribak, 1998). GAT-3 in astrocytes regulates inhibitory synapses by controlling extrasynaptic GABA concentrations (Shigetomi et al., 2011). In conclusion, astrocytes are essential to maintaining CNS homeostasis by buffering substances released by neurons.

1.4. ASTROCYTES REGULATE SYNAPSE FORMATION AND ELIMINATION.

Sherrington proposed the concept of the "synapse"- a structure that allows a neuron to pass an electrical or chemical signal to another cell. The ability to form synapses was thought to be intrinsic to neurons (Ullian et al., 2004). Evidence that neurons alone were not sufficient to mediate synaptogenesis first came from the observation of a week-long delay between axons reaching their target and synapse formation, indicating that some other cell type had to be involved in synaptogenesis (Lund and Lund, 1972).

Astrocytes are involved in inducing and stabilizing CNS synapses (Ullian et al., 2001). While neurons cultured alone form very few synapses, the addition of a feeder layer of astrocytes or astrocyte-conditioned media (ACM) to the neuronal culture leads to high levels of functional synapses (Pfrieger and Barres, 1997). ACM's ability to promote synaptogenesis suggests that an astrocyte-secreted factor is involved. Indeed, later work showed that astrocytes secrete thrombospondins and cholesterol to promote synapse formation

(Christopherson et al., 2005; Mauch et al., 2001). Thrombospondins mediate synaptogenesis by binding neuronal α2δ-1 receptors (Eroglu et al., 2009). Synapse elimination is also crucial during development (Katz and Shatz, 1996); astrocytes trigger complement-mediated synapse elimination by releasing an unidentified molecular signal (Stevens et al., 2007).

1.4.1. CNS injury causes gliosis.

Astrocytes perform all of the above functions as part of normal physiology. During injury, astrocytes undergo reactive gliosis (or astrogliosis). Reactive astrocytes undergo hypertrophy, proliferate, and upregulate markers such as GFAP (Maxwell and Kruger, 1965; Latov et al., 1979).

Reactive astrocytes are compromised in their ability to perform many of their normal physiological functions. For instance, glutamine synthetase expression is downregulated in reactive astrocytes in patients suffering from temporal lobe epilepsy (Eid et al., 2004). *In vivo* microdialysis studies have shown that extracellular glutamate levels rise to neurotoxic concentrations just before a seizure and remain elevated for at least fifteen minutes after seizure cessation (During and Spencer, 1993). Decreased glutamine synthetase disrupts the glutamate-glutamine cycle causing elevated extracellular glutamate; this can lead to seizures.

Electrophysiological studies show that inducing gliosis *in vitro* causes astrocytes to switch their potassium channel expression from inwardly rectifying to outwardly rectifying (MacFarlane and Sontheimer, 1997). Kir channels are key to astrocyte potassium buffering (Ransom and Sontheimer, 1995). Gliosis results in decreased Kir expression, which could explain why gliotic tissue in an experimental model of epilepsy had impaired potassium buffering (Lewis et al., 1977).

Astrogliosis occurs in a wide range of neuropathological conditions including epilepsy (Wetherington et al., 2008), Alzheimer's disease (Dickson et al., 1988; Schechter et al., 1981), and chronic pain (Garrison et al., 1991; Tanga et al., 2004). It is controversial whether astrogliosis is detrimental or beneficial. Preventing gliosis with glial metabolic inhibitors blocks chronic pain (Meller et al., 1994). This suggests that astrogliosis is detrimental. By contrast, ablation of reactive astrocytes in a mouse model of traumatic brain injury exacerbated neural injury suggesting a beneficial role for gliosis (Myer et al., 2006). The observation that astrogliosis occurs in so many different kinds of neurological disorders suggests that glia may prove to be valuable therapeutic targets. The development of therapeutics that target glia will require a better understanding of glial cell signaling.

1.5. ASTROCYTE GPCRS RESPOND TO AND MODULATE NEURONAL ACTIVITY.

1.5.1. Review of heterotrimeric guanine nucleotide-binding protein (G-protein) coupled receptor (GPCR) pathways.

GPCRs comprise a superfamily of receptors that bind ligands to activate intracellular signaling cascades (Stoddard et al., 1992). GPCRs are activated by a variety of ligands including hormones, neurotransmitters, odorants, and even photons (Kristiansen, 2004). The importance of GPCRs is reflected by the fact that roughly 30% of all pharmaceuticals work by modulating GPCRs (Hopkins and Groom, 2002).

The human genome contains an estimated 900 GPCRs (Besnard et al., 2012). They regulate a wide range of physiological processes including metabolism, neurotransmission, growth, and visual perception (Wong, 2003). While the primary amino acid sequence of GPCRs varies widely, all GPCRs share certain structural features. Solving the crystal structure for rhodopsin confirmed that GPCRs are composed of seven-transmembrane α-

helices separated by three intracellular and three extracellular loops; the helices are oriented with an extracellular N-terminus and an intracellular C-terminus (Palczewski et al., 2000). GPCRs undergo cycles of activation and inactivation (Iiri et al., 1998). In the inactive state, the heterotrimeric G-protein is composed of tightly associated GDP (guanosine-5'-diphosphate)-bound Gα and Gβγ subunits. Upon ligand binding, GPCRs undergo a conformational change, catalyzing Gα to exchange GDP for GTP (guanosine-5'-triphosphate) (Rhee, 2001). Then, GTP-bound Gα and Gβγ dissociate from the GPCR and activate downstream signaling effectors. Hydrolysis of Gα-GTP to Gα-GDP causes the reassociation of the heterotrimeric G-protein with the GPCR.

Heterotrimeric G proteins signal through four classes of Gα proteins: $G_{s/olf}$, $G_{i/o}$, $G_{12/13}$, and G_q (Cabrera-Vera et al., 2003). $G_{s/olf}$ stimulates adenylyl cyclase (AC) whereas $G_{i/o}$ inhibits it (Taussig and Zimmermann, 1998). $G_{12/13}$ activates RhoGEFs (Ras homology guanine nucleotide exchange factors) (Hart et al., 1998). The G_q subfamily contains four types of α-subunits ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$) (Simon et al., 1991); they all stimulate phospholipase C β (PLC-β) (Knall and Johnson, 1998); $G\alpha_{q/11}$ has also been shown to activate p63RhoGEF (Rojas et al., 2007; Lutz et al., 2005). While early work focused on Gα signaling, Gβγ also activates signal transduction cascades (Clapham and Neer, 1997).

In the late 1950s, Sutherland and Rall were the first to characterize how GPCRs activate second messenger cascades. They showed that stimulation of various tissues with epinephrine or glucagon (both GPCR agonists) promoted the production of cyclic adenosine monophosphate (cAMP) by AC (SUTHERLAND et al., 1962; RALL and SUTHERLAND,

1958; SUTHERLAND and RALL, 1958). Mammals express at least nine different isoforms of AC that vary in their pattern of tissue expression and regulation (Simonds, 1999).

The production of cAMP by AC can be inhibited as well as stimulated. G_i-coupled GPCRs inhibit AC (Rodbell, 1995; Hildebrandt et al., 1983). Pertussis toxin catalyzes the ADP-ribosylation of G_i, which prevents GPCR coupling and inhibition of AC (Gilman, 1995).

Heterotrimeric G proteins and monomeric Ras GTPases both share the feature of being inactive in the GDP-bound state and active in the GTP-bound state (Vetter and Wittinghofer, 2001). Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP to generate the active Ras GTPase (Schmidt and Hall, 2002). Ras GTPase signaling is terminated via GTPase activating proteins (GAPs), which accelerate the intrinsic GTPase activity of Ras family members.

The Ras-related small GTPase protein super family contains the Rho GTPase sub family (Jaffe and Hall, 2005). The human Rho GTPase sub family contains 22 members; the most extensively characterized members of this family are RhoA, Rac1, and CDC42 due to their ability to cause striking changes in cell morphology upon activation (Rojas et al., 2007). RhoGTPases have been implicated in a variety of cellular responses including gene transcription, embryogenesis, and actin cytoskeletal dynamics (Hall, 1998).

Stimulation of $G_{12/13}$-coupled GPCRs leads to $G\alpha$-GTP binding of the RH domain of RhoGEF (Kozasa et al., 2011). This induces a conformational change in RhoGEF, activating it (Hart et al., 1998). Activated RhoGEF can then activate the RhoGTPase by promoting the release of bound GDP, which then allows the subsequent binding of GTP (Schmidt and Hall, 2002).

Most GPCRs that couple to $G_{12/13}$ also couple to G_q (Kozasa et al., 2011). G_q-coupled GPCRs stimulate PLC-β, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and diacylglycerol (DAG) (Smrcka et al., 1991; Taylor et al., 1991). IP_3 binds IP_3 receptors on the endoplasmic reticulum (ER) and induces calcium release (Streb et al., 1983). PLC-β promotes the termination of G_q-GPCR signaling complexes by stimulating GTP hydrolysis of the GTP-bound G_q (Harden et al., 2011).

While PLC-β has been thought of as being the canonical G_q effector, evidence from the last decade has shown that G_q-GPCRs are also potent activators of p63RhoGEF (Rojas et al., 2007; Lutz et al., 2005). Northern blot analysis and immunohistochemistry suggest that p63RhoGEF is expressed in human heart and brain tissue (Lutz et al., 2004; Souchet et al., 2002). Several groups of investigators have shown that p63RhoGEF activates the small GTPase RhoA, but not Rac1 or CDC42 (Lutz et al., 2004; Souchet et al., 2002).

Astrocytes express GPCRs that signal through all four classes of Gα proteins. Astrocyte signaling via three of those classes— G_s, G_i, and $G_{12/13}$-coupled GPCRs— is poorly understood. Astrocytic G_q-GPCRs have been the most extensively studied because of the early development of calcium indicator dyes that allow the monitoring of cytoplasmic calcium concentrations in real time (Tsien, 1988).

Unlike neurons, astrocytes are electrically non-excitable. Instead, they employ a range of second messengers, including calcium, in their signaling cascades (Cornell-Bell et al., 1990b). In 1996, Porter and McCarthy demonstrated that hippocampal astrocytes respond to synaptically released glutamate with calcium increases (Porter and McCarthy, 1996). Astrocytes not only respond to neuronal activity; they modulate neuronal activity via calcium

elevations that cause the release of gliotransmitters including ATP (Guthrie et al., 1999), glutamate (Parpura et al., 1994), and D-serine (Schell et al., 1995).

Astrocytic release of gliotransmitters has been proposed to modulate several forms of synaptic transmission including heterosynaptic depression, inhibitory neurotransmission, and Long Term Potentiation (LTP, the sustained enhancement of excitatory synaptic transmission associated with memory and learning (Bliss and Lomo, 1973; Pascual et al., 2005; Kang et al., 1998). Astrocyte-mediated heterosynaptic depression has been suggested to occur in the hippocampus (Zhang et al., 2003). High frequency stimulation of the Schaffer collateral-pyramidal neuron synapse induces calcium increases in an interposed astrocyte, which then releases ATP. ATP is converted to adenosine, which binds presynaptic adenosine A1 receptors at a neighboring synapse, causing heterosynaptic depression.

Astrocytes also have been reported to potentiate inhibitory synaptic transmission in the hippocampus (Kang et al., 1998). GABA release from inhibitory neurons binds $GABA_B$ receptors on astrocytes. This increases astrocytic calcium and causes astrocytes to release glutamate onto the GABAergic interneuron, increasing inhibitory drive.

Hippocampal LTP depends on activation of post-synaptic N-methyl-$_D$-aspartic acid (NMDA) receptors in the presence of a co-agonist— either glycine or D-serine (Johnson and Ascher, 1987; Bashir et al., 1990). LTP induction in the hippocampus and somatosensory cortex may depend on astrocyte calcium increases stimulating the release of D-serine (Henneberger et al., 2010; Takata et al., 2011).

Despite these findings, gliotransmission is a controversial issue. Our lab has published several papers that question whether gliotransmission occurs under physiological conditions

(Agulhon et al., 2010; Fiacco et al., 2007; Petravicz et al., 2008). Figure 1 shows the tripartite synapse and astrocytic release of gliotransmitters.

Astrocyte calcium increases have also been linked to control of cerebrovascular tone (Straub and Nelson, 2007). Over a century ago, Sherrington posited the notion of functional hyperemia: the coupling of temporal and spatial regulation of blood flow to support neuronal activity (Roy and Sherrington, 1890). Astrocytes are well-positioned to modulate blood flow in the CNS due to their extensive contacts with the vasculature (Newman, 1986). Moreover, the purinergic G_q-coupled GPCRs, $P2Y_2$ and $P2Y_4$ are highly expressed at astrocyte endfeet (Simard et al., 2003). Arteriole dilation triggered by neuronal activity depends on astrocyte calcium increases (Zonta et al., 2003). A major caveat of research linking astrocyte calcium increases to gliotransmission and modulation of the cerebrovasculature is the difficulty in selectively stimulating astrocyte G_q-GPCR signaling pathways.

Astrocytes and neurons express many of the same GPCRs. As a result, conventional G_q-GPCR ligands stimulate calcium increases in both neurons and astrocytes. Alternate approaches for stimulating astrocyte calcium include mechanical stimulation, electrical stimulation, or uncaging caged calcium or IP_3 (Fiacco et al., 2009). All of these approaches have serious limitations. Neither mechanical nor electrical stimulation are physiological stimuli. Likewise, uncaging calcium or IP_3 bypasses the endogenous G_q-GPCR signaling pathways. To address the role of astrocyte G_q-GPCR signaling pathways in physiology, we have developed new genetic mouse models that allow the selective stimulation of glial G_q-GPCRs. In chapter 4, I will describe the development of this novel tool.

CHAPTER 2.

OVERVIEW OF PAIN AND ITCH BIOLOGY

2.1. ANATOMY OF THE PAIN AND ITCH PROCESSING SYSTEM

Pain and itch (pruritus) are both noxious sensory perceptions that alert an organism to potential harm. More than 2,300 years ago, Aristotle argued that the heart is the seat of emotions, including pain (Aristotle, 350 BCE). Descartes' reflex theory, the first neurobiological model for pain processing, (Descartes, 1972) posited that painful stimuli activate a linear pathway that terminates in the brain where pain is perceived.

Descartes' theory was the dominant paradigm for more than 330 years until Melzack and Wall proposed their revolutionary "gate control" theory (Melzack and Wall, 1965). Gate theory states that pain perception is based on the operation of a neural gate in the dorsal horn whose opening is determined by the balance between noxious and innocuous input from the periphery. Melzack and Wall also recognized that there is dynamic regulation within and between the periphery, spinal cord, and supraspinal regions.

Painful stimuli elicit a withdrawal response whereas itch elicits a scratch response. It has long been known that scratching blocks the sensation of itch, but the mechanism by which this occurs has only recently been discovered. Itch is a distinct sensory phenomenon from pain, but has a large degree of overlap with pain in its anatomical basis.

2.1.1. The peripheral pain and itch neurocircuitry.

Sensory information is conveyed from the periphery to the spinal cord by primary afferents, which have their cell bodies in dorsal root ganglia (DRG). Primary afferents comprise three major classes: Aα/Aβ, Aδ, and C fibers. Aα/Aβ fibers are fast conducting, heavily myelinated, and generally convey innocuous and proprioceptive sensory information (Djouhri et al., 1998). Aβ fibers are low-threshold mechanoreceptors that are activated by gentle touch (Todd, 2010).

In 1906, Sherrington conceived the idea of "specialized detectors" for pain, or nociceptors (Sherrington, 1906). Nociceptors fall into two major categories: Aδ fibers and C fibers (COLLINS et al., 1960). Aδ fibers are lightly myelinated and conduct more rapidly than unmyelinated C fibers (Burgess and Perl, 1967). Aδ fibers convey cold pain, mechanical pain, and well-localized pain sensations whereas C fibers are polymodal responding to noxious heat, mechanical, and chemical stimuli (DeLeo, 2006). C fibers can be further divided into peptidergic and non-peptidergic subtypes (Lawson et al., 1997; Snider and McMahon, 1998). Peptidergic C fibers contain neuropeptides such as substance P (Lawson et al., 1997); they innervate deep layers of the skin as well as other tissues (Bennett et al., 1996; Perry and Lawson, 1998). Non-peptidergic C fibers are restricted to the epidermis (Taylor et al., 2009).

Nociceptors innervate sites throughout the body including skin, muscle, joints, and viscera (Julius and Basbaum, 2001). By contrast, itch is a dermatological phenomenon and, itch-sensing fibers are restricted to the skin. The sensation of itch is transmitted by a subset of mechanically insensitive, very slowly conducting C fibers (Schmelz et al., 1997). Both

nociceptors and pruritoceptors (itch-sensing primary afferents) have their central projections in the dorsal horn of the spinal cord, a central hub of sensory processing.

2.1.2. The dorsal horn of spinal cord.

Sensory information is processed in the spinal cord dorsal horn whereas the ventral horn contains motor neurons. The spinal cord is a laminated structure; the grey matter is divided into ten laminae. The dorsal horn contains laminae I-V whereas the ventral horn contains lamina VI-X (REXED, 1952). Lamina I and II compose the superficial dorsal horn. Lamina II is often subdivided into inner and outer sections (lamina IIi and IIo).

Primary afferents have their central terminations in the dorsal horn. Aβ fibers are low-threshold mechanoreceptors that arborize in lamina III-lamina V (Todd, 2010). Peripheral nociceptors terminate in lamina I and lamina II (also known as the marginal zone and the substantia gelatinosa, respectively) and lamina V (Kumazawa and Perl, 1978). More specifically, Aδ fibers terminate in lamina I, IIo, and V. Peptidergic C fibers terminate in lamina IIo, and non-peptidergic C fibers terminate in lamina II (Lawson et al., 1997; Light and Perl, 1979a; Lorenzo et al., 2008). Gastrin-releasing peptide receptor (GRPR) is restricted to lamina I and has been shown to mediate itch sensation in the spinal cord (Sun and Chen, 2007).

The vast majority of neurons in the dorsal horn are interneurons: neurons whose axons remain in the spinal cord and arborize locally. Dorsal horn interneurons respond to a variety of stimuli. Wide dynamic range (WDR) neurons respond to noxious and innocuous stimuli whereas nociceptive-specific (NS) neurons selectively respond to potentially tissue-damaging stimuli (Price et al., 2003). Histamine, a classic pruritogen, also activates WDR neurons in the dorsal horn (Carstens, 1997). NS neurons dominate in the superficial dorsal horn whereas

WDR neurons comprise the majority of lamina V neurons (Willis, 1985). Lamina II contains a high density of both excitatory and inhibitory interneurons (Todd, 2010; Moore et al., 2002). The inhibitory interneurons use GABA and/or glycine as inhibitory neurotransmitters.

The interneurons of the dorsal horn vary widely in their morphology and their electrophysiological properties, making them difficult to classify. Perl and colleagues developed the most widely accepted classification scheme for lamina II interneurons (Grudt and Perl, 2002; Hantman et al., 2004; Lu and Perl, 2003; Lu and Perl, 2005). They identified four classes of interneurons: islet, central, vertical, and radial. This classification scheme still leaves roughly 30% of interneurons as being "unclassified," which further illustrates the complexity of this region (Grudt and Perl, 2002). Lamina I contains both interneurons— which have been categorized based on morphology into pyramidal, fusiform, and multipolar categories— and projection neurons (Han et al., 1998).

The dorsal horn receives sensory information from the periphery and transmits it to supraspinal sites via projection neurons. The majority of projection neurons reside in lamina I with a few scattered across laminae III-VI (Todd, 2010). Most projection neurons have axons that cross the midline to terminate in contralateral supraspinal structures. There are three major pain-selective ascending pathways from the dorsal horn: spinothalamic (to the thalamus and then on to the cortex), spinoreticular (to the medulla and pons), and spinomesencephalic (to the periaqueductal grey (PAG)) (Tracey and Dickenson, 2012). Lamina I also contains spinothalamic projection neurons that are selectively sensitive to histamine (Andrew and Craig, 2001).

Pain and itch can be reduced by counter-stimuli. For example, a person might rub his arm after bumping his "funny bone" or put ice on burned skin. Counter-stimuli, such as

scratching, heat, cold, and pain-inducing chemicals, can inhibit itch in humans (Ross, 2011). Conversely, inhibition of nociceptors with opiates can cause itch (Ballantyne et al., 1988). Painful stimuli override itch-inducing stimuli; this seems favorable from an evolutionary standpoint, since pain-generating stimuli can cause massive tissue damage much faster than most itch-generating stimuli.

The inhibition of itch by counter-stimuli occurs within the spinal cord dorsal horn since counter-stimuli inhibit itch even when applied outside the receptive field of the activated pruritoceptors (Nilsson et al., 1997). Scratching activates nociceptors, which synapse on spinal interneurons. These interneurons then inhibit itch spinothalamic projection neurons (Ross et al., 2010; Davidson et al., 2009).

2.1.3. Supraspinal processing of pain and itch.

Most of our knowledge about how the brain processes noxious stimuli is derived from functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies. While the PNS and spinal cord transmit noxious sensory information, supraspinal structures ultimately create the sensation of pain or itch. Supraspinal processing of pain and itch show large degrees of overlap. There are sensory-discriminative and affective-motivational components to both pain and itch perception.

Incoming sensory information from the spinal cord is relayed first to the thalamus and then to cortical regions. Lateral thalamic nuclei send projections to the somatosensory cortex where the spatial, temporal, and intensity aspects of pain and itch are processed (Tracey and Dickenson, 2012). Medial thalamic nuclei send projections to the anterior cingulate cortex (ACC) and to the insular cortex where the aversive aspect of pain and itch is processed (Vogt, 2005).

Imaging studies reveal that pain and itch stimuli cause activation of the prefrontal cortex (PFC) (Hsieh et al., 1994; Kwan et al., 2000; Moulton et al., 2005). The PFC has been linked to attention as well as to preparation of the motor response (Pardo et al., 1991). Both pain and itch activate the premotor area (PMA) and the supplementary motor area (SMA) (Hsieh et al., 1994; Kwan et al., 2000; Moulton et al., 2005), yet they generate distinct motor responses. Pain causes withdrawal, whereas itch generates scratching. Motor areas control movement on the contralateral side of the body. Hence, pain activates contralateral SMA to cause withdrawal and itch activates ipsilateral SMA to cause scratching (Hsieh et al., 1994). Pain and itch differentially activate the somatosensory cortex; pain activates the primary and secondary somatosensory cortices (S1 and S2) whereas itch only activates S1 (Ikoma et al., 2006).

2.1.4. Descending modulation.

Primary afferents transmit noxious sensory information from the periphery to ascending pathways. Supraspinal structures can likewise modulate incoming sensory information through a process called descending modulation. For example, descending inhibition occurs in badly injured soldiers who do not appear to be in pain while on the battlefield, only to start complaining about pain after being taken to a hospital. Descending modulatory pathways can also facilitate pain perception (Fields, 2004).

Descending modulation employs two major pathways (Fields, 2004). In the first, the ACC sends projections first to the PFC and then to the PAG. The second pathway directs input from the insula cortex to the PAG. The PAG then sends projections to the rostral ventromedial medulla (RVM), which transmits those projections via the spinal cord dorsolateral funiculus to the dorsal horn (North and Yoshimura, 1984). While descending

modulation was first discovered in the 1960s in relation to nociception (Wall, 1967), it also modulates itch processing. For example, stimulation of the PAG suppresses histamine-evoked responses in WDR neurons in the dorsal horn (Carstens, 1997). In conclusion, pain and itch perception arise from the summation of complex ascending and descending peripheral, spinal, and supraspinal neurocircuitry. Understanding the anatomical basis of pain and itch processing provides insight into the changes that occur in pathological pain and itch states.

2.2. HOW DO ACUTE PAIN AND ITCH DIFFER FROM CHRONIC PAIN AND ITCH?

2.2.1. Acute pain and itch are adaptive.

Our pain and itch sensing systems have evolved to protect us from possible threats. Aristotle reportedly said that, "we cannot learn without pain." Mutations in genes critical to the normal development and/or functioning of pain neurocircuitry can result in congenital insensitivity to pain (CIP) (Cox et al., 2006). Patients with this disorder suffer repeated injuries due to painless tissue damage (Sandroni et al., 2006). Most CIP patients do not live past childhood due to neglect of injuries and illnesses (Nagasako et al., 2003). These severe impairments highlight how important normal processing of noxious stimuli is for survival.

2.2.2. Chronic pain and itch are pathological.

While acute pain and itch perception is beneficial, chronic pain and itch do not serve any purpose and cause great suffering to patients. Acute pain and itch alert an organism to the presence of a potential threat. By contrast, chronic pain and itch can occur in the absence of any noxious stimuli. Chronic pain and chronic itch both involve long-term changes at the cellular and molecular levels in both the PNS and CNS.

PNS plasticity that leads to hypersensitivity is called peripheral sensitization. Inflammatory mediators, bradykinin, serotonin (5-HT), and prostaglandin, can all induce peripheral sensitization in both chronic pain and itch (Ikoma et al., 2006). Nerve growth factor (NGF), a neurotrophin, is upregulated in chronic pain syndromes such as vulvar vestibulitis and in chronic itch conditions such as atopic dermatitis (AD) (Bohm-Starke et al., 1998; Hefti et al., 2006). NGF induces peripheral sensitization by causing sprouting of nociceptors and pruritoceptors (Bohm-Starke et al., 1998; Urashima and Mihara, 1998).

Another mechanism for peripheral sensitization is alterations in the subcellular localization of voltage-gated sodium channels. Voltage-gated sodium channels generate and conduct action potentials in neurons (Dib-Hajj et al., 2009). Injury to peripheral nerves can lead to redistribution of tetrodotoxin-resistant voltage-gated sodium channels in nociceptors from the soma to the site of axonal injury (Novakovic et al., 1998). This has been linked to hyperactivity and abnormal spontaneous activity in human nociceptors (Ochoa et al., 1982).

CNS plasticity that leads to enhanced pain and itch is called central sensitization. Homeostasis depends on the balance of excitatory and inhibitory neurotransmission. Hyperexcitability at glutamatergic synapses in lamina II has been observed in animal models of neuropathic pain (Balasubramanyan et al., 2006). In addition, activation of silent glutamatergic synapses in the superficial dorsal horn has been linked to chronic pain (Li and Zhuo, 1998).

Decreases in inhibitory neurotransmission in the CNS can also lead to central sensitization. Tonic GABA tone is required for homeostasis, and intrathecal injection of the $GABA_A$ antagonist, bicuculline, produces thermal hyperalgesia and mechanical allodynia in naïve rats (Malan et al., 2002). Hence, losing GABAergic inhibitory tone in lamina II can

contribute to enhanced pain sensitivity in patients with neuropathic pain (Moore et al., 2002; Malan et al., 2002; Yamamoto and Yaksh, 1993).

Several mechanisms have been proposed to explain diminished GABAergic tone during neuropathic pain. Decreased levels of glutamate decarboxylase 65 (GAD65), a rate-limiting enzyme for GABA synthesis, were detected in the dorsal horn of rats that had undergone two models of neuropathic pain: CCI (chronic constriction injury) and SNI (spared nerve injury) (Moore et al., 2002). Changes in GAD expression could affect presynaptic GABA levels. Dorsal horn GABA levels appear to decrease in rats with CCI as compared to sham-operated rats (Ibuki et al., 1997). In addition, CCI reduces excitatory drive to and membrane excitability of GABAergic tonic central cells in rats (Balasubramanyan et al., 2006). Loss of inhibitory neurons in the dorsal horn could also contribute to chronic itch. Mice lacking the transcription factor Bhlhb5 show a selective loss of inhibitory interneurons in the spinal cord and show abnormally heightened itch (Ross et al., 2010).

2.2.3. Traits of chronic pain and itch.

The International Association for the Study of Pain defines chronic pain as “pain that persists past the healing phase following an injury” (Merskey and Bogduk, 1994). Clinicians generally consider itch and/or pain to be chronic when it lasts for at least three to six months (Ikoma et al., 2006; Verhaak et al., 1998). Chronic pain and itch have very different phenotypes than acute pain and itch.

The psychophysical traits of chronic pain and itch can be subdivided into spontaneous and evoked categories. Ectopic C fiber activity and the sensitization of dorsal horn neurons are thought to cause spontaneous pain (Woolf and Mannion, 1999). Patients assess spontaneous pain and itch through self-reported rating scales. The Visual Analog Scale

(VAS), which rates pain along a continuum (usually from 0-10) (Scott and Huskisson, 1976), is one of the most common clinically used measures of pain due to its simplicity (Carlsson, 1983).

The VAS is limited primarily by its inability to either describe pain quality or detect changes in pain quality over time (Elman et al., 2010). Pain has a range of qualities that cluster in three major categories: paroxysmal (shooting, sharp, and radiating), superficial (cold, sensitive, tingling) and deep (aching, throbbing, cramping) (Victor et al., 2008). To address these limitations, questionnaires that measure pain qualities have been developed. The McGill Pain Questionnaire (MPQ) was the first comprehensive pain quality measure (Melzack, 1975); it measured intensity, temporal qualities, spatial location, and sensory and affective qualities. Measurements of spontaneous pain are usually restricted to humans since animals obviously cannot speak. However, Mogil and colleagues have developed an intriguing measure of spontaneous pain in rodents, the "Grimace Scale," which quantifies pain using facial expressions (Langford et al., 2010; Sotocinal et al., 2011). Figure 2 shows the Rat Grimace Scale.

Itch, like pain, is a multidimensional, subjective experience and therefore faces similar measurement challenges. Again, the VAS is widely used to measure itch, but the limitations it faces in measuring itch are similar to those limiting its use in measuring pain (Elman et al., 2010). For adult patients, the 5-D Pruritus Scale is a validated measurement tool; the "5 Ds" are duration, degree, direction, disability, and distribution (Elman et al., 2010). The Itch Man Scale, developed for use in pediatric patients, measures both itch severity and impact on quality of life through a series of cartoons (Figure 3) which show the "Itch Man" becoming increasingly itchy and distressed (Blakeney and Marvin, 2000).

Studies of humans measure evoked pain and itch as well as spontaneous pain and itch. Evoked measures of thermal and mechanical sensitivity have the benefit of being objective measurements as opposed to the strictly subjective measurements of spontaneous pain and itch. Evoked pain and itch are the chief tools for measuring noxious sensory processing in laboratory animals.

Evoked responses to both mechanical and thermal pain are usually measured to find the threshold at which mechanical, hot and cold stimuli are perceived as noxious. Mechanical allodynia and hyperalgesia are hallmarks of chronic pain patients. The former is the perception of a formerly non-noxious mechanical stimulus as being noxious; the latter is an exaggerated reaction to a painful thermal or mechanical stimulus. Central sensitization is thought to cause both allodynia and hyperalgesia. Allodynia is generated by low-threshold nociceptors or Aβ fibers (Woolf and Mannion, 1999; Sandkuhler, 2009). Hyperalgesia is thought to be due to sensitization of peripheral nociceptors (Sandkuhler, 2009).

Chronic itch also has patterns of central sensitization; these patterns result in phenotypes analogous to that observed in chronic pain. Alloknesis, the itch analogue of allodynia, is when innocuous touch triggers itch (Ikoma et al., 2006). Hyperknesis— the itch analogue of hyperalgesia— is an exaggerated itch reaction evoked by mechanical stimuli (Atanassoff et al., 1999). Acute itch is a common sensation relieved by scratching. By contrast, scratching does not alleviate chronic itch; indeed, it exacerbates the problem by causing tissue damage (Yosipovitch et al., 2003).

2.2.4. Major categories of chronic pain and itch.

A broad range of conditions can result in chronic pain and itch. The major categories for chronic pain are determined by the site of injury (back, head, viscera) or the type of injury

(cancer, neuropathic, inflammatory, arthritis) (Apkarian et al., 2009). Damage to the nervous system – either centrally or peripherally- can result in neuropathic pain (Woolf and Mannion, 1999). A range of nervous system injuries, caused by such things as trauma, stroke, infection, diabetes, autoimmune disease and exposure to chemotherapeutic agents, can all result in neuropathic pain (Ji and Strichartz, 2004; Campbell and Meyer, 2006).

Chronic itch is also a heterogeneous disease caused by a broad spectrum of pathologies. Clinically, chronic itch can be subdivided into itch caused by skin disorders, itch caused by systemic disorders, neuropathic itch, and psychogenic itch. Skin disorders that can cause itch include atopic dermatitis (AD), psoriasis and parasitic infections (Ikoma et al., 2006). While itch is a skin sensation, dysfunction of other organ systems can cause chronic itch. Systemic disorders such as renal failure, hyperthyroidism, lymphoma, cholestasis (a chronic gall bladder disease), solid tumors and HIV/AIDS can cause chronic itch (Twycross et al., 2003). The same sorts of nervous system injury that result in neuropathic pain can also cause neuropathic itch (Yosipovitch et al., 2003). Psychogenic itch can be generated by delusions of parasitosis, stress, and depression (Ikoma et al., 2006). Understanding the clinical aspects of chronic pain and itch is essential to make the proper diagnosis and to prescribe appropriate treatments.

2.3. DIAGNOSIS AND TREATMENT

2.3.1. How are chronic pain and itch diagnosed?

The first step in diagnosing chronic pain and itch is to obtain a patient history and a detailed description of spontaneous pain and/or itch using a validated questionnaire (Yosipovitch et al., 2003). Next, a full physical examination including testing of reflexes and motor skills should be performed (Harden, 2005). Another important diagnostic tool is

sensory testing. These tests include measurements of mechanical, heat pain, and cold pain thresholds (Oaklander et al., 2002).

Small-fiber polyneuropathy (SFPN) refers to damage to the axons of Aδ fibers and C fibers (Oaklander, 2011). It is a common cause of neuropathic pain and neuropathic itch (Mendell and Sahenk, 2003). The American Academy of Neurology endorses two tests to diagnose SFPN: distal-leg skin biopsies and autonomic-function testing (England et al., 2009). Skin biopsies are stained with the panaxonal marker anti-PGP9.5 in order to quantify small cutaneous sensory fibers; patients with SFPN have a reduced density of intra-epidermal fibers compared to controls (McCarthy et al., 1995) Autonomic-function testing is used because autonomic nervous system deficits such as anhidrosis (the inability to sweat normally) have strong predictive value for SFPN (Low et al., 2006).

2.3.2. Statistics and costs to treat chronic pain and itch.

Chronic pain has tremendous costs to society in addition to the toll it exacts on patients. Chronic pain has been estimated to affect one-sixth of the population (Campbell and Meyer, 2006). Neuropathic pain, caused by damage to the nervous system, accounts for an estimated 3.5 million cases in the United States alone (Harden, 2005). Chronic inflammatory pain is also a major public health issue. For example, osteoarthritis is a leading cause of disability worldwide (Sharma et al., 2006). In the United States workforce, lost productivity due to chronic pain conditions is estimated to cost $61.2 billion per year (Stewart et al., 2003).

Chronic itch also places a large burden on society, both in terms of cost and human suffering. Chronic itch has been estimated to occur in 50-90% of patients with renal or liver disease and in patients with severe burns, AD, and other dermatological diseases (Carstens, 2008). The global prevalence of AD in children has been estimated at 20%, and it has an

annual cost of almost $1 billion in the United States alone (Carroll et al., 2005). The best-known complication of herpes zoster (shingles) is postherpetic neuralgia (PHN), a type of neuropathic pain. Postherpetic itch (PHI), a type of neuropathic itch, has been reported to occur in 17-58% of postherpetic patients instead of or accompanying PHN (Oaklander et al., 2003). Both chronic pain and itch create additional suffering due to the inadequacy of current treatments.

2.3.3. Major classes of analgesics and antipruritics.

Pharmacological treatment is the mainstay for managing chronic pain. The first drugs developed to treat pain came from serendipitous observations of analgesic properties of plants. Opioids were first derived from the poppy and salicylic acid, an early non-steroidal anti-inflammatory drug (NSAID), was extracted from willow bark (Scholz and Woolf, 2002). The use of opioids is limited by sedation as well as the risk for addiction and the development of opioid-induced hyperalgesia, a state of heightened pain sensitivity (Mendell and Sahenk, 2003; Hartrick et al., 2012). Since pain is known to inhibit itch, opioid treatment can cause uncontrollable itchiness (Ballantyne et al., 1988). Further evidence implicating opioidergic neurotransmission in itch comes from patients with chronic itch due to liver disease. These patients have abnormally high levels of endogenous opioids (Swain et al., 1992), and they experience reduced scratching following treatment with the opioid receptor antagonist, naloxone (Jones et al., 2002). A recent study suggests that opioid-induced itch occurs when the mu-opioid receptor, MOR1D, heterodimerizes with GRPR in the spinal cord to relay itch information (Liu et al., 2011). Inhibiting downstream signaling effectors of GRPR blocked morphine-induced itch while preserving morphine-induced analgesia thus

demonstrating that selective targeting of MOR isoforms might preserve the analgesic effects of opioids while bypassing the side effect of itchiness (Liu et al., 2011).

Patients with neuropathic pain do not respond to NSAIDs and are often insensitive to opioids (Woolf and Mannion, 1999). Neuropathic pain is treated primarily with antidepressants (selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants) and anticonvulsants, including gabapentin (Carter and Galer, 2001). These drugs have limited efficacy and often undesired side effects (Woolf and Mannion, 1999; Kingery, 1997).

Unlike acute itch, neuropathic itch is resistant to treatment with antihistamines, corticosteroids, and most pain medications (Oaklander, 2011). Scratching in chronic itch patients can often cause severe tissue damage; one patient with PHI eventually scratched through her own skull (Oaklander et al., 2002). Surprisingly, some chronic itch patients may not understand that their skin lesions are due to their scratching. Patient education therefore can be an important intervention. Some patients use protective garments to protect their skin from involuntary scratching during sleep (Oaklander, 2011).

Capsaicin, the active ingredient in chili peppers, has been shown to be an effective treatment for the itch of AD (Yosipovitch et al., 2003). However, it also produces irritation at the site of application, which reduces patient compliance. Local anesthetics such as lidocaine that block selectively pruritoceptor firing can be beneficial (Oaklander, 2011; Fishman et al., 1997).

Clearly, chronic pain and itch patients would benefit greatly from the development of more effective treatments. Historically, pain and itch research has been neurocentric. During the last two decades, a role for glia in pain modulation has been uncovered and may provide future potential therapeutic targets.

CHAPTER 3.

THE EVOLVING ROLE OF GLIA IN PAIN MODULATION.

3.1. ARE GLIA "GOOD GUYS OR BAD GUYS" IN THE SETTING OF CHRONIC PAIN?

3.1.1. The role of astrocytes in chronic pain.

Pain researchers have traditionally focused on neurons, since they transmit nociceptive sensory information. But, a growing body of research implicates glia in the development and maintenance of chronic pain. While there are other types of glia (microglia, oligodendrocytes, NG2 glia, Schwann cells), our focus is on the role that astrocytes, satellite glial cells (SGCs), and non-myelinating Schwann cells play in chronic pain. Astrocytes are present throughout the spinal cord and brain. In the PNS, SGCs surround the cell bodies of sensory neurons in DRG, while non-myelinating Schwann cells contact the axons of C fibers. The glial marker GFAP is expressed by astrocytes, SGCs, and non-myelinating Schwann cells.

Many neurological diseases cause astrocytes to become reactive, a process also known as astrogliosis. Reactive astrocytes exhibit cellular hypertrophy and increased levels of GFAP (Figure 5) (Hansson, 2006). Garrison and coworkers were the first to show that inducing chronic pain in adult rats causes astrogliosis in the ipsilateral dorsal horn of the spinal cord (Garrison et al., 1994) while the contralateral astrocytes remain quiescent. Since then, a range of chronic pain models including peripheral nerve injury, inflammation, and tumor invasion have been shown to cause astrogliosis (Gao and Ji, 2010; Colburn et al., 1999; Raghavendra

et al., 2004). In addition, chronic pain causes astrogliosis at supraspinal sites. For example, CCI causes astrogliosis in the RVM, whereas injection of complete Freund's adjuvant (CFA) —an inflammatory agent— causes astrogliosis in the forebrain (Raghavendra et al., 2004; Guo et al., 2007; Wei et al., 2008). However, most studies of astrogliosis have focused on the spinal dorsal horn since it is an extensively studied site of nociceptive processing.

It has been demonstrated that blocking astrogliosis also prevents chronic pain. For example, both fluoroacetate and its metabolite fluorocitrate are selectively taken up by glia and are general inhibitors of astrocytes (Gao and Ji, 2010; Largo et al., 1997). Fluoroacetate and fluorocitrate attenuate metabolic activity in astrocytes by interfering with ATP production in the Krebs cycle (Hassel et al., 1992). Intrathecal administration of fluorocitrate blocks thermal and mechanical hyperalgesia in an inflammatory pain model (Meller et al., 1994) as well as in a mirror-image neuropathic pain model (Milligan et al., 2003). L-α-aminoadipate— a homolog of glutamate that naturally occurs in the brain— has also been proposed as an astrocyte-selective inhibitor (Huck et al., 1984; Khurgel et al., 1996). Intrathecal administration of L-α-aminoadipate reversed mechanical allodynia generated by spinal nerve ligation (SNL), a model of chronic neuropathic pain (Zhuang et al., 2006). While these observations that inhibition of astrocytes could block chronic pain were made more than twenty years ago, only in the past decade have researchers found evidence for specific glial mediators of chronic pain.

Tissue or nerve injury results in the release of various neurotransmitters and inflammatory mediators— including ATP, glutamate, and tumor necrosis factor-alpha (TNF-α)— which go on to activate spinal cord astrocytes (Gao and Ji, 2010). Reactive astrocytes then activate several intracellular signaling pathways including the c-Jun N-terminal kinase

(JNK) and extracellular signal-related kinase (ERK) pathways (Zhuang et al., 2005; Ma and Quirion, 2002; Gao et al., 2009). Finally, astrocytic JNK and ERK activation leads to the release of inflammatory cytokines and chemokines, which cause hypersensitivity in pain processing pathways.

Spinal cord astrocytes upregulate interleukin-1 beta (IL-1β)— an inflammatory cytokine— in several pain conditions including bone cancer, nerve injury, and masseter inflammation (Guo et al., 2007; Wei et al., 2008; Zhang et al., 2005). In addition, intrathecal injection of IL-1β is sufficient to generate pain hypersensitivity (Kawasaki et al., 2008b). The IL-1 receptor colocalizes with neuronal NMDA receptors in the spinal cord, trigeminal nucleus, and RVM (Guo et al., 2007; Wei et al., 2008; Zhang et al., 2008). Thus, IL-1β released from astrocytes can bind to neuronal receptors and directly modulate neuronal activity. In patch clamp studies of lamina II neurons in spinal cord slices, application of IL-1β enhanced both the frequency and amplitude of NMDA-mediated spontaneous excitatory post-synaptic currents (sEPSCs) and decreased the frequency and amplitude of spontaneous inhibitory post-synaptic currents (Kawasaki et al., 2008a). This suggests that IL-1β causes central sensitization by increasing excitatory synaptic transmission and decreasing inhibitory synaptic transmission.

The chemokine monocyte chemoattractant protein-1 (MCP-1, also called CCL2), which has also been implicated in astrocytic promotion of pain hypersensitivity, is upregulated in spinal cord astrocytes after SNL (Gao and Ji, 2010; Gao et al., 2009). CCR2 is the major receptor for MCP-1 (Abbadie et al., 2003), and nerve injury increases CCR2 expression in DRG and spinal cord neurons (Gao et al., 2009; White et al., 2005). MCP-1 potentiates glutamatergic synaptic transmission and inhibits GABAergic synaptic transmission in spinal

cord neurons, and thus may enhance central sensitization (Gao et al., 2009; Gosselin et al., 2005).

Changes in glutamate transporters have also been linked to the astrocyte-mediated enhancement of chronic pain states. Astrocytic glutamate transporters facilitate clearance of extracellular and synaptic glutamate in order to maintain homeostasis (Huang and Bergles, 2004). Nerve injury causes an initial increase in astrocytic glutamate transporters, GLT-1 and GLAST, followed by a persistent decrease (Sung et al., 2003; Xin et al., 2009). While gene transfer of GLT-1 into the spinal cord of rats did not affect acute pain, transgenic GLT-1 was anti-nociceptive in chronic inflammatory and neuropathic pain models (Maeda et al., 2008). These data highlight the importance of astrocytic glutamate transporters and raise the possibility that they may be viable therapeutic targets.

Finally, reactive astrocytes have been linked to the phenomenon of allodynia, a widespread and intractable symptom of neuropathic pain. The spinal cord has a tonic inhibitory tone due to the neurotransmitters, GABA and glycine (Willcockson et al., 1984). Acute intrathecal strychnine— a glycine receptor antagonist— results in morphine-insensitive allodynia resembling the sensory abnormalities observed in neuropathic pain patients (Sherman and Loomis, 1994). Strychnine-evoked allodynia is blocked by the NMDA receptor antagonist, MK-801, suggesting that it depends on NMDA receptors (Yaksh, 1989). NMDA receptor activation requires binding by glutamate and its co-agonist, D-serine (Miraucourt et al., 2011). D-serine has also been shown to function as a gliotransmitter (Schell et al., 1995). Recent work shows that intrathecal strychnine blocks the spinal cord's tonic inhibitory tone, which may result in touch-mediated astrogliosis. This potentially causes reactive astrocytes to then release D-serine, activating NMDA receptors and resulting

in allodynia (Miraucourt et al., 2011). Clearly, numerous mediators have been implicated in the ability for reactive astrocytes to enhance chronic pain states. More research is required to fully understand the role of astrocytes in chronic pain.

3.1.2. The role of SGCs in chronic pain.

The glial research community has emphasized the role of astrocytes in chronic pain. SGCs respond to both nerve injury and chronic compression of the DRG (CCD), an animal model of low back pain, in the same manner as astrocytes— by proliferating and upregulating GFAP (Elson et al., 2004; Zhang et al., 2009). SGCs appear to modulate chronic pain through changes in potassium buffering.

Neuronal excitability is largely determined by the extracellular concentration of potassium ions (Orkand et al., 1966). Astrocytes and SGCs regulate the extracellular potassium concentration via the inward rectifying channel Kir4.1 (Takumi et al., 1995; Vit et al., 2006). CCD causes SGCs to exhibit decreased inwardly rectifying potassium currents, which leads to neuronal hyperexcitability (Zhang et al., 2009). In addition, peripheral nerve injury causes the downregulation of Kir4.1 in SGCs. Silencing Kir4.1 in the trigeminal ganglia of naïve rats leads to spontaneous and evoked facial pain (Vit et al., 2008). This suggests that potassium buffering by astrocytes and SGCs is crucial to maintaining homeostasis, and that it decreases in multiple models of chronic pain.

3.2. THE LIMITATIONS OF CURRENT APPROACHES FOR STUDYING GLIA AND PAIN

While the research to date has shed a great deal of light on the role of glia in chronic pain, previously used approaches have limitations. For instance, glial inhibitors such as fluorocitrate have been extensively used to show that blocking gliosis prevents the

development of chronic pain. However, these inhibitors are crude pharmacological tools; fluorocitrate is thought to disrupt both astrocytic and microglial metabolism (McMahon and Malcangio, 2009). Moreover, microglia have been shown to contribute to chronic pain states through the release of inflammatory cytokines. Current approaches lack the ability to distinguish the role of astrocytes in chronic pain from the roles of other glia. Finally, there is the "chicken and egg" problem: which came first, the chronic pain or the astrogliosis? Almost all current approaches involve inducing chronic pain and then measuring changes in astrocytes. Establishing that astrocytes are causal agents— rather than merely correlated— is difficult using this approach. In Chapter 4, we will show how our lab used a genetic approach to examine whether the G_q-DREADD (Designer Receptor Exclusively Activated by a Designer Drug) model can be used to selectively stimulate glial G_q-GPCRs and then measure changes in pain and itch sensitivity.

CHAPTER 4.

THE G_Q-DREADD MOUSE MODEL CAN BE USED TO SELECTIVELY STIMULATE G_Q-GPCR PATHWAYS IN $GFAP^+$ GLIA.

4.1. INTRODUCTION: DEVELOPMENT OF THE G_Q-DREADD MOUSE MODEL

Selective stimulation of astrocytic GPCRs is difficult because astrocytes and neurons often express overlapping subsets of receptors. As discussed in Chapter 1, previous approaches that have been used include conventional pharmacological agents, mechanical stimulation, and uncaging Ca^{2+}or IP_3 (Fiacco et al., 2009). All of these approaches are either non-physiological stimuli or lack astrocytic selectivity. Transgenic mouse models were developed as an improvement over these past techniques.

The first engineered GPCRs were the RASSLs (Receptor Activated Solely by a Synthetic Ligand). The Ro1 RASSL is a modified human kappa-opioid receptor (KOR) with dramatically reduced affinity for the natural peptide ligand, but retained affinity for synthetic ligands such as spiradoline (Coward et al., 1998). Ro1 is a chimeric receptor of KOR containing the second extracellular loop of the delta-opioid receptor. Stimulation of Ro1 with spiradoline activates G_i-coupled signaling pathways.

A tetracycline-inducible system was used to express Ro1 selectively in $GFAP^+$ cells (Sweger et al., 2007). When such mice were taken off doxycycline, a tetracycline derivative, 100% of the mice developed hydrocephalus (Sweger et al., 2007; McMullen et al., 2012). The fact that hydrocephalus developed without Ro1 stimulation with spiradoline is consistent with Ro1 having high levels of constitutive activity. While this was a powerful model for

studying the role of glia in hydrocephalus, the high basal RASSL activity was undesirable for studying GPCR pathways. Moreover, spiradoline is a KOR agonist; thus the Ro1 mouse model had to be maintained on a KOR knockout background. Ideally, an engineered receptor would be activated by a synthetic ligand that did not also activate endogenous GPCRs.

The first transgenic mouse model that allowed the selective stimulation of astrocytic G_q-GPCRs was the MrgA1 (Mas-related genes receptor A1) model (see Figure 6) (Fiacco et al., 2007). The MrgA1 receptor is endogenously expressed in peripheral sensory neurons (Dong et al., 2001). Stimulation of MrgA1 with the Phe-Met-Arg-Phe (FMRF) peptide activates G_q-coupled GPCR signaling pathways (Dong et al., 2001).

Expression of the MrgA1 receptor in astrocytes is achieved by breeding a tetracycline-regulated tet operon (tetO MrgA1) mouse to a transgenic mouse expressing an astrocyte-selective tetracycline transactivator (GFAP tTA) line; expression of the MrgA1 protein occurs in the absence of tetracycline or doxycycline. A major advantage of this approach is that the timing of expression of MrgA1 can be easily controlled. Keeping the mice on doxycycline until post-natal day 30 avoids any possible phenotype due to transgenic MrgA1 expression in the developing nervous system. Our laboratory showed that the MrgA1 peptide ligand, FMRF, selectively causes calcium increases in astrocytes, but not in neurons in acutely isolated hippocampus slices (Fiacco et al., 2007).

While the MrgA1 model was a major technical advance for the glial biology field, no research tool is without limitations. The peptide, FMRF, does not cross the BBB. Thus, the MrgA1 model cannot be used for *in vivo* studies of astrocyte G_q-GPCR signaling pathways. Similarly, the MrgA1 model is not appropriate for pain or itch studies on peripheral $GFAP^+$ glial G_q-GPCR signaling due to the endogenous receptors on peripheral nociceptors.

Because of these technical limitations, we turned to the recently developed DREADD (Designer Receptor Exclusively Activated by a Designer Drug) model (Armbruster et al., 2007). The native M_3 muscarinic acetylcholine receptor (M_3AChR) is a G_q-coupled GPCR. M_3AChR is activated by acetylcholine (ACh), but not the pharmacologically inert ligand, clozapine N-oxide (CNO). DREADD was engineered to respond to CNO, and not ACh using a directed molecular evolution approach in yeast (Armbruster et al., 2007). First, libraries of randomly mutated muscarinic receptors were created by mutagenic PCR. Then, yeast-expressing mutant receptors activated by CNO were selected for growth on nutrient-deficient media and verified in liquid growth assays. Clones were retransformed into yeast and those with desirable properties were remutagenized for subsequent rounds of selection using lower concentrations of CNO to select for receptors with higher potency. The final mutant, DREADD (hM_3D) has two point mutation in the 3rd and 5th transmembrane regions of the endogenous M_3 muscarinic receptor (hM_3): Y149C and A239G (Figure 7). Importantly, DREADD appears to have low basal activity in the absence of CNO. While DREADDs have been engineered that couple to $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$ (Guettier et al., 2009), this dissertation will focus on the G_q-DREADD model.

To determine the effect of the selective stimulation of glial G_q-GPCR signaling cascades on nociception and itch, we utilized a transgenic mouse line that expresses G_q-DREADD under the control of the GFAP promoter. CNO crosses the BBB and lacks appreciable affinity for endogenous GPCRs (Armbruster et al., 2007). Selective activation of G_q-DREADD *in vivo* can be achieved easily by intraperitoneal injection of CNO. Our data strongly suggest that the G_q-DREADD mouse model allows selective stimulation of G_q-GPCR signaling pathways in GFAP-positive glia. Both immunocytochemistry and calcium

imaging data show that G_q-DREADD is expressed in astrocytes and non-myelinating peripheral glial but not in neurons. Thus, this model is a powerful new tool for studying the role of glial signaling pathways in pain and itch transmission neurocircuitry.

4.2. MATERIALS AND METHODS

4.2.1. Generation of transgenic mice.

To generate the G_q-DREADD mice, the coding sequence for G_q-DREADD (obtained courtesy of B. Roth lab, UNC-CH; (Armbruster et al., 2007) was placed downstream of β-globin intron containing splice donor/acceptor sites and upstream of simian virus 40 (SV40) polyA sequence (intron and SV40 sequence are contained in pTg1 (courtesy of R. Thresher, UNC-CH)). The 2 kilobase (kB) human GFAP promoter (courtesy of M. Brenner, UA Birmingham) was cloned upstream of the intron. G_q-DREADD has a human influenza hemagglutinin (HA) epitope tag on the N terminus. The expression construct was isolated from prokaryotic sequence and injected into C57 × C3H hybrid oocytes by the Animal Models Core Facility at UNC-CH. Founder mice were identified by polymerase chain reaction (PCR), bred to C57/BL/6J mice and screened for transgene expression. G_q-DREADD mice were then backcrossed with C57/BL6/6J mice for at least 7 generations before testing.

The tdTomato protein (Shaner et al., 2004) was expressed in transgenic mice using standard molecular biology techniques. Briefly, the 2.2 kB human GFAP promoter was cloned upstream of a β-globin intron in pTg1. Then, the tdTomato sequence was excised from pdtTomato vector (Clontech) and cloned downstream of the β-globin intron. The polyA signal from SV40 followed the tdTomato sequence. Finally, GFAP-Tomato-pA was excised

from the vector and injected into fertilized oocytes. Founder mice were identified by PCR, bred to C57/BL/6J mice, and screened for transgene expression.

G_q-DREADD mice were bred to GFAP-tdTomato mice, and GFAP-GFP mice (Zhuo et al., 1997). All mice were housed with a 12-h light-dark cycle and were given food and water *ad libitum*. Mice were kept in the animal facilities at the University of North Carolina, Chapel Hill in accordance with Institutional Animal Care and Use Committee guidelines.

4.2.2. Calcium imaging.

Acute transverse (500 μm) spinal cord slices were prepared from 10- to 17-day old GFAP-tdTomato and G_q-DREADD × GFAP-tdTomato mice. Briefly, the lumbar spinal cord was quickly removed from anesthetized mice and sectioned on a Leica VT1000s Vibratome (Bannockburn, IL) in oxygenated ice-cold slicing buffer containing (in millimolar (mM)): 75 sucrose, 2.5 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 1.3 ascorbic acid, 3 pyruvate, and 0.1 kynurenic acid. The slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 10 glucose, 1.25 NaH_2PO_4 × H_2O, 26 $NaHCO_3$, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, and 0.1 Trolox plus 0.07% pluronic acid and 11 μM calcium indicator fluo-4 AM at 37°C for 17 minutes and then at room temperature for 43 minutes. The slices were then washed in ACSF to remove unincorporated dye. Slices were then transferred to a recording chamber (Warner Instruments, Hamden, CT) of an upright microscope (Olympus, Melville, NY) and continuously perfused with oxygenated, room temperature ACSF containing 1μM tetrodotoxin (TTX).

Calcium imaging was performed using a custom-made two-photon microscope. Two-photon confocal microscopy was used to visualize fluo-4 and GFAP-Tomato-positive cells in laminae II and V. A Coherent (Santa Clara, CA) Chameleon Ultra Ti:sapphire diode-pumped

laser was tuned to 830 nm to excite fluo-4 AM and 930 nm to excite tdTomato. A 525/50 bandpass filter (Chroma) was used to detect fluo-4 AM, and a 620/52 bandpass filter (Chroma) was used to detect tdTomato. Images were acquired by using an Olympus (Tokyo, Japan) IR-LUMPlanFl water-immersion objective (60x; 0.9 numerical aperture). To monitor changes in fluorescence over time, Olympus Fluoview v.5 software was used to place region of interest (ROI) boxes over the soma of astrocytes. Increases in calcium concentration were measured as increases in average fluorescence intensity over baseline fluorescence ($\Delta F/F_0$) within the ROI.

4.2.3. Immunoprecipitation/Western blot.

Fresh spinal cord tissue was dissected in ice-cold phosphate buffered saline (PBS) and tissue was homogenized in HEPES/sucrose buffer, sonicated on ice, and centrifuged to precipitate cell membranes. Membranes were resuspended in RIPA buffer and incubated at 4°C with mouse anti-HA affinity matrix beads (Roche). Beads were incubated at 55°C for 10 minutes in Laemli buffer, run on an SDS-PAGE gel, and transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk–TBS/0.1% Tween 20 (TBST) for 2 h at room temperature and probed overnight at 4°C with 1:1000 mouse anti-HA antibody (Roche) in 1% milk/TBST. Membranes were washed in TBST, probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000), and processed for enhanced chemiluminescence (ECL).

4.2.4. Immunofluorescence

Mice were anesthetized with avertin and then perfused with ice-cold 4% paraformaldehyde. Spinal cords were removed, post-fixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose overnight before freezing in Optimum Cutting Temperature

Medium (Tissue-Tek; Sakura, Torrance, CA). Frozen tissue was sectioned at 14 μm thickness and fixed to slides. Sections were further incubated with primary antibody [1:250 rabbit anti-HA (Invitrogen); 1:500 mouse anti-NeuN (Millipore); 1:500 mouse anti-GFP (Sigma)]. Slices were washed in PBS and the fluorescent secondary antibodies Alexa 594 conjugated goat anti-mouse, Alexa 594 conjugated goat anti-rabbit, Alexa 488 conjugated goat anti-mouse, and Alexa 488 conjugated goat anti-rabbit were used. Slides were washed, dried, and cover slipped. Colocalization of HA and GFP or NeuN was visualized with a confocal microscope. For GFAP-tdTomato characterization, colocalization of tdTomato with GFP, NeuN, and HA was visualized with a confocal microscope.

4.3. RESULTS

4.3.1. G_q-DREADD is expressed in spinal cord astrocytes, but not neurons.

G_q-DREADD was derived from the G_q-coupled M3 muscarinic receptor (Armbruster et al., 2007), which, upon stimulation, activates G_q-GPCR driven signal cascades, including IP_3-dependent calcium increases. We prepared a transgenic mouse line that expresses G_q-DREADD under the control of the GFAP promoter (Brenner et al., 1994). G_q-DREADD contains a human influenza hemagglutinin (HA) epitope tag to facilitate immunolabeling. In the spinal cord, G_q-DREADD was expressed throughout the grey matter of the dorsal and ventral horns (Figure 8A). G_q-DREADD-expressing cells in the spinal cord showed the classic "bushy" morphology of astrocytes. Spinal cord expression was confirmed by western blot analyses that showed G_q-DREADD expression in spinal cord tissue from G_q-DREADD but not WT mice (Figure 8B). Immunohistological studies indicated that G_q-DREADD is restricted to astrocytes in the brain, satellite glia in the DRG, and non-myelinating Schwann cells in the peripheral nervous system (data not shown.)

GFAP-GFP mice have been extensively used to identify astrocytes (Zhuo et al., 1997). GFAP-GFP mice were crossed with G_q-DREADD mice to quantify the percentage of astrocytes expressing G_q-DREADD in the spinal cord. In the dorsal horn of G_q-DREADD × GFAP-GFP mice, 55% (20/36) of GFAP-GFP-positive cells expressed G_q-DREADD (Figure 8C-E). There were some G_q-DREADD-positive cells that did not express GFAP-GFP. However, these cells all had typical astrocyte morphology. The neuronal nuclei marker, NeuN, was used to label neurons; 0% (0/277) of NeuN-positive cells in the spinal cord expressed G_q-DREADD (Figure 8F-H). These results indicate that G_q-DREADD expression is restricted to GFAP-positive glia, and that any phenotype seen upon G_q-DREADD stimulation can be attributed to activating glial G_q-GPCR signaling cascades.

To confirm that G_q-DREADD is restricted to astrocytes, we made the GFAP-tdTomato transgenic mouse and crossed it to the G_q-DREADD mouse. Cells expressing GFAP-tdTomato had typical astrocyte morphology (Figure 9A, D, and G). GFAP-tdTomato (Figure 9A) and the astrocyte marker, GFAP (Figure 9B) were highly colocalized in the dorsal horn of spinal cord (Figure 9C). By contrast, GFAP-tdTomato (Figure 9D) and the neuronal marker, NeuN, (Figure 9E) did not colocalize in the dorsal spinal cord (Figure 9F). These findings suggest that GFAP-tdTomato$^+$ cells are astrocytes. When GFAP-tdTomato mice were crossed with G_q-DREADD mice, the vast majority (91%; 21/23) of spinal cord GFAP-tdTomato$^+$ cells co-expressed G_q-DREADD. (Figure 9G-I).

4.3.2. G_q-DREADD stimulation causes calcium increases in G_q-DREADD, but not WT spinal cord astrocytes.

In the spinal cord, both lamina II and V are involved in nociceptive information processing (Light and Perl, 1979a; Besson et al., 1972). Calcium-imaging studies were

performed to determine whether glial G_q-DREADD stimulation induces calcium increases in lamina II and V astrocytes (Figure 10). G_q-DREADD mice were bred to GFAP-tdTomato mice to use tdTomato to identify spinal cord astrocytes. Acute spinal cord slices were prepared and loaded with the calcium indicator dye fluo-4. In G_q-DREADD × GFAP-tdTomato slices, application of 10 μM CNO caused calcium increases in lamina II astrocytes (13/27; n = 2 mice, Figure 10A) and lamina V astrocytes (10/20; n = 2 mice, Figure 10B). In GFAP-tdTomato slices, application of 10 μM CNO failed to increase calcium in lamina II astrocytes (0/18; n= 2 mice, Figure 10C) or lamina V astrocytes (0/8; n = 2 mice, Figure 10D). As a positive control, we measured calcium increases in response to an agonist cocktail (10 μM each: (S)-3,5-Dihydroxyphenylglycine (DHPG), histamine, and carbachol). The majority of astrocytes in both G_q-DREADD × GFAP-tdTomato (19/27 in lamina II; 11/20 in lamina V) and GFAP-tdTomato (11/18 in lamina II; 5/8 in lamina V) slices responded to the agonist cocktail (Figure 10A-D). Quantification of astrocytes responding is summarized in Table 1. Overall, our findings demonstrate that G_q-DREADD is localized to astrocytes in laminae II and V and activates G_q-GPCR signaling cascades.

Table 1. Quantification of GFAP-tdTomato-positive astrocytes responding to CNO and cocktail in spinal cord slices from G_q-DREADD × GFAP-tdTomato mice.

Genotype/lamina	% cells responding to CNO	% cells responding to agonist cocktail
G_q-DREADD × GFAP-tdTomato Lamina II	13/27 (48%)	19/27 (70%)
G_q-DREADD × GFAP-tdTomato Lamina V	10/20 (50%)	11/20 (55%)
GFAP-tdTomato Lamina II	0/18 (0%)	11/18 (61%)
GFAP-tdTomato Lamina V	0/8 (0%)	5/8 (63%)

Many lamina II and V astrocytes in both GFAP-tdTomato and G_q-DREADD × GFAP-tdTomato slices responded to the positive control agonist cocktail with calcium increases. Astrocytes in lamina II and V from G_q-DREADD × GFAP-tdTomato slices responded to CNO while astrocytes in both lamina II and V from GFAP-tdTomato slices failed to respond to CNO with calcium increases.

4.4. DISCUSSION

Both immunohistochemistry and Western blot data demonstrated that G_q-DREADD is selectively expressed in glia. Stimulation of glial G_q-DREADD with CNO elicited calcium increases in lamina II and V spinal cord astrocytes. This finding suggests that G_q-DREADD signaling recapitulates that of endogenous glial G_q-GPCRs and further validates our

experimental model. Future studies will examine calcium signaling in peripheral $GFAP^+$ glia to bolster our observations by immunohistochemistry for G_q-DREADD expression in DRG and peripheral nerves.

All of our data suggests that G_q-DREADD is expressed in $GFAP^+$ glia. We however cannot exclude the possibility that G_q-DREADD is expressed in a small population of neurons not examined in our studies. Furthermore, we are assuming that G_q-DREADD signaling is comparable to that of endogenous GPCRs. Stimulation of G_q-DREADD expressed in cell lines activates MAPK signaling through interactions with β-arrestin similarly to activated native GPCRs (Armbruster et al., 2007; Armbruster and Roth, 2005). In our mouse model, G_q-DREADD may be expressed at significantly higher levels than endogenous G_q-GPCRs, and therefore the possibility exists that signaling might be altered. Now that the G_q-DREADD mouse model has been validated, we can investigate how glial G_q-GPCRs modulate pain and itch processing.

CHAPTER 5.

STIMULATION OF GLIAL G_Q-DREADD BLOCKS ACUTE PAIN.

5.1. INTRODUCTION

Excitatory and inhibitory synaptic drive must be exquisitely balanced to maintain CNS homeostasis. In chronic pain, dysregulation of synaptic drive occurs at multiple sites in the nervous system, including the dorsal horn of the spinal cord (Andrew and Craig, 2001; Basbaum, 1999). The causes of this dysregulation remain elusive, and a better understanding of the mechanisms of neuropathic pain may lead to novel treatments.

Changes in intracellular signaling systems lead to sensitization of the cells within the dorsal horn. While neurons have historically been the focus of studies on neuropathic pain, it is now clear that glia play an important role in the establishment and maintenance of chronic pain states (Scholz and Woolf, 2007). Glial fibrillary acidic protein positive (GFAP-positive) glia release a variety of molecules including inflammatory cytokines that lead to the chronic sensitization of nociceptive neurons (Milligan and Watkins, 2009). Astrocytes— a type of GFAP-positive glial cell— envelope tens of thousands of synapses (Oberheim et al., 2009). Astrocytes also regulate synaptic activity through the release of neuroactive molecules (Schell et al., 1995; Zhang et al., 2003; Eroglu and Barres, 2010; Bezzi et al., 2001; Bowser and Khakh, 2004) and by buffering potassium (D'Ambrosio et al., 1999) and glutamate (Huang et al., 2004). Thus, GFAP-positive glia are well-positioned to modulate synaptic activity related to pain processing.

The role that GFAP-positive glial G_q-GPCRs play in nociception is unknown. As neurons and GFAP-positive glia express many of the same GPCRs, it is difficult to selectively stimulate signaling pathways in GFAP-positive glia to evaluate the role of these pathways in physiology and pathology. Recently, a novel G_q-GPCR (G_q-DREADD or *Designer Receptor Exclusively Activated by Designer Drug*) was engineered to respond only to the pharmacologically inert ligand CNO (Armbruster et al., 2007). To determine how selective stimulation of glial G_q-GPCR signaling cascades affects nociception, we utilized a transgenic mouse line that expresses G_q-DREADD under the control of the GFAP promoter.

Our data strongly suggest that the G_q-DREADD mouse model allows selective stimulation of G_q-GPCR signaling pathways in GFAP-positive glia. Thus, the G_q-DREADD mouse is a powerful new tool for studying the role of glial signaling pathways in pain transmission neurocircuitry. The Hargreaves test was used to measure the effect of glial G_q-GPCR signaling on acute thermal nociceptive processing. Our findings suggest that GFAP-positive glial G_q-GPCR signaling pathways mediate antinociception in response to acute noxious thermal stimuli.

5.2. MATERIALS AND METHODS

5.2.1. Transgenic mice.

G_q-DREADD mice were bred to IP_3R2 KO mice (Li et al., 2005), and FosGFP mice (Barth et al., 2004).

5.2.2. Hargreaves test.

Each mouse was placed in a small clear container on an elevated glass plate. After 60 minutes of habituation, when the mice were inactive, but not asleep, a low intensity thermal

beam was applied to the footpad of each hind paw. The latency for each mouse to respond to this beam with a definite paw flick response was recorded. The beam was cut off after a maximum of 20s to prevent tissue damage. CNO was injected either intrathecally or intraperitoneally. For intrathecal injections, unanesthetized mice were injected using the direct lumbar puncture method (Fairbanks, 2003).

5.2.3. Von Frey test.

Each mouse was placed in a small clear container on an elevated mesh floor. Calibrated Von Frey hairs were applied to the plantar surface of the hind paws and the frequency to respond to noxious and innocuous stimuli as well as the paw withdrawal threshold were measured. For the frequency measurements, Von Frey hairs were applied ten times and the number of paw withdrawal responses was counted. The "up down" method was used to measure paw withdrawal threshold, ie. the force in grams at which the mouse withdrew its paw fifty percent of the time (Chaplan et al., 1994).

5.2.4. **C-fos Induction**.

For formalin induction, mice were injected in one plantar hind paw with 10 μL of 2% formalin. Two hours after formalin injection, anesthetized mice were perfused with 4% paraformaldehyde. Spinal cords were removed, post fixed, and cryoprotected in 20% sucrose/PBS before freezing. Frozen tissue was sectioned and fixed to slides. For visualization of c-fos-positive nuclei in FosGFP sections, sections were incubated with 1:500 mouse anti-GFP (Sigma), washed in PBS, and incubated with the fluorescent secondary antibody Alexa 594 conjugated goat anti-mouse. Quantification of c-fos positive nuclei was performed with ImageJ software with the cell counter plug-in.

5.2.5. Dorsal root stimulation.

Acute transverse (800-900 μm) spinal cord slices with one dorsal root were prepared (Wang and Zylka, 2009). The lumbar spinal cord was quickly removed from anesthetized mice and sectioned on a vibratome in oxygenated ice-cold slicing buffer. The slices were incubated in oxygenated ACSF at 37°C for 1 hour and then maintained at room temperature in oxygenated ACSF. For recordings, slices were placed in a chamber that was continuously perfused with room temperature, oxygenated ACSF. A suction-stimulating electrode was used to stimulate peripheral nociceptors in the dorsal root. A recording electrode was pulled from borosilicate glass to a resistance of 1-2 MΩ, filled with ACSF, and placed in lamina II to record evoked field excitatory post synaptic potentials. The amplitude of field potentials elicited by a 200 μs pulse was monitored and the strength of the stimulation was adjusted to achieve a half-maximal response. The root was stimulated at 0.1 Hz and every six signals were averaged to give a single point per minute of recording. Data analysis was performed using pClamp 10 software. The field potential waveform consisted of an early, narrow peak mediated by A-delta fibers and a later, broader peak mediated by C-fibers and polysynaptic activity. For each G_q-DREADD spinal cord slice, baseline data was recorded for five minutes, after which data were recorded for five minutes as 10 μM CNO was bath-applied and for the following twenty-minute wash period.

5.3. RESULTS

5.3.1. G_q-DREADD mice do not have a basal difference in mechanical sensitivity compared to WT littermates.

To determine whether G_q-DREADD mice have a basal difference in sensitivity to mechanical stimuli compared to WT littermates, we measured mechanical sensitivity to

punctate stimuli using calibrated Von Frey filaments (Chaplan et al., 1994). We found no difference in sensitivity to either a low-intensity, innocuous (0.4 g) mechanical stimulus (Frequency of paw withdrawal, WT = 2.3 ± 0.3, G_q-DREADD = 2.0 ± 0.2, WT n = 12, G_q-DREADD n = 24, student's T test p = 0.26, Figure 11A) or a high intensity, noxious (1.4 g) mechanical stimulus (Frequency of paw withdrawal, WT = 7.6 ± 0.4, G_q-DREADD = 7.4 ± 0.3, WT n = 12, G_q-DREADD n = 24, student's T test p = 0.63, Figure 11B) between G_q-DREADD mice and WT littermates. Finally, we measured paw withdrawal threshold and found no difference between G_q-DREADD mice and WT littermates (Paw withdrawal threshold, WT = 1.1 ± 0.1 g, G_q-DREADD = 1.2 ± 0.1 g, WT n = 12, G_q-DREADD n = 24, student's T-test, p = 0.48, Figure 11C).

5.3.2. Stimulation of spinal and/or dorsal root ganglia (DRG) glial G_q-DREADD causes an increase in withdrawal latency in an acute thermal nociceptive assay.

We next examined the effect of stimulating G_q-GPCR pathways in GFAP-positive glia on acute noxious thermal sensitivity using the Hargreaves method (Hargreaves et al., 1988). No differences in paw withdrawal latencies were seen in G_q-DREADD or WT mice prior to injection of CNO (Paw withdrawal latency at 0 minutes, WT = 10.8 ± 1.5 s, G_q-DREADD = 9.9 ± 1.6 s, Figure 12A). Injection of CNO (1 mg/kg intraperitoneally (I.P.)) caused large increases in paw withdrawal latency in G_q-DREADD but not WT mice. The effect occurred within 15 minutes of the injection and lasted for the 4-hour measurement period. (Paw withdrawal at 60 minutes, WT = 9.1 ± 1.6 s, G_q-DREADD = 16.0 ± 1.8 s; WT n = 9, G_q-DREADD n = 10, 2-way Repeated Measures ANOVA: genotype, $p < 0.001$, time course × genotype, p = 0.01). When G_q-DREADD and WT mice received 0.5 mg/kg CNO I.P., the same rapid and dramatic increase in paw withdrawal latency appeared in G_q-DREADD but

not WT mice (Paw withdrawal at 30 minutes, WT = 9.9 ± 1.5 s, G_q-DREADD = 16.9 ± 1.7 s; WT n = 9, G_q-DREADD n = 10, 2-way Repeated Measures ANOVA: genotype, $p < 0.01$, Figure 12B). However, the effect subsided after 2.5 hours. These data indicate that stimulation of G_q-GPCR pathways in GFAP-positive glia is antinociceptive in an acute model of thermal pain.

Nociceptive processing occurs in the peripheral nervous system, spinal cord, and supraspinal regions (Burgess and Perl, 1967; Harris, 1921; Brooks and Tracey, 2005; Urban and Gebhart, 1999). G_q-DREADD is expressed in GFAP-positive glia in the spinal cord as well as in supraspinal regions, DRG, and peripheral nerves (unpublished observations). To determine whether supraspinal regions were required for the antinociceptive thermal phenotype seen upon systemic glial G_q-DREADD stimulation, we performed the Hargreaves test following intrathecal (I.T.) injections of CNO or saline. I.T. injections are used to deliver drugs to both the spinal cord (Hylden and Wilcox, 1980) and DRG (Michael et al., 1997) while excluding supraspinal regions. No significant differences in paw withdrawal latencies were seen in G_q-DREADD or WT mice prior to I.T. injection of CNO or saline (Paw withdrawal latency at 0 minutes, WT/saline = 10.1 ± 1.3 s, G_q-DREADD/saline = 9.4 ± 0.9 s, WT/CNO = 11.7 ± 1.0 s, G_q-DREADD/CNO = 11.2 ± 1.1 s, Figure 12C). I.T. injection of saline did not affect paw withdrawal latency for either G_q-DREADD or WT mice (Paw withdrawal latency at 60 minutes, WT/saline = 8.0 ± 1.3 s, G_q-DREADD/saline = 9.0 ± 0.9 s, WT/saline n = 10, G_q-DREADD/saline n = 18). Injection of CNO (30 nmol, I.T.) caused a marked increase in paw withdrawal latency in G_q-DREADD but not WT mice, an effect that lasted for 4 hours (Paw withdrawal latency at 60 minutes, WT/CNO = 9.3 ± 1.6 s, G_q-DREADD/CNO = 15.9 ± 1.2 s, WT/CNO n = 10, G_q-DREADD/CNO n = 17, 2-way

Repeated Measures ANOVA: genotype, $p < 0.01$, time course × genotype, $p < 0.001$). A lower dose of CNO (1 nmol, I.T.) did not affect paw withdrawal latency for either G_q-DREADD or WT mice (Paw withdrawal latency at 60 minutes, WT = 11.2 ± 0.6 s, G_q-DREADD = 11.3 ± 0.7 s, WT n = 8, G_q-DREADD n = 10, 2-way Repeated Measures ANOVA: genotype, $p = 0.62$, Figure 12D). Thus, the ability of intrathecal CNO to decrease thermal sensitivity for G_q-DREADD mice is dose-dependent. Our findings support an antinociceptive role for G_q-GPCR pathways in GFAP-positive glia in the spinal cord and/or DRG in regards to acute thermal sensitivity.

5.3.3. Stimulation of glial G_q-DREADD causes antinociception in an IP_3R2-independent manner.

Calcium increases are a major mode of excitability for astrocytes (Cornell-Bell et al., 1990b). Astrocyte calcium increases are linked to several physiological phenomena ranging from gliotransmission— the release of neuroactive molecules such as glutamate, adenosine triphosphate (ATP), and D-serine from glia— (Parpura et al., 1994) and modulation of cerebrovascular dynamics (Straub and Nelson, 2007) to pathophysiological states such as epilepsy (Kang et al., 2005). Stimulation of G_q-GPCRs triggers phospholipase C (PLC) hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and diacylglycerol (DAG) (Michell, 1975). IP_3 binds IP_3 receptors on the endoplasmic reticulum (ER) and induces calcium release (Streb et al., 1983). IP_3R2 is necessary for astrocytic, but not neuronal, calcium increases in the hippocampus (Petravicz et al., 2008).

To test whether IP_3R2 mediates astrocytic calcium increases in the spinal cord dorsal horn, we performed calcium-imaging experiments in spinal cord slices from IP_3R2 knockout (KO) mice. Cells in WT and KO spinal cord slices were loaded with the calcium indicator

dye fluo-4 (Figure 13A). While an agonist cocktail elicited calcium increases from the majority of cells (91.9%; 34/37 cells) in lamina II in WT slices, none of the cells in lamina II in slices from IP_3R2 KO mice responded (0%; 0/27 cells; Figure 13B-C.) These results support our hypothesis that calcium increases in the spinal cord dorsal horn depend on the presence of IP_3R2.

Gliotransmission modulates baseline mechanical nociception (Foley et al., 2011). To test whether the G_q-DREADD-mediated antinociception to an acute noxious thermal stimulus depended on the ability of astrocytes to produce calcium increases from the ER, we bred G_q-DREADD mice to IP_3R2 KO mice. In the Hargreaves test, none of the mice exhibited significant differences in basal thermal sensitivity (Paw withdrawal latency at 0 minutes, WT/saline = 9.7 ± 1.3 s, WT/CNO = 9.8 ± 1.4 s, G_q-DREADD/saline = 9.0 ± 1.3 s, G_q-DREADD/CNO = 8.0 ± 1.4 s, IP_3R2 KO/saline = 10.1 ± 1.4 s, IP_3R2 KO/CNO = 9.6 ± 2.1 s, G_q-DREADD × IP_3R2 KO/saline = 7.4 ± 1.0 s, G_q-DREADD × IP_3R2 KO/CNO = 11.1 ± 1.7 s, WT/saline n = 12, WT/CNO n = 12, G_q-DREADD/saline n = 8, G_q-DREADD/CNO n = 8, IP_3R2 KO/saline n = 5, IP_3R2 KO/CNO n = 5, G_q-DREADD × IP_3R2 KO/saline n = 5, G_q-DREADD × IP_3R2 KO/CNO n = 5, Figure 13D-E). Mice then received intrathecal injections of either saline or 30 nmol CNO. Saline did not affect the withdrawal latency of G_q-DREADD, WT, IP_3R2 KO, or G_q-DREADD × IP_3R2 KO mice (Paw withdrawal latency at 60 minutes, WT/saline = 8.1 ± 1.3 s, G_q-DREADD/saline = 8.5 ± 1.8 s, IP_3R2 KO/saline = 10.7 ± 1.5 s, G_q-DREADD × IP_3R2 KO/saline = 6.3 ± 1.4 s). CNO had no discernable effect on WT or IP_3R2 KO mice but caused a long lasting increase in paw withdrawal latency for both G_q-DREADD and G_q-DREADD × IP_3R2 KO mice (Paw withdrawal latency at 60 minutes, WT/CNO = 9.5 ± 1.4 s, G_q-DREADD/CNO = 17.4 ± 1.4 s, IP_3R2 KO/CNO = 8.3 ±

1.4 s, G_q-DREADD × IP_3R2 KO/CNO = 18.0 ± 1.8 s, 2-way Repeated Measures ANOVA: genotype, $p < 0.05$). The antinociceptive effect of G_q-DREADD stimulation on thermal sensitivity appears to be independent of the ability of G_q-GPCRs on astrocytes to generate calcium increases from the ER.

5.3.4. G_q-DREADD stimulation decreases the response to an acute noxious heat stimulus.

A variety of noxious stimuli have been shown to induce c-fos in the dorsal horn of the spinal cord (Hunt et al., 1987). To test the hypothesis that c-fos is a marker for changes in dorsal horn neuronal activity mediated by G_q-DREADD activation, we performed intraplantar injection of the hind paw with formalin to induce c-fos in the dorsal horn (Figure 14). Intraplantar formalin is well validated in generating acute inflammatory pain (Tjolsen et al., 1992). FosGFP transgenic mice (Barth et al., 2004) were bred with G_q-DREADD mice to allow fluorescent labeling of c-fos positive cells.

Intraplantar formalin injection induced c-fos in the ipsilateral— but not contralateral— superficial dorsal horn for both G_q-DREADD × FosGFP mice pretreated with saline (n=2) or CNO (n=2) and control FosGFP mice pretreated with saline (n=1). Regardless of genotype or drug, significantly more c-fos was induced in the ipsilateral dorsal horn than in the contralateral dorsal horn (Fold change in c-fos positive nuclei in ipsilateral vs. contralateral dorsal horn, control FosGFP/saline = 3.3 ± 0.5, G_q-DREADD × FosGFP /saline = 3.2 ± 0.4, G_q-DREADD × FosGFP/CNO = 2.9 ± 0.6). These data suggest that while noxious stimuli induce c-fos in the dorsal horn, c-fos is not a useful marker for neuronal changes elicited by stimulation of G_q-GPCR pathways in GFAP-positive glia.

5.3.5. DREADD stimulation does not affect excitatory synaptic drive from the periphery to lamina II.

Primary afferents have their central projections in laminae I and II of the dorsal horn of the spinal cord (Light and Perl, 1979a; Light and Perl, 1979b). In acutely isolated spinal cord slices, the dorsal root can be stimulated to generate evoked field potentials in lamina II (Figure 15A) (Street et al., 2011). To test whether the antinociceptive phenotype seen upon DREADD stimulation was due to modulation of excitatory drive from peripheral nociceptors, we measured the effect of DREADD stimulation on evoked excitatory field potentials in lamina II (Figure 15B). Applying CNO to DREADD spinal cord slices did not affect either the magnitude or kinetics of evoked field potentials in lamina II. Therefore, stimulation of G_q-GPCR pathways in $GFAP^+$ glia does not appear to modulate excitatory drive from peripheral nociceptors.

5.4. DISCUSSION

In this study, we examined glial signaling in acute pain using the novel GFAP G_q-DREADD mouse model. G_q-DREADD and WT mice do not differ in basal mechanical sensitivity. Stimulation of glial G_q-DREADD caused antinociception in response to an acute noxious heat stimulus. This antinociceptive phenotype involves spinal and/or DRG glial G_q-DREADD. Astrocyte calcium increases via IP_3 receptors have been implicated in astrocyte modulation of neuronal activity (Sheppard et al., 1997; Weerth et al., 2007). Removal of IP_3R2 obliterated G_q-GPCR-mediated calcium increases in acute spinal cord slices. However, CNO-injection of G_q-DREADD × IP_3R2 KO mice caused antinociception of a similar magnitude as G_q-DREADD mice in response to acute noxious heat. Stimulation of glial G_q-DREADD modulates nociceptive neurocircuitry resulting in our observed phenotype.

C-fos has been used as a marker of dorsal horn neural activity in response to nociceptive stimuli. Intraplantar injection of formalin induced c-fos in the ipsilateral dorsal horn; c-fos expression was unaffected by stimulation of G_q-DREADD. Primary afferents release glutamate at synapses in lamina II of the dorsal horn; this can be detected as field potentials mediated by AMPA receptors (Street et al., 2011). G_q-DREADD stimulation did not modulate excitatory neurotransmission at nociceptor-lamina II synapses.

The observation that stimulation of glial G_q-GPCR pathways is antinociceptive surprised us, as most researchers have described a pro-nociceptive role for glia (Guo et al., 2007; Watkins and Maier, 2005). However, these studies have focused on chronic pain rather than the acute pain paradigm that we studied. Furthermore, chronic pain models often cause astrogliosis (Sweitzer et al., 2001). Reactive astrocytes, unlike quiescent astrocytes, release inflammatory cytokines (Milligan et al., 2003; Raghavendra et al., 2004; Guo et al., 2007; Ledeboer et al., 2005) and experience impaired potassium and glutamate buffering (D'Ambrosio et al., 1999; Rao et al., 1998; Fine et al., 1996); these changes all lead to increased pain sensitivity. Our study provides insight into the role of glial signaling in acute pain.

What could GFAP-positive glia do that would result in an antinociceptive phenotype? Stimulation of astrocyte G_q-GPCR pathways has been linked to a number of processes that modulate neuronal activity; these include gliotransmission, potassium and glutamate buffering, and synapse formation. Blocking G_q-GPCR-mediated, calcium-dependent, vesicular release from glia caused an increase in basal mechanical sensitivity (Foley et al., 2011). This finding — that gliotransmission and pain sensitivity are inversely related—

corresponds to our observation that stimulating glial G_q-GPCR pathways, which would presumably enhance gliotransmission, is antinociceptive.

ATP— which has been proposed as a gliotransmitter— is converted to adenosine in the extracellular space. Activation of the adenosine A1 receptor has been shown to be antinociceptive in both humans (Eisenach et al., 2003; Karlsten and Gordh, 1995) and rodents (Gomes et al., 1999; Sawynok et al., 1986). In addition, astrocyte G_q-GPCR signaling has been shown to cause potassium uptake into astrocytes (Wang et al., 2012), which could decrease neuronal excitability and thus block pain transmission.

Glia modulate pain by releasing neuroactive substances (Guo et al., 2007; Scholz and Woolf, 2007; Ohtori et al., 2004), yet the location in the pain processing neurocircuitry where glia exert their effects is unknown. The fact that intrathecal CNO causes antinociception in G_q-DREADD mice suggests that spinal cord astrocytes and/or satellite glia in DRG are responsible for the reduction in sensitivity to noxious heat. While most of the glial biology literature has focused on the role of spinal cord astrocytes in modulation of pain transmission, it is clear that satellite glial cells may also play a role in pathogenesis of pain (Vit et al., 2008; Jasmin et al., 2010). Additional study will be needed to determine whether G_q-GPCR signaling in astrocytes, satellite glial cells, or both regulate acute noxious thermal sensitivity.

IP_3R2-dependent astrocyte calcium increases are implicated in modulating neuronal excitability, synaptic plasticity, and cerebrovascular tone (Scemes and Giaume, 2006). Indeed, removal of IP_3R2 abolishes astrocyte calcium increases in the brain (Takata et al., 2011; Petravicz et al., 2008) and spinal cord. Yet, stimulation of G_q-GPCR pathways on GFAP-positive glia decreases thermal pain sensitivity in an IP_3R2-independent manner.

G_q-GPCR calcium-independent effectors such as Gβγ or DAG may prove to be responsible for the decrease in thermal pain sensitivity upon glial G_q-DREADD stimulation. Activation of p63RhoGEF-dependent pathways is another calcium-independent G_q-GPCR signaling pathway (Rojas et al., 2007).

Alternatively, there may be other sources for astrocyte calcium besides release from intracellular stores. For example, TRPA1 channels function as a transmembrane calcium source for astrocytes and may be sufficient to maintain calcium-dependent signaling (Shigetomi et al., 2011). Clearly, the role of calcium in glial biology has yet to be fully resolved.

While G_q-DREADD does not appear to modulate excitatory synaptic transmission from the periphery to lamina II, this does not exclude the possibility that our phenotype is being mediated by modulation of inhibitory synaptic transmission or by modulation of wide dynamic range neurons in lamina V. A loss of inhibitory synaptic tone in the dorsal horn has been shown to contribute to neuropathic pain (Moore et al., 2002; Ibuki et al., 1997). Astrocyte calcium signaling has been shown to potentiate inhibitory synaptic transmission in the hippocampus (Kang et al., 1998). If glial GPCR signaling increased inhibitory drive in the spinal cord, this would be antinociceptive. Future studies will examine how glial GPCR signaling changes synaptic transmission.

In conclusion, our data demonstrate that GFAP-positive glial G_q-GPCR pathways are antinociceptive for acute noxious thermal stimuli. This is the first study to demonstrate that GFAP-positive glial G_q-GPCR pathways play a role in pain. Further study will be needed to uncover the mechanism by which glial G_q-GPCRs cause antinociception.

CHAPTER 6.

STIMULATION OF GLIAL G_Q-DREADD BLOCKS ACUTE ITCH.

6.1. INTRODUCTION

Chronic itch is a significant clinical problem that lacks effective treatments (Oaklander, 2011). New therapeutics will require understanding of signaling mechanisms involved in itch. To this end, we used the histamine-induced itch assay (Wilson et al., 2011) to measure the effect of glial G_q-GPCR signaling on acute itch processing. Our findings suggest that GFAP-positive glial G_q-GPCR signaling pathways block acute histamine-dependent itch.

6.2. METHODS

6.3. ITCH BEHAVIORAL TEST.

Intradermal histamine was used to generate acute itch in mice (Wilson et al., 2011). Mice were habituated to the testing environment for 30 minutes before testing and then injected intradermally in the nape of the neck with 500 μg of histamine dissolved in 50 μL saline. Finally, the number of scratching bouts over 30 minutes was counted. A scratching bout was defined as the mouse using its hind paw to scratch the site of histamine injection.

6.4. RESULTS

6.4.1. Glial G_q-DREADD stimulation blocks histamine-induced itch.

Many pathologies that cause neuropathic pain also cause neuropathic itch (Oaklander, 2011). As glial G_q-DREADD stimulation is antinociceptive for acute noxious thermal

stimuli, we conducted acute itch assays to determine whether glial G_q-DREADD stimulation also modulates itch-related behaviors. We injected histamine intradermally into the nape of the neck, as this has been demonstrated to induce scratching in rodents (Lee et al., 2011). Intradermal histamine robustly caused itch in WT mice pretreated with saline or CNO (0.5 mg/kg, I.P.) and G_q-DREADD mice pretreated with saline (total number of scratches: WT/saline = 98.1 ± 14.1, WT/CNO = 76.2 ± 11.5, G_q-DREADD/saline = 130.7 ± 17.9, WT/saline n = 6, WT/CNO n = 6, G_q-DREADD/saline n = 7, Figure 16). Pretreatment of G_q-DREADD mice with CNO (0.5 mg/kg, I.P.) completely blocked histamine-induced itch (total number of scratches: G_q-DREADD/CNO = 0.7 ± 0.7, G_q-DREADD/CNO n = 7, 2-way ANOVA: CNO factor, $p < 0.001$ Figure 16).

6.4.2. Systemic inhibition of glial G_q-DREADD restores histamine-induced itch.

To confirm that the antipruritic phenotype is mediated through G_q-DREADD, we used scopolamine—a CNS-permeable muscarinic antagonist— to rescue histamine-induced itch in CNO-injected G_q-DREADD mice (Figure 18A). Again, CNO injection of G_q-DREADD mice blocked histamine-induced itch. While scopolamine treatment alone had no effect at a dose of 1 mg/kg, it slightly decreased histamine-dependent itch at a dose of 10 mg/kg. When blocking peripheral and central glial G_q-DREADD, 1 mg/kg scopolamine did not rescue histamine-induced itch in CNO-injected G_q-DREADD mice (total number of scratches: saline/saline = 126.9 ± 20.8, saline/CNO, = 0.5 ± 0.4, 1 mg/kg scopolamine/saline = 125.8 ± 30.2, 1 mg/kg scopolamine/CNO = 3.3 ± 1.2, saline/saline n = 8, saline/CNO n = 8, 1 mg/kg scopolamine/saline n = 4, 1 mg/kg scopolamine/CNO n = 4, 2-way ANOVA: CNO treatment, $p < 0.0001$, Scopolamine treatment, $p = 0.63$, Figure 17A). However, 10 mg/kg scopolamine partially rescued histamine-induced itch in CNO-injected G_q-DREADD mice

(total number of scratches: 10 mg/kg scopolamine/saline = 63.7 ± 33.7, 10 mg/kg scopolamine/CNO = 31.8 ± 12.3, 10 mg/kg scopolamine/saline n = 3, 10 mg/kg scopolamine/CNO n = 6, 2-way ANOVA: interaction between CNO and scopolamine treatment p = 0.04, Figure 17A). The fact that 10 mg/kg scopolamine partially rescued histamine-induced itch in CNO-injected G_q-DREADD mice confirms that glial G_q-GPCR signaling can modulate acute itch.

To measure the effect of scopolamine itself on histamine-induced itch, we measured histamine-induced itch responses in WT mice pretreated with saline or scopolamine. Histamine-dependent itch in WT mice was significantly affected by pretreatment with 10 mg/kg scopolamine but not by a 1 mg/kg dose (total number of scratches: WT/saline = 99.3 ± 12.0, WT/1 mg/kg scopolamine = 77.9 ± 11.3, WT/10 mg/kg scopolamine = 46.9 ± 11.3, WT/saline n = 12, WT/1 mg/kg scopolamine n = 7, WT/10 mg/kg scopolamine n = 8, 1-way ANOVA: scopolamine treatment, p = 0.02, Figure 17B). The decrease in histamine-induced itch in WT mice at the higher dose of scopolamine compared to saline-injected mice is likely due to scopolamine's effects at endogenous muscarinic receptors.

6.4.3. Inhibition of peripheral glial G_q-DREADD restores histamine-induced itch.

We next determined whether the antipruritic phenotype was centrally and/or peripherally mediated. Scopolamine methyl bromide (SMB)— a central nervous system (CNS) impermeable muscarinic antagonist— also blocks G_q-DREADD. G_q-DREADD mice were injected with either saline or SMB (1 or 10 mg/kg, I.P.) prior to CNO/saline and histamine injections. As expected, G_q-DREADD mice pretreated with saline or either dose of SMB and then injected with saline experienced normal histamine-induced itch (total number of scratches: saline/saline = 108.4 ± 20.7, 1 mg/kg SMB/saline = 129.6 ± 15.4, 10 mg/kg

SMB/saline = 139.8 ± 21.0, saline/saline n = 5, 1 mg/kg SMB/ saline n = 5, 10 mg/kg SMB/ saline n = 5, Figure 18A). As seen previously (Figure 16), G_q-DREADD mice pretreated with saline prior to CNO injection did not experience histamine-induced itch (total number of scratches: saline/CNO = 11.0 ± 5.8, n = 7, 2-way ANOVA: CNO treatment, $p < 0.0001$, Figure 18A). When blocking peripheral glial G_q-DREADD, 1 mg/kg SMB did not rescue histamine-induced itch and 10mg/kg SMB partially restored histamine-induced itch in CNO-injected G_q-DREADD mice (total number of scratches: 1 mg/kg SMB/CNO = 16.8 ± 8.7, 10 mg/kg SMB/CNO = 47.0 ± 11.6, 1 mg/kg SMB/CNO n = 6, 1 mg/kg SMB/CNO n = 5, two-way ANOVA: SMB treatment, $p = 0.06$).

To determine whether SMB by itself effects histamine-induced itch, we performed acute itch experiments with WT mice and two doses of SMB. WT mice injected with 1 mg/kg SMB or 10 mg/kg SMB experienced less histamine-induced itch than saline-injected mice (total number of scratches: WT/saline = 151.7 ± 10.0, WT/1 mg/kg SMB = 86.0 ± 17.0, WT/10 mg/kg SMB = 83.2 ± 11.1, WT/saline n = 6, WT/1 mg/kg SMB n = 6, WT/10mg/kg SMB n = 5, 1-way ANOVA with Bonferroni's post-hoc tests were used to compare each drug treatment to saline: $p < 0.05$, Figure 18B). The decrease in histamine-induced itch in SMB-injected mice compared to saline-injected mice is likely due to SMB's effects at endogenous muscarinic receptors.

The fact that SMB and scopolamine had similar effects on histamine-induced itch in CNO-injected G_q-DREADD mice indicates that stimulation of G_q-DREADD in peripheral GFAP-positive glia— non-myelinating Schwann cells and/or satellite glial cells— blocks acute histamine-dependent itch. The small but significant reduction in histamine-induced itch in SMB-treated and scopolamine-treated WT mice may explain why both scopolamine and

SMB gave a partial, rather than full, rescue of histamine-induced itch in CNO-injected G_q-DREADD mice.

6.5. DISCUSSION

Almost nothing is currently known about the role of glia in itch perception. Stimulation of G_q-GPCR pathways in GFAP-positive glia obliterated acute histamine-dependent itch. Pharmacological data suggest that peripheral glial G_q-GPCR signaling pathways are involved in blocking histamine-induced itch. Peripheral C-fibers, which have been implicated in the transmission of pruritogenic stimuli, are closely associated with non-myelinating Schwann cells (Schmelz et al., 1997). Non-myelinating Schwann cells express GFAP (Jessen et al., 1990) and respond to stimulation of purinergic receptors by releasing calcium from intracellular stores (Mayer et al., 1998). Non-myelinating Schwann cells also provide trophic support to C-fibers (Chen et al., 2003). Since G_q-DREADD has been detected in both non-myelinating Schwann cells and in satellite glial cells in G_q-DREADD mice (data not shown), it seems likely that G_q-GPCR signaling in peripheral GFAP-positive glia mediates the anti-itch phenotype. Further study will be needed to determine the mechanism by which glia inhibit itch.

There are no effective therapeutics for chronic itch. Our research focused on the role of glial signaling on acute itch. Future study will be needed to determine whether glial G_q-GPCR signaling pathways modulate chronic itch states. The majority of therapeutically useful agents target GPCRs (Pierce et al., 2002). A greater understanding of glial modulation of itch neurocircuitry may lead to improved treatments for itch.

CHAPTER 7.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1. CURRENT PROBLEMS IN PAIN AND ITCH RESEARCH

Researchers have developed few effective treatments for chronic pain and itch over the last 50 years, largely because despite a tremendous basic research effort, we still do not completely understand the mechanisms of these disorders. This is astounding when we consider that chronic pain and itch affect 30.7% and 23% of adults, respectively (Johannes et al., 2010; Matterne et al., 2009).

The vast majority of studies on pain and itch rely on animal models for ethical reasons. Unfortunately, animal models of pain face serious limitations that hamper our ability to study these phenomena. Mechanical allodynia is hypersensitivity to normally innocuous tactile stimuli whereas exaggerated responses to noxious thermal stimuli is the definition of thermal hyperalgesia. Since animals cannot speak, it is difficult for pain researchers to assess spontaneous pain in animals. Researchers are generally limited to measuring hypersensitivity evoked by mechanical or thermal stimuli. However, the primary complaint of pain patients is spontaneous pain, not mechanical allodynia or thermal hyperalgesia (Mogil and Crager, 2004). While the Grimace Scale has been recently developed to measure spontaneous pain in rodents, it remains laborious to use and therefore is unlikely to be widely adapted by basic pain researchers (Langford et al., 2010; Sotocinal et al., 2011). The basic pain research community would benefit greatly from the development of a simple, high-throughput, non-invasive method for measuring spontaneous pain in animal models.

Itch research using animal models faces technical limitations similar to that those encountered in pain research. Applying a pruritic stimulus to a human subject elicits two kinds of response: a verbal response (i.e. "I feel itchy") and a site-directed response (ie. scratching the site of pruritogen application). Obviously, in animals, researchers can only measure site-directed responses. The standard method for measuring acute itch in mice is to intradermally inject a pruritogen, such as histamine, into the nape of the neck and measure the resulting scratch bouts (Green et al., 2006). Algesic agents such as capsaicin also elicit scratching in mice when applied in this manner since no other behavioral response is possible at this site (Shimada and LaMotte, 2008). Thus, the nape of the neck cannot be used to behaviorally differentiate between chemicals that evoke itch and those that cause pain.

These technical limitations may be partially bypassed by a "cheek model of itch" recently developed for use in mice (Shimada and LaMotte, 2008). When histamine and capsaicin are injected into the mouse cheek, they produce two site-directed behaviors: the former evokes scratching with the hind limb whereas the latter elicits wiping with the forelimb. Future studies of pain and itch using mouse models would be well advised to employ behavioral assays that can distinguish between pain and itch.

7.2. THE G_Q-DREADD MOUSE MODEL

7.2.1. Advantages of the G_q-DREADD mouse model.

The G_q-DREADD mouse can be used as a model for studying the effects of selectively stimulating G_q-GPCR pathways in $GFAP^+$ glia *in vivo*. A major advantage of the G_q-DREADD mouse model is that injection of the DREADD agonist, CNO, is easy and relatively non-invasive. *In vitro* studies show that stimulating G_q-DREADD with CNO causes calcium increases in spinal cord glia and other regions of the CNS (unpublished

observations). While we have yet to examine other components of the G_q-GPCR signaling pathway, our calcium imaging data suggests that G_q-DREADD signaling recapitulates endogenous GPCR signaling. In addition, the ability to control glial G_q-GPCR signaling raises the tantalizing prospect of translational applications for G_q-DREADD. A majority of FDA approved drugs are taken orally and act through GPCRs (Rogan and Roth, 2011). Perhaps in the future, G_q-DREADD technology will be harnessed to control glial G_q-GPCR signaling in humans in order to treat nervous system disorders. For example, if glial G_q-GPCR signaling is found to block chronic pain as well as acute pain, then chronic pain patients might benefit from intrathecal injections of viral vectors that drive G_q-DREADD expression in glia.

7.2.2. Disadvantages of the G_q-DREADD mouse model.

The greatest disadvantage of the G_q-DREADD mouse model is that systemic injection of CNO causes a range of phenotypes—increased heart rate, increased saliva formation, sedation—that are unrelated to pain or itch. While intrathecal delivery of CNO appears to cause antinociception without these additional phenotypes, this delivery method is only useful for measuring pain and itch sensitivity in the hind limbs. Another disadvantage of the G_q-DREADD mouse model is its low temporal resolution. Systemic delivery of CNO causes G_q-DREADD activation in tens of minutes which is much slower than the activation of endogenous GPCRs. Cannulas can be placed in the brain to reduce the time needed to deliver CNO to selected brain regions, but this method of drug delivery is neither easy nor non-invasive.

While this dissertation focuses on the role of G_q-GPCR signaling, G_i-coupled and G_s-coupled DREADDs have also been developed. As CNO is the only known agonist for all

three DREADD signaling systems, multiple DREADDs cannot be studied in the same cell or mouse model. Finally, while CNO is a good ligand in mice— it is highly bioavailable, penetrates the BBB, and does not undergo back-metabolism to clozapine, which has activity at more than 50 neuronal targets (Rogan and Roth, 2011) — CNO is not appropriate for use in humans because of significant back-metabolism to clozapine. Therefore, any therapeutic use of DREADD technology in humans will require the development of novel DREADD ligands.

7.3. POSSIBLE SIGNALING MECHANISMS

The G_q-DREADD receptor was generated by making point mutations in the 3rd and 5th transmembrane regions of the endogenous M_3 muscarinic receptor (Armbruster et al., 2007). Since the M_3 muscarinic receptor is a G_q-coupled GPCR, it is likely that G_q-DREADD also signals through G_q-coupled GPCR pathways. An activated G_q-coupled GPCR stimulates PLC, which metabolizes PIP_2 into the second messengers IP_3 and DAG. Some of the downstream signaling effectors that could be responsible for mediating the antinociceptive and antipruritic phenotypes seen in the G_q-DREADD mouse have been discussed in Chapters 5 and 6 respectively.

In the G_q-DREADD mouse, G_q-DREADD is presumably localized to the plasma membrane of GFAP-positive cells. A recently developed approach allows researchers to target DREADD to distinct neuronal subdomains (Dong et al., 2010). Glia also have distinct subdomains where GPCR activation could possibly differentially activate signaling cascades. For example, astrocytes have fine processes that envelope synapses and contact the cerebrovasculature (Halassa et al., 2007; Petzold and Murthy, 2011; Giaume et al., 2010); there may be different local signaling machinery and scaffolds at these subdomains. The

antinociceptive and antipruritic phenotypes that we observed in the G_q-DREADD mouse may depend on activation of G_q-GPCRs in specific glial subdomains.

Finally, while much of the glial research community has focused on G protein-dependent aspects of G_q-GPCR signaling, G protein-independent signaling via β-arrestins may mediate the effects of certain drugs (Rogan and Roth, 2011). For example, clinically effective antipsychotics appear to antagonize GPCR signaling through β-arrestins (Masri et al., 2008). It is possible that G protein-independent signaling pathways could play a role in the ability of G_q-DREADD stimulation to block acute pain and itch.

7.4. FUTURE DIRECTIONS

The G_q-DREADD mouse model represents a unique tool for studying the role of glia in pain and itch. In Chapter 4, we used immunohistochemistry and calcium imaging studies to demonstrate that the G_q-DREADD mouse is a useful tool for studying G_q-GPCR signaling in GFAP-positive glia. While G_q-DREADD expression has been extensively characterized in the PNS and CNS by immunohistochemistry, functional characterization of G_q-DREADD by calcium imaging has largely been limited to the spinal cord dorsal horn, hippocampus, and visual cortex. Furthermore, with the exception of *in vivo* studies performed in the visual cortex, most of these calcium imaging studies have used acutely isolated slices. Future studies to examine G_q-DREADD-mediated calcium signaling in peripheral glia would add to our understanding of glial GPCR signaling in the PNS. In addition, it would be interesting to compare *in vivo* calcium imaging of the dorsal horn with *in vitro* acute slice calcium imaging data. In acute slice experiments, agonist-mediated receptor desensitization occurs within minutes of agonist application; this event terminates calcium increases. However, preliminary evidence from *in vivo* calcium imaging suggests that an I.P. injection of CNO in

G_q-DREADD mice causes calcium increases in cortical astrocytes that continue for hours. CNO injection in G_q-DREADD mice also causes antinociception in response to noxious heat that lasts for hours. Further study will be needed to resolve this seeming disconnect between *in vivo* and *in vitro* data.

In Chapter 5, we examined the role of glial GPCR signaling in acute pain. Future pain studies should focus on elucidating signaling pathways involved in the antinociceptive phenotypes. One potential method is to use pharmacological antagonists to block the antinociceptive phenotype seen in the Hargreaves test. Potential candidates for the antinociceptive phenotype include adenosine and endorphins.

While our finding that glial GPCRs modulate acute pain is novel and exciting, chronic— rather than acute— pain is clinically relevant. Our lab is proficient in using the spared nerve injury (SNI) model of chronic neuropathic pain in mice (Shields et al., 2003). This model causes long lasting, robust mechanical allodynia, which can easily be assessed using calibrated Von Frey hairs. G_q-DREADD mice do not appear to differ from WT littermates in their mechanical sensitivity. Insofar as we have observed antinociception to noxious heat, it seems worth investigating whether CNO injection of naïve G_q-DREADD mice causes also antinociception to mechanical stimuli. Then the role of glial G_q-GPCR signaling in chronic pain could be assessed by performing SNI on G_q-DREADD mice and measuring mechanical sensitivity at various time points after post-surgery injection of CNO or saline.

In Chapter 6, we examined the role of glial G_q-GPCR signaling in acute itch. Future studies would expand on our understanding of the antipruritic phenotype seen upon stimulation of glial G_q-DREADD in the acute histamine-induced itch assay. While our

preliminary data suggest that peripheral G_q-DREADD signaling mediates the antipruritic phenotype, the molecular mechanism remains unknown. The isolated skin-nerve preparation allows thermal, mechanical, and chemical stimuli to be applied to the skin and the evoked action potentials of single fibers to be measured (Reeh, 1986). This paradigm could be used to test the hypothesis that glial G_q-DREADD stimulation blocks acute histamine-induced itch by decreasing the activity of C-fibers. This would require making a skin-nerve preparation from G_q-DREADD mice, applying histamine to the skin to evoke C fiber responses, and then applying CNO or saline to the skin and recording any changes in C fiber responses.

7.5. **SUMMARY AND CONCLUSIONS**

Chronic pain and itch are highly prevalent and devastating disorders that lack effective treatments. The G_q-DREADD mouse model represents a powerful new tool for selectively activating glial G_q-GPCR pathways *in vivo*. The experiments presented herein provide insight into the previously unknown role of glial G_q-GPCR pathways in acute pain and itch. The current state of research generally supports a role for astrocytes in enhancing chronic pain states. Our finding that glial G_q-GPCR pathways can block acute pain and itch provides insight into an additional physiological role for glia. Further examination of the role of glial GPCRs in sensory disorders may lead to the identification of novel targets for drug development.

APPENDIX A.

FIGURES

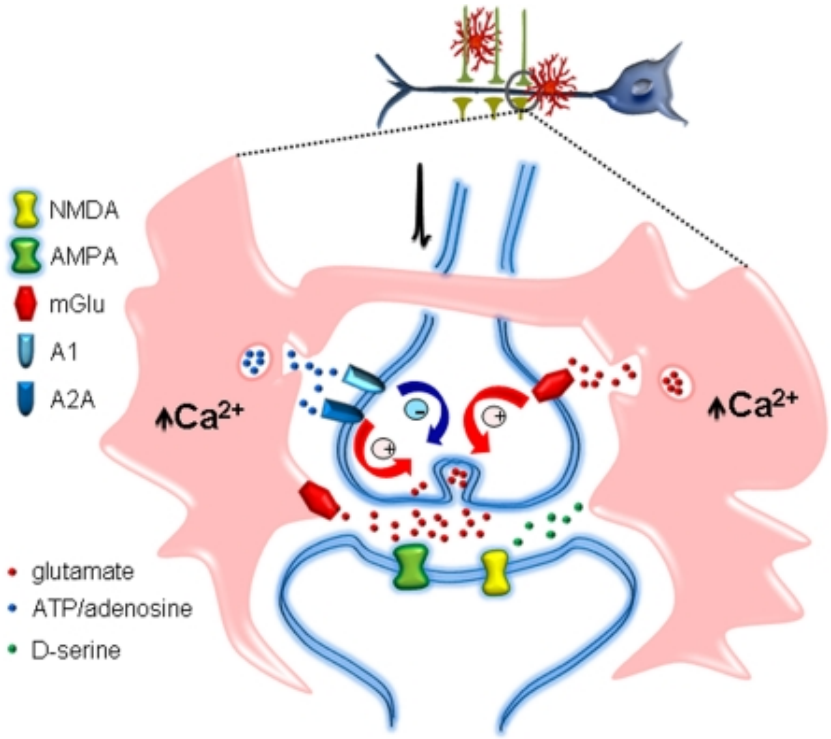

Figure 1. The Tripartite System.

Astrocyte fine processes contact the presynaptic and postsynaptic neurons. Stimulation of astrocyte calcium is thought to lead to release of gliotransmitters, glutamate, ATP/adenosine, and D-serine. Gliotransmitters act at Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), NMDA, metabotropic glutamate (mGluR), and A1/A2 adenosine receptors located on either the pre- or post-synaptic neuron. Image reprinted from Zorec et al., 2012.

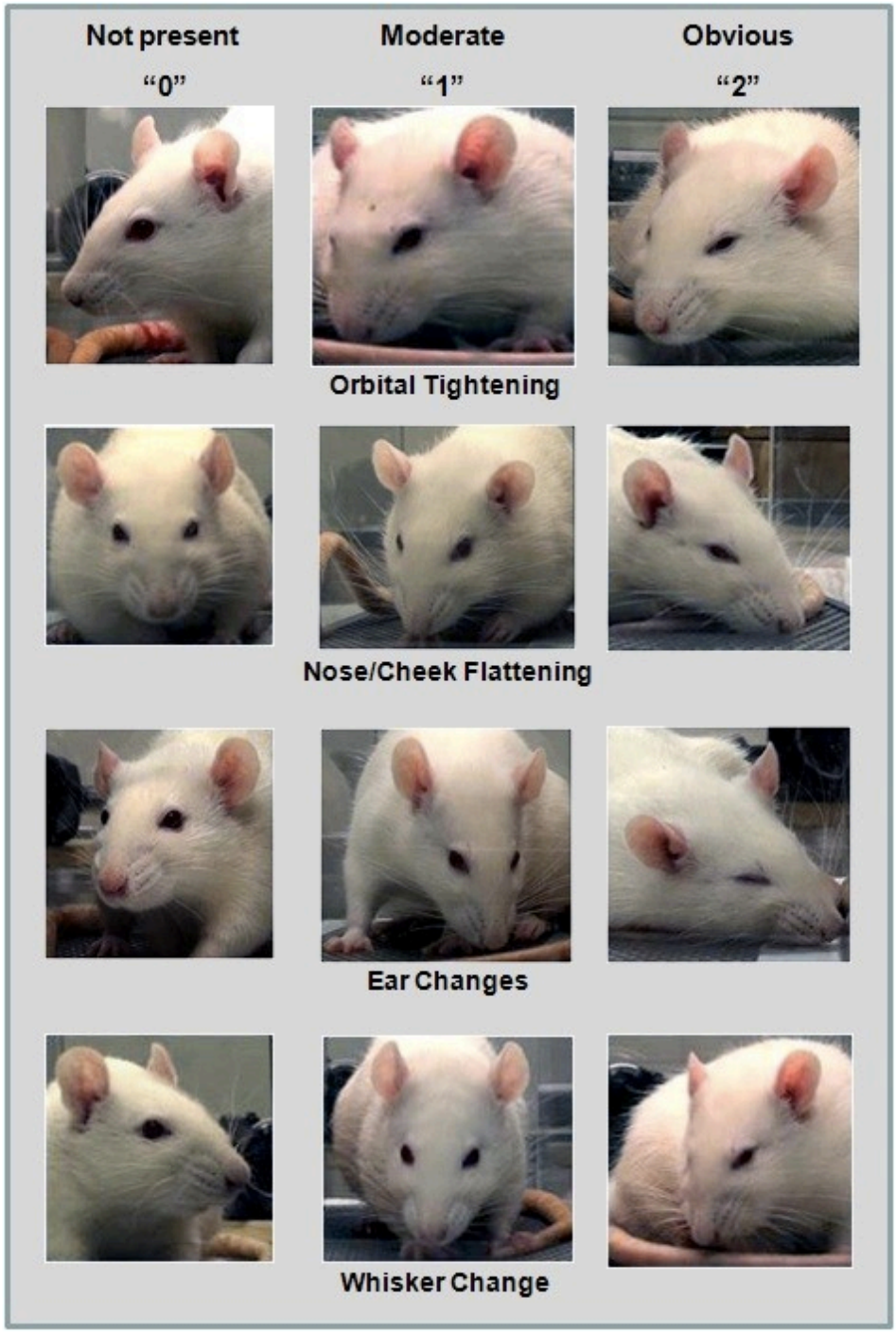

Figure 2. The Rat Grimace Scale.

The four action units of the Rat Grimace Scale are depicted. These correspond to the "pain face" that a rat makes when in pain. Image reprinted from Sotocinal et al., 2011.

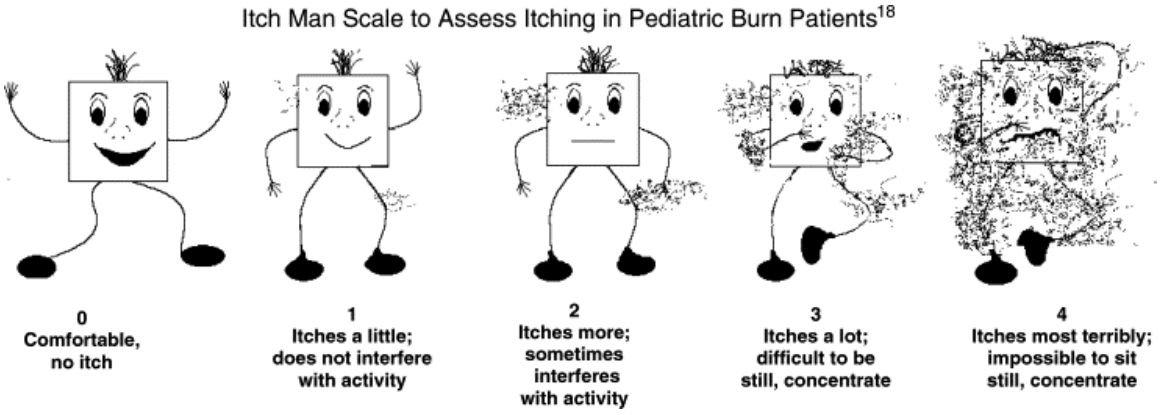

Figure 3. The Itch Man Scale.

The Itch Man scale was developed to assess itch in pediatric burn patients. Image reprinted from Blakeney and Marvin, 2000.

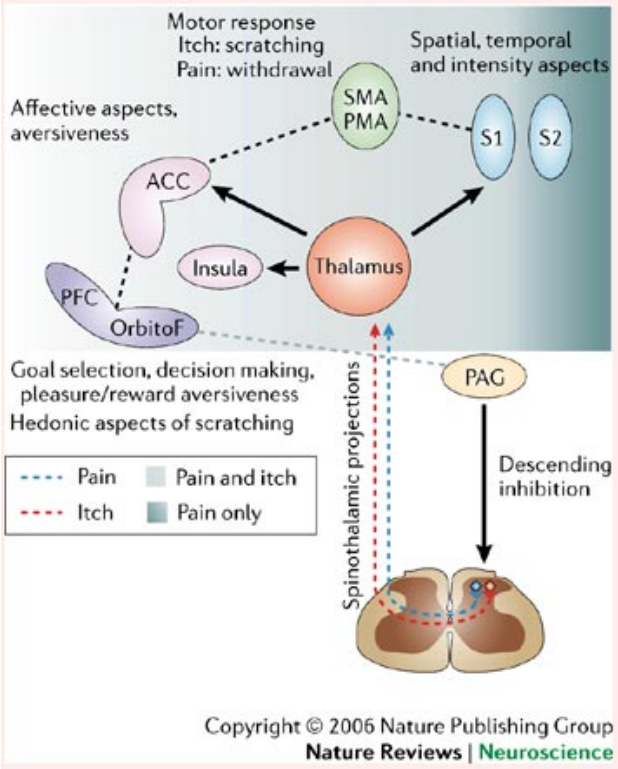

Figure 4. The anatomical basis for pain and itch processing.

The neurocircuitry used to process painful and pruritic stimuli has a large degree of overlap. Image reprinted from Ikoma et al,, 2006.

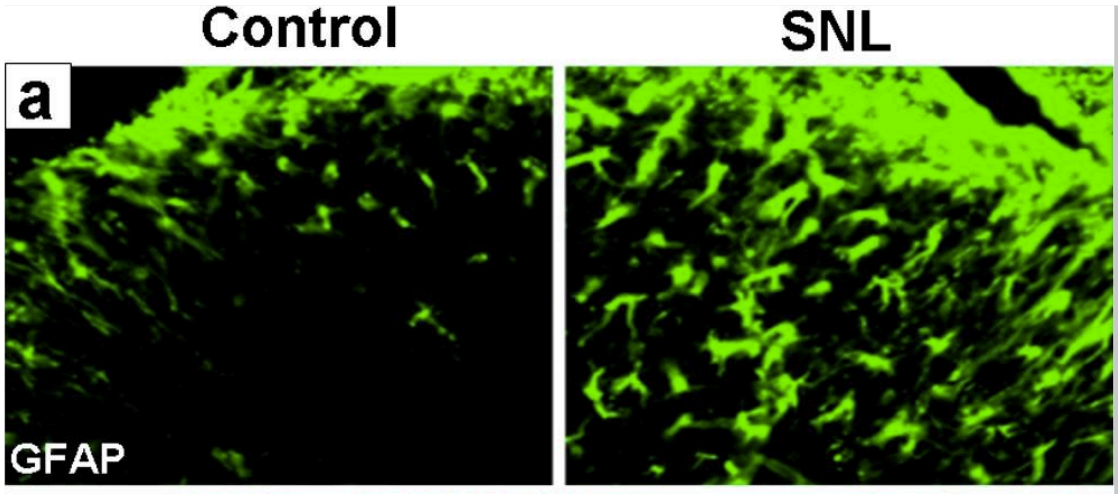

Figure 5. Peripheral injury causes astrogliosis in the dorsal horn of the spinal cord.

A variety of injuries including nerve damage, tumor invasion, or tissue damage cause astrogliosis in the spinal cord. After spinal nerve ligation (SNL), reactive astrocytes undergo hypertrophy and upregulate expression of GFAP. Image reprinted from Gao and Ji, 2010.

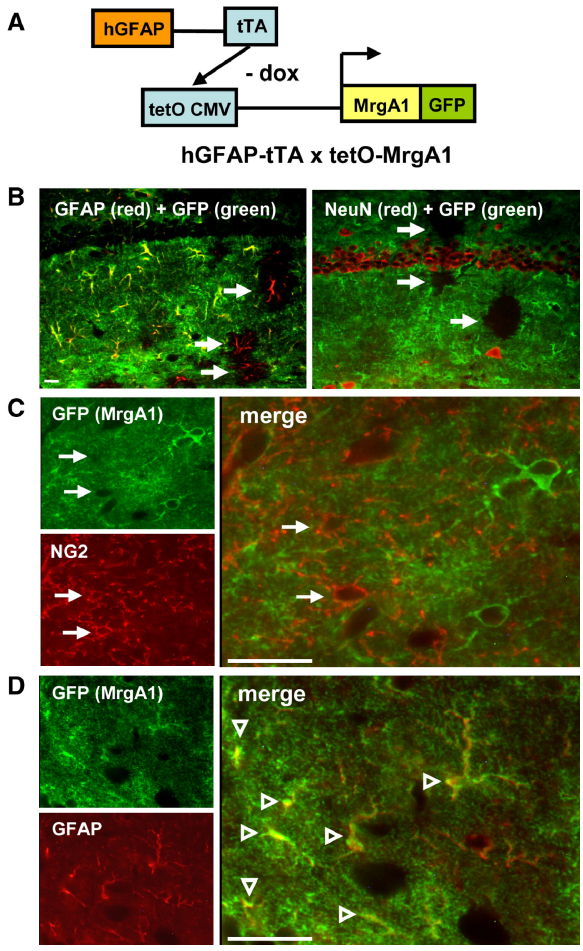

Figure 6. The MrgA1 transgenic mouse model allows selective activation of astrocytic G_q-GPCRs.

(A) Crossing hGFAP-tTA to tetO-MrgA1 mice resulted in the expression of MrgA1 in $GFAP^+$ cells in the absence of doxycycline. (B) MrgA1 is fused to GFP. In the hippocampus, astrocytes (GFAP), but not neurons (NeuN), expressed the MrgA1 receptor. (C) $NG2^+$ glia did not express the MrgA1 receptor. (D) A higher magnification shows overlapping expression of GFP (MrgA1) and GFAP (red) in both the astrocyte soma and major processes. Image reprinted from Fiacco et al., 2007

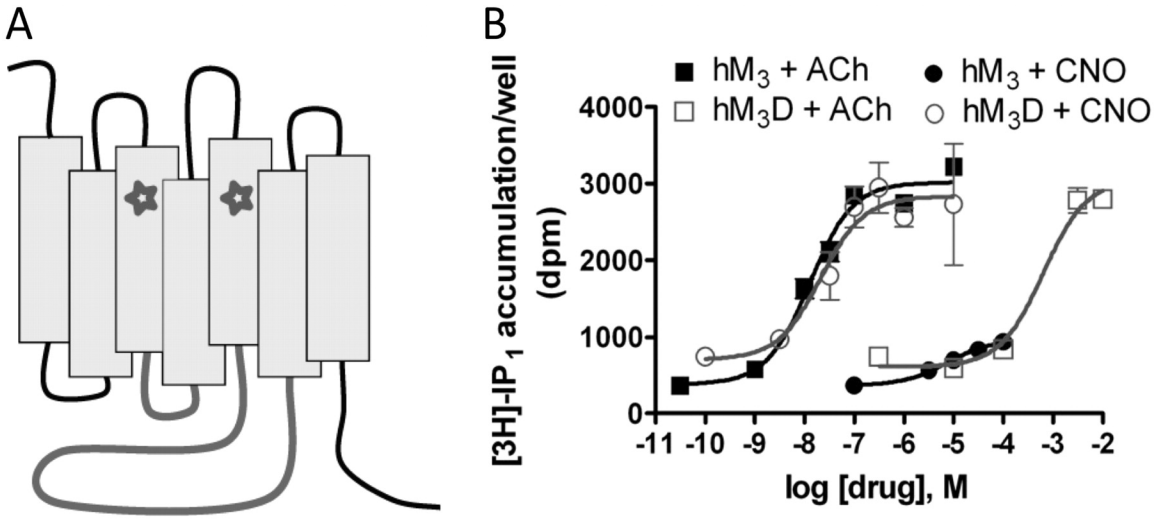

Figure 7. DREADDs are mutated muscarinic receptors.

(A) DREADD (hM_3D) is formed by making two point mutation in the 3rd and 5th transmembrane regions of the endogenous M_3 muscarinic receptor (hM_3): stars; Y149C and A239G). (B) In human pulmonary artery smooth muscle cells, the wild-type hM_3 is potently activated by acetylcholine (ACh), but not CNO, resulting in PIP_2 hydrolysis. Conversely, hM_3D is activated by CNO, but not ACh. Image reprinted from Rogan and Roth, 2011.

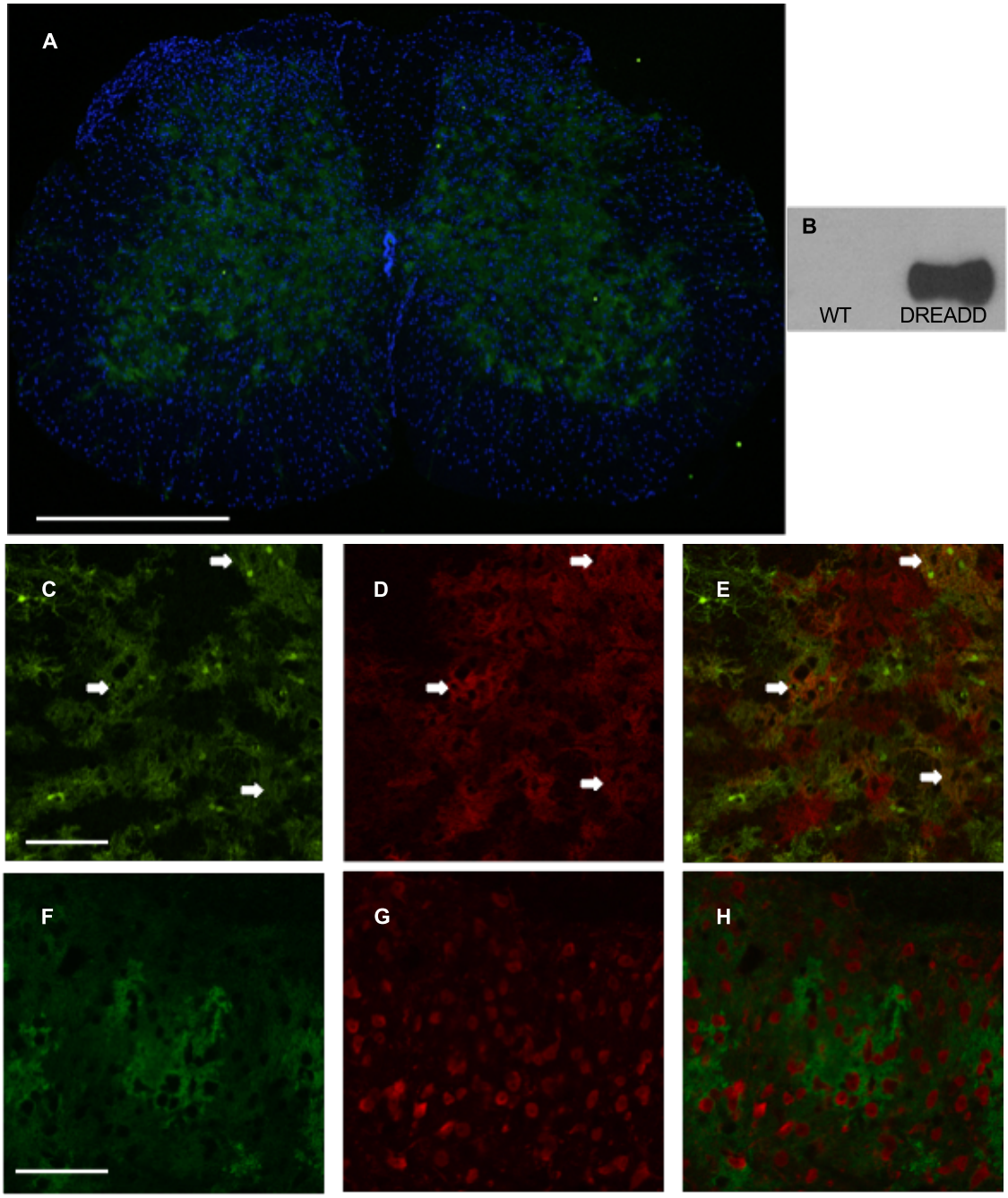

Figure 8. G_q-DREADD is expressed in spinal cord astrocytes, but not neurons.

(A) HA-tagged G_q-DREADD (green) and DAPI (blue) staining of the spinal cord (Scale bar = 500 μM). (B) Western blot of spinal cord from G_q-DREADD and WT mice. (C) The dorsal horn of the spinal cord from a GFAP-GFP mouse bred to a G_q-DREADD mouse. GFP-positive spinal cord astrocytes are shown in green. Scale bar = 100 μm. (D)

Immunostaining for HA-tagged G_q-DREADD is shown in red. (E) GFP-positive astrocytes showed colocalization (marked with arrows) with the HA-epitope tagged G_q-DREADD. (F) HA-tagged G_q-DREADD immunostaining (green). Scale bar = 50 μm. (G) The dorsal horn of the spinal cord was immunostained for the neuronal marker, NeuN (red, marked with arrows). (H) No colocalization was found between NeuN and G_q-DREADD.

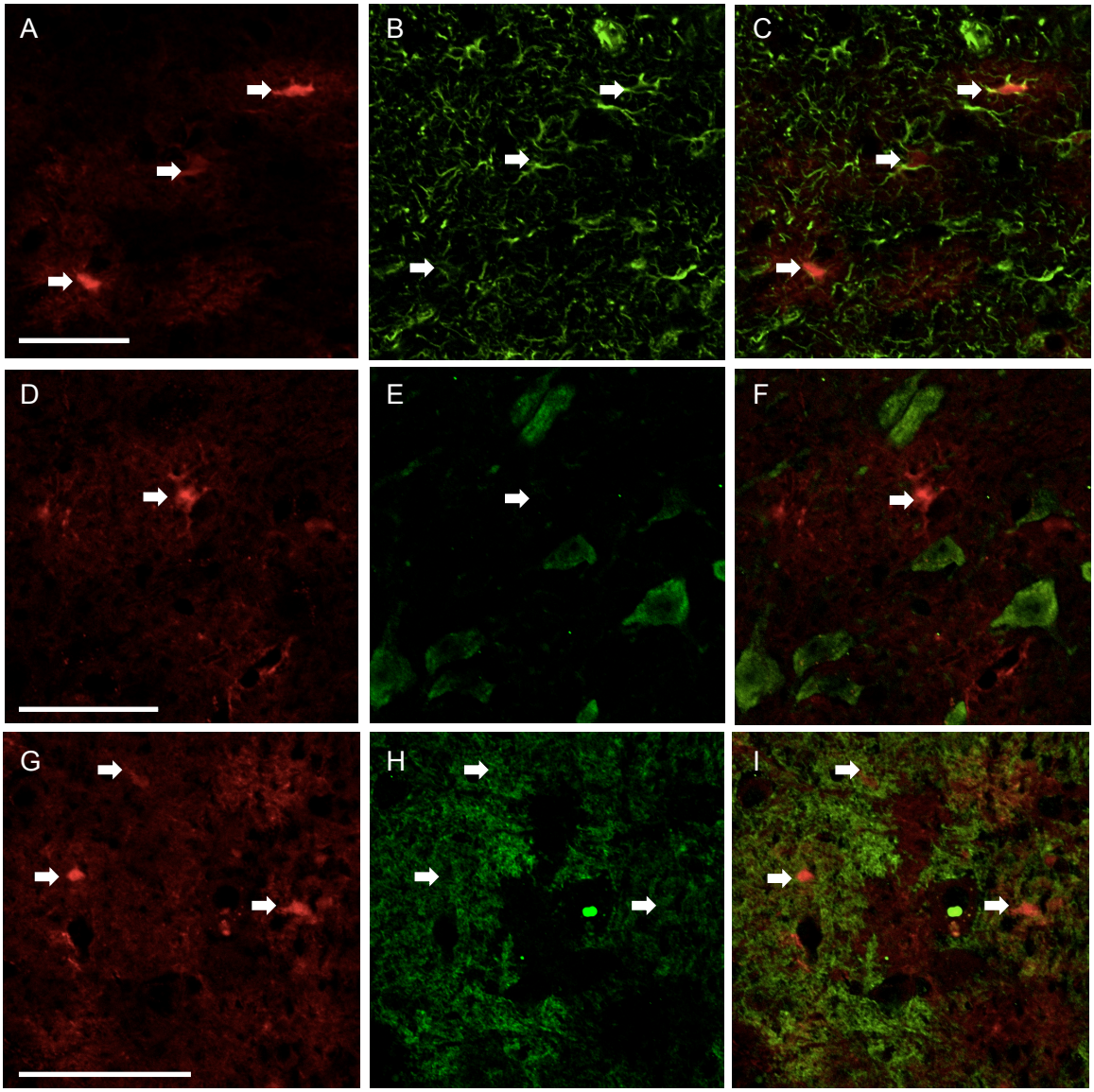

Figure 9. GFAP-tdTomato is expressed in spinal cord astrocytes, but not neurons.

(A) GFAP-tdTomato^{+} cells (arrows) in the spinal cord. (Scale bar = 50 μm). (B) GFAP staining of spinal cord (C) Merge of A and B. (D) GFAP-tdTomato^{+} cell is marked with an arrow. (Scale bar = 50 μm) (E) NeuN (F) merge of D and E. (G) GFAP-tdTomato^{+} cells (arrows, Scale bar = 50 μm) (H) HA-G_q-DREADD. (I) Merge of G and H.

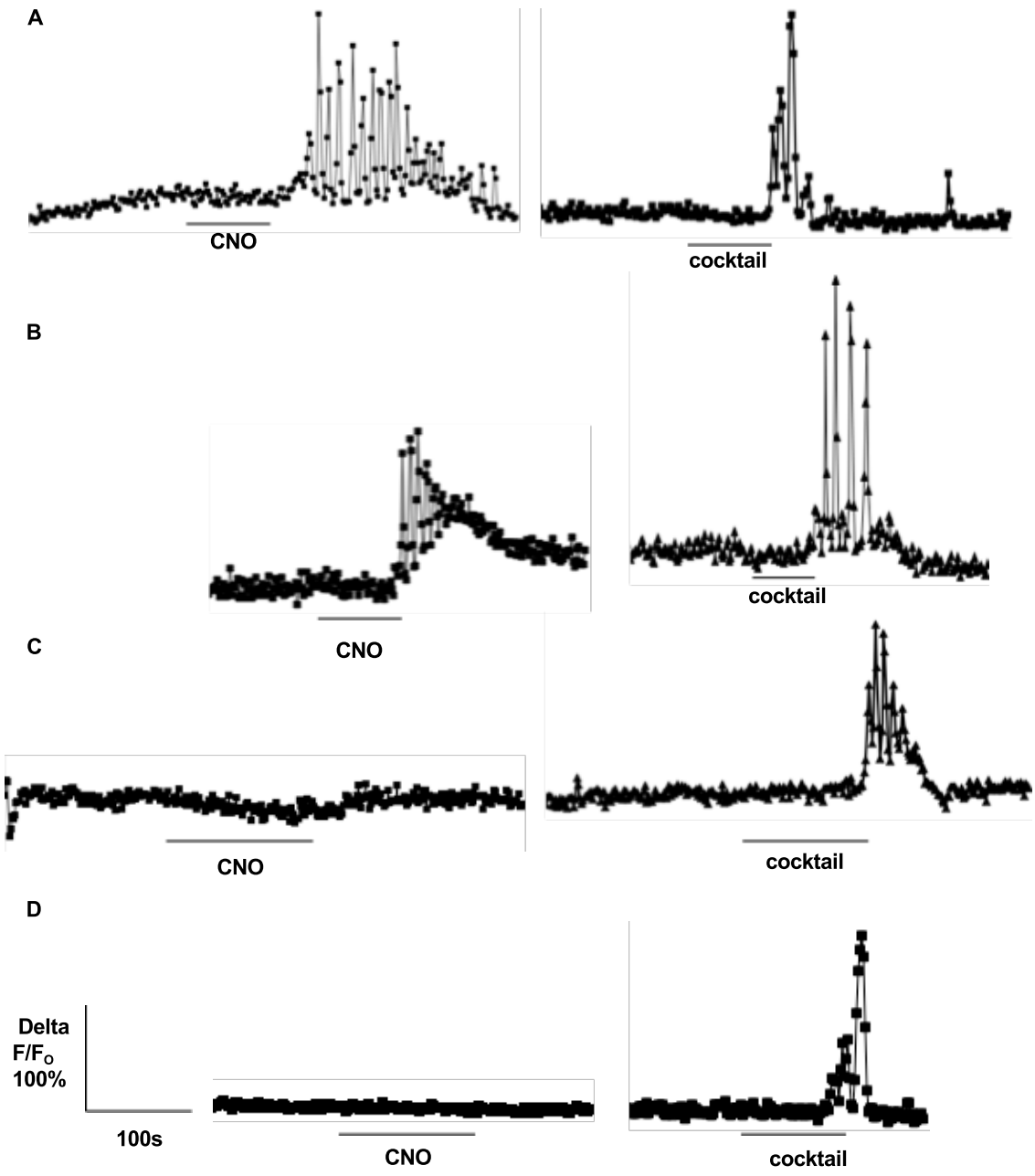

Figure 10. G_q-DREADD stimulation causes calcium increases in G_q-DREADD but not WT spinal cord astrocytes.

(A) GFAP-tdTomato × G_q-DREADD-positive astrocytes in lamina II respond to CNO and a positive control agonist cocktail (10 μm each DHPG, histamine, carbachol). (B) GFAP-tdTomato$^+$ × G_q-DREADD lamina V astrocytes respond to CNO and the cocktail with calcium increases. (C) GFAP-tdTomato positive astrocytes in lamina II do not respond to

CNO but do respond to the cocktail. (D) GFAP-tdTomato astrocytes in lamina V do not respond to CNO but do respond to the cocktail.

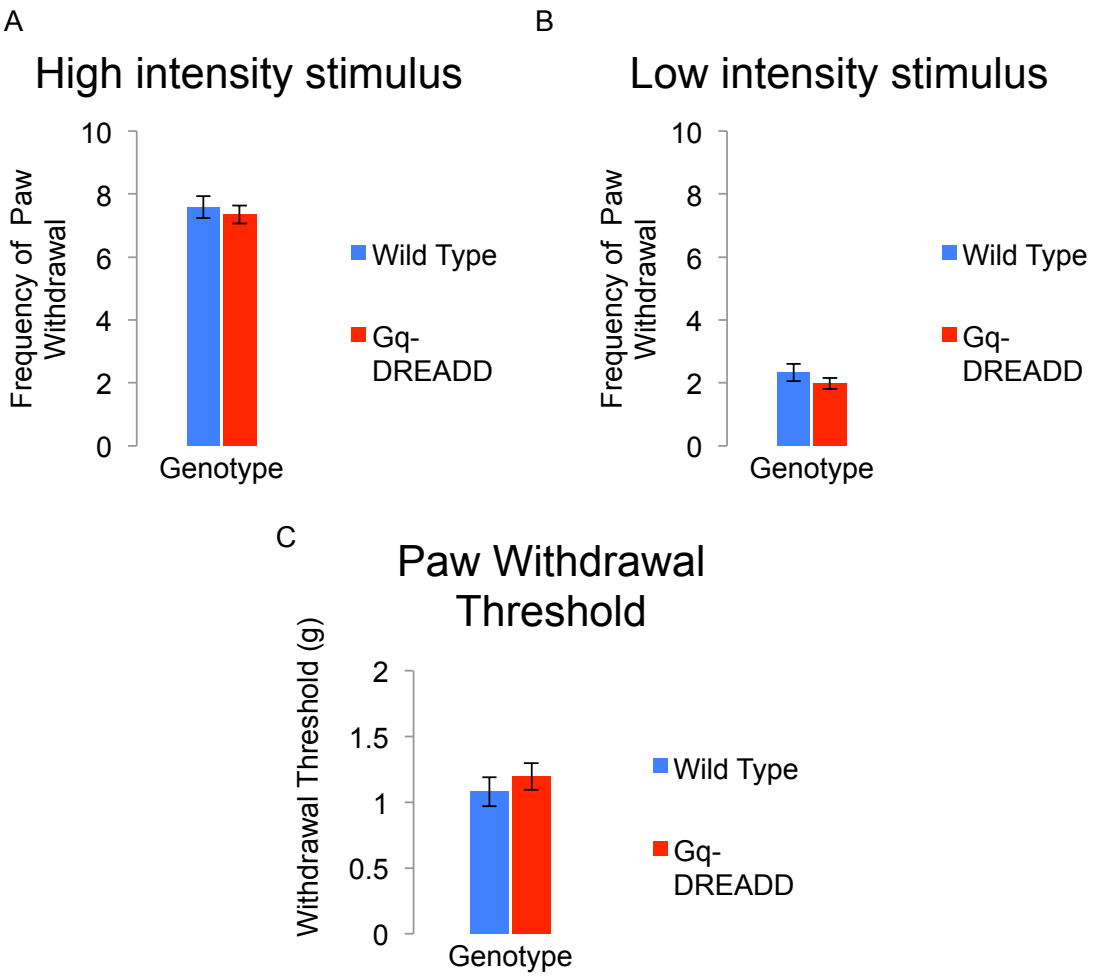

Figure 11. G_q-DREADD and WT littermates have similar basal mechanical sensitivity.

(A) Frequency of paw withdrawal in response to a low-intensity mechanical stimulus. (B) Frequency of paw withdrawal in response to a high-intensity mechanical stimulus. (C) Paw withdrawal threshold.

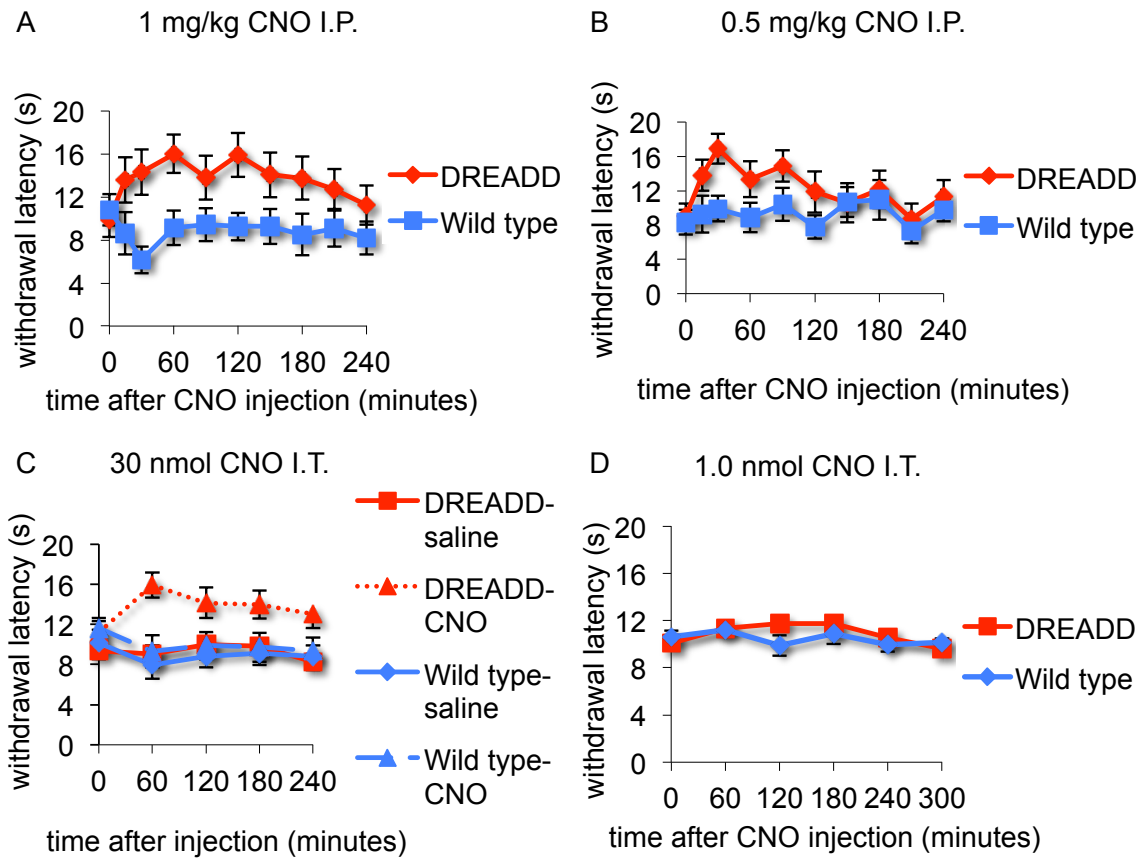

Figure 12. Stimulation of spinal and DRG glial G_q-DREADD causes an increase in withdrawal latency in an acute thermal nociceptive assay.

(A) CNO (1 mg/kg, I.P.) caused an increase in withdrawal latency for G_q-DREADD mice that lasted for almost 4 hours. (B) A lower dose of CNO (0.5 mg/kg, I.P.) caused an increase in withdrawal latency for G_q-DREADD mice that lasted for more than 2 hours. (C) While there was no difference in baseline paw withdrawal latency, intrathecal injection of CNO (30 nmol) caused an increase in paw withdrawal latency in G_q-DREADD but not WT mice in the Hargreaves test. Intrathecal injection of saline did not affect withdrawal latency for either G_q-DREADD or WT mice. (D) A lower dose of CNO (1 nmol, I.T.) did not cause a difference in paw withdrawal latency for G_q-DREADD or WT mice in the Hargreaves test.

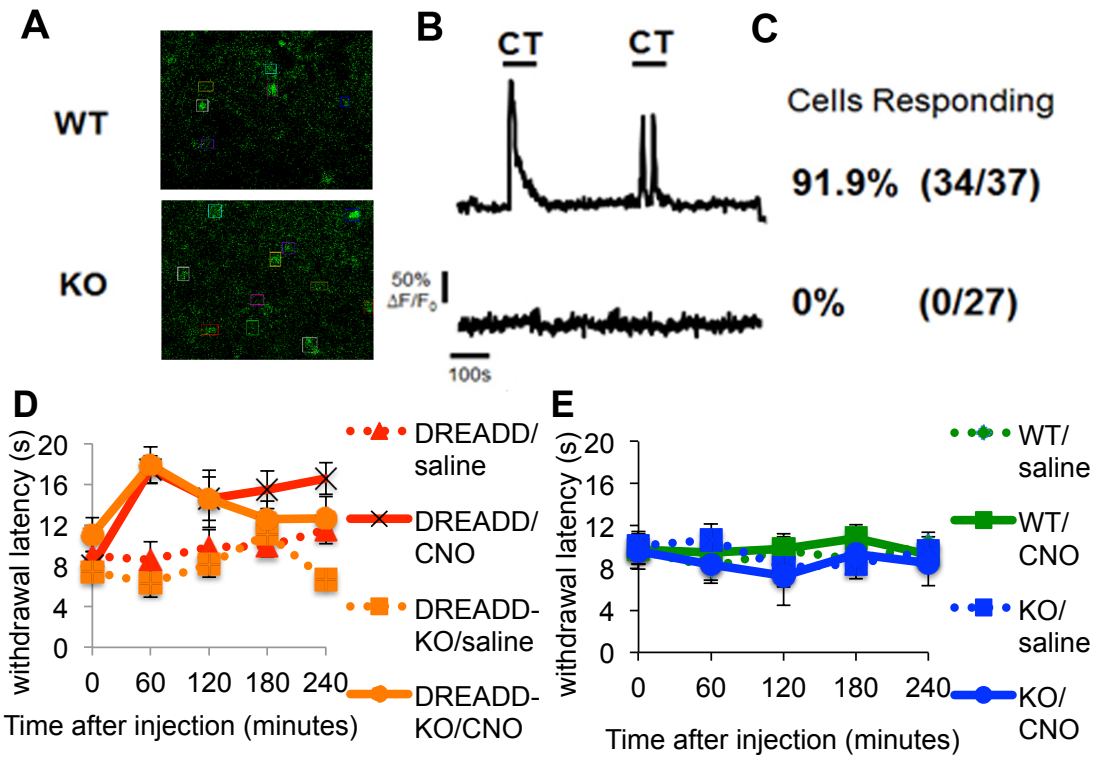

Figure 13. Stimulation of G_q-GPCR pathways on GFAP-positive glia causes antinociception in an IP_3R2-independent manner.

(A) Representative slices in which astrocytes were loaded with fluo-4 calcium indicator dye. Regions of interest were placed over cell bodies of bulk-loaded cells in lamina II of the dorsal horn to record their responses to agonist application. (B) Representative calcium traces to bath application of an agonist cocktail (histamine, carbachol and DHPG; 10 µm each). (C) The cocktail elicited robust calcium responses in astrocytes from WT littermate control slices (91.9% of 37 cells; n = 3 mice) but not in IP_3R2 KO slices (0% of 27 cells; n = 3 mice). (D) G_q-DREADD and G_q-DREADD × IP_3R2 KO mice experienced a dramatic and long-lasting increase in paw withdrawal latency in response to intrathecal injection of 30 nmol CNO, but not in response to saline. (E) The paw withdrawal latency for IP_3R2 KO and WT mice did not change in response to intrathecal injection of either 30 nmol CNO or saline.

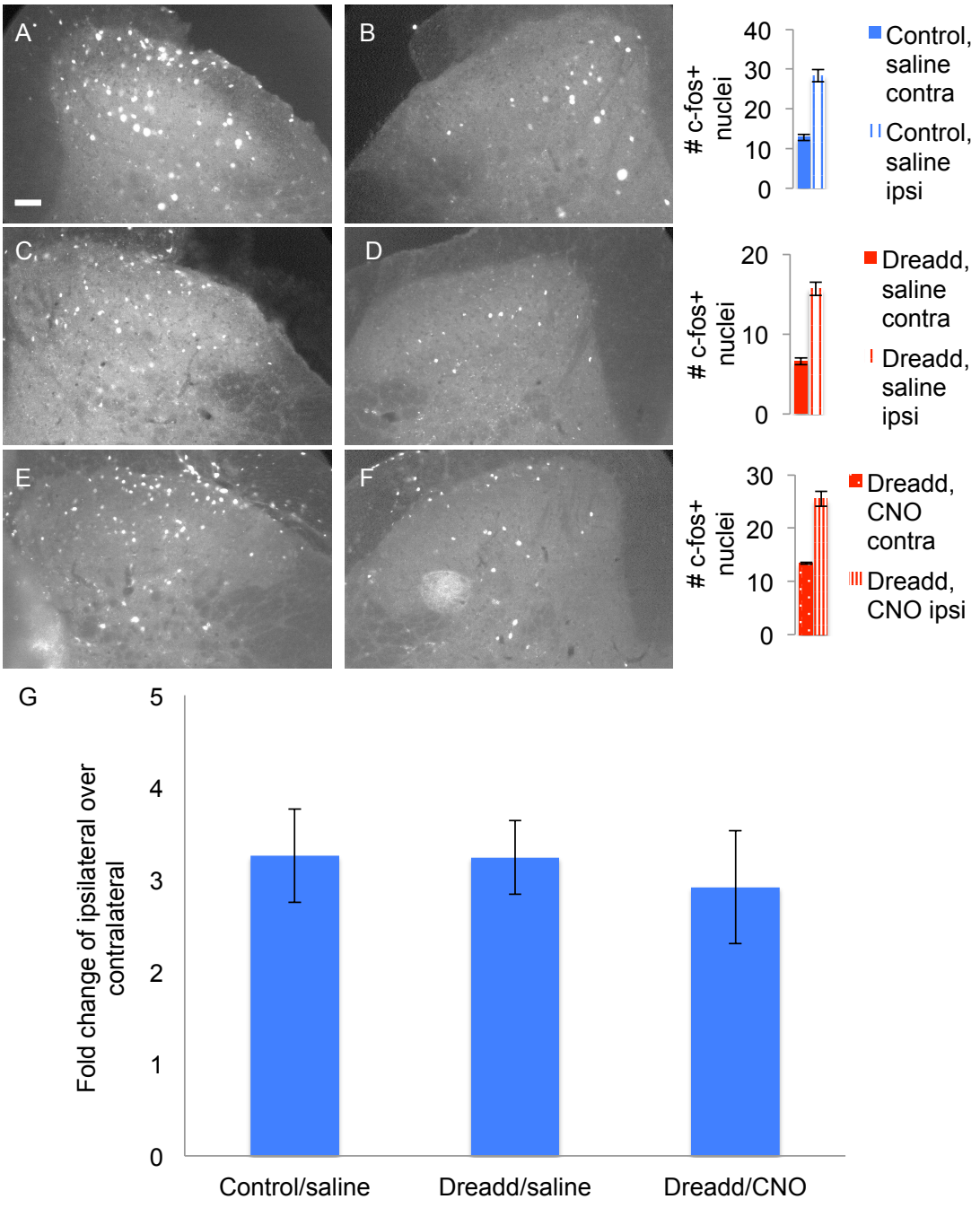

Figure 14. G_q-DREADD stimulation did not alter induction of c-fos in the dorsal horn in response to intraplantar formalin.

Formalin injected into the plantar surface of the hind paw of a control FosGFP mouse induced c-fos in the ipsilateral (A) but not contralateral (B) superficial dorsal horn and was not affected by an i.p. saline injection. Formalin injected into the plantar surface of the hind

paw of a G_q-DREADD × FosGFP mouse induced c-fos in the ipsilateral but not contralateral superficial dorsal horn and was not affected by i.p. saline (ipsilateral – C, contralateral – D) or 0.5 mg/kg i.p. CNO (ipsilateral – E, contralateral - F). (G) Quantification of fold difference of c-fos induction in the ipsilateral vs. contralateral dorsal horn. (Scale bar = 50 μm).

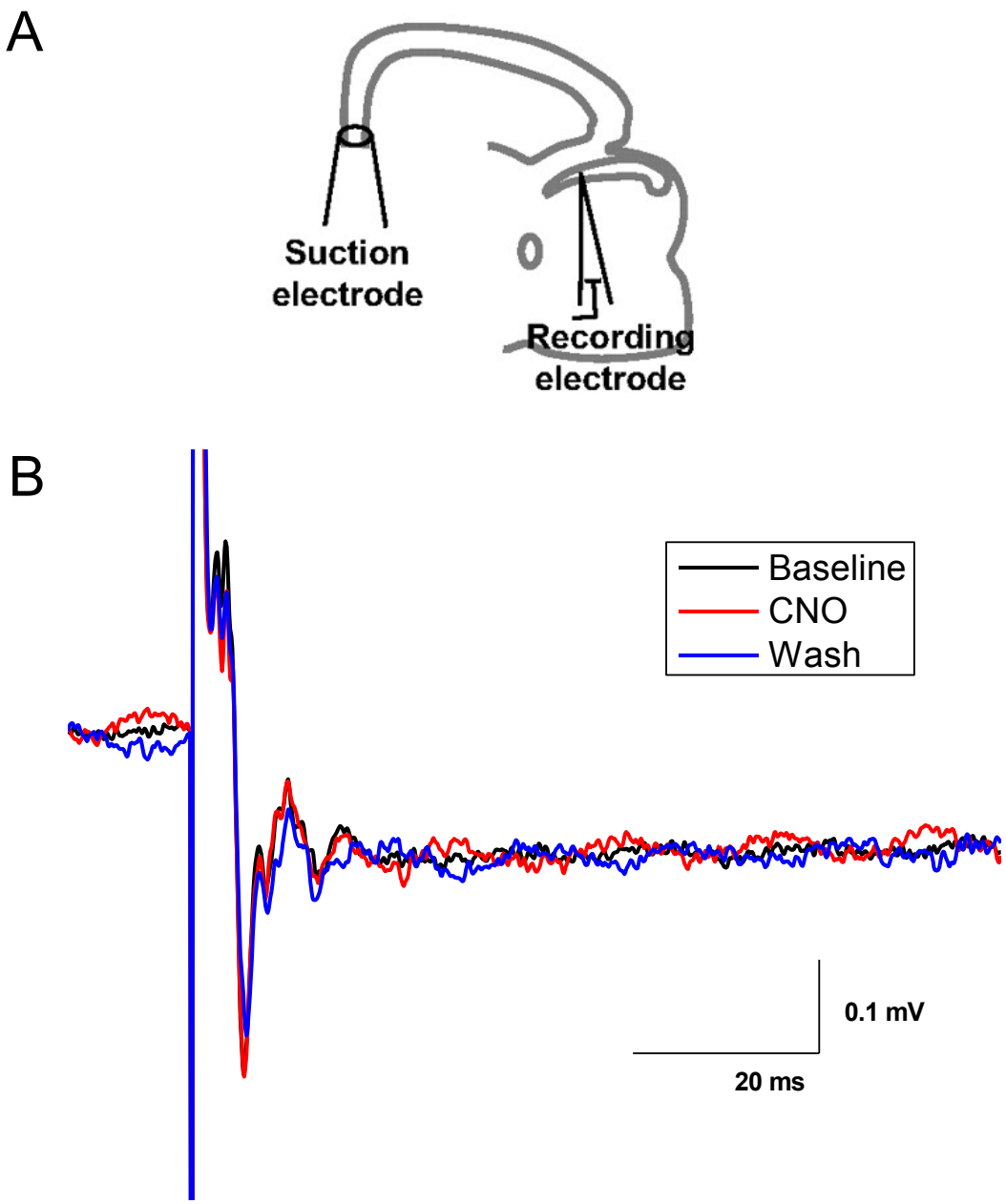

Figure 15. G_q-DREADD stimulation did not affect excitatory synaptic drive from the peripheral primary afferents to lamina II.

(A) Schematic of experimental set up. A suction electrode stimulated the dorsal root while evoked field potentials were recorded in lamina II. (B) Representative traces of evoked

field potentials during the baseline, CNO, and wash periods. Data were collected from three G_q-DREADD spinal cord slices from two mice. No difference was seen in the evoked field potentials recorded during the baseline, CNO application and the wash periods.

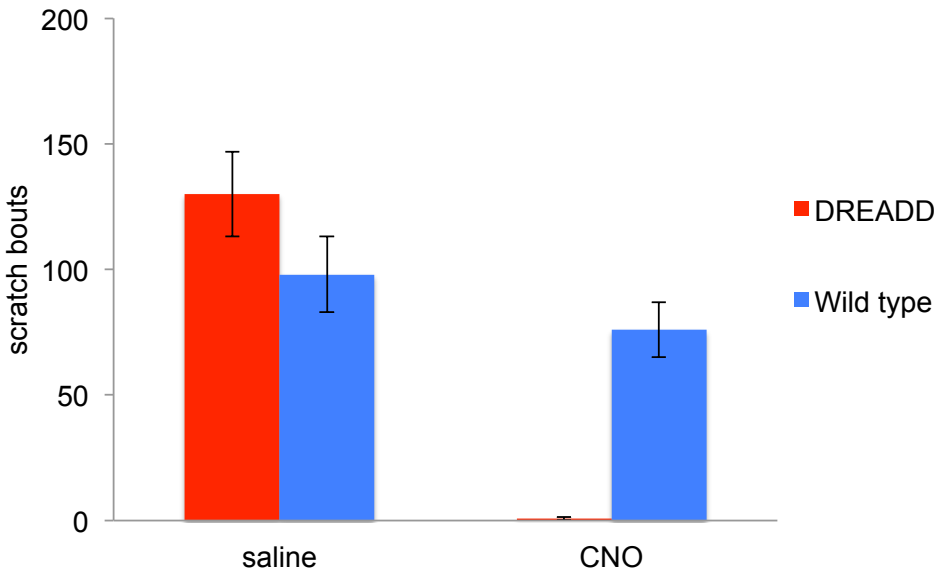

Figure 16. Glial G_q-DREADD stimulation blocks histamine-dependent itch.

Pretreatment with saline did not affect histamine-induced itch in WT or G_q-DREADD mice. Pretreatment with CNO (0.5 mg/kg I.P.) blocked histamine-induced itch in G_q-DREADD mice but not WT mice.

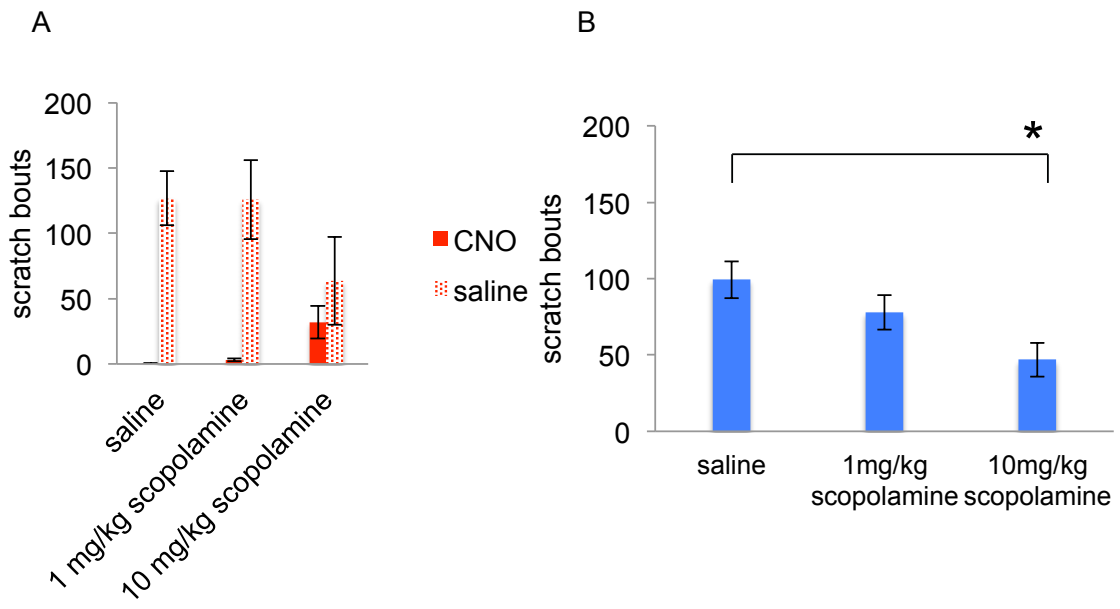

Figure 17. Systemic inhibition of glial G_q-DREADD restores histamine-induced itch.

(A) Histamine-induced itch in G_q-DREADD mice was not affected by pretreatment with saline, 1 mg/kg scopolamine, or 10 mg/kg scopolamine followed by saline injection. G_q-DREADD mice pretreated with saline or 1 mg/kg scopolamine and then injected with 0.5 mg/kg CNO did not experience histamine-induced itch. Pretreatment with 10 mg/kg scopolamine partially rescued histamine-induced itch in CNO-injected G_q-DREADD mice. (B) Histamine-induced itch in WT mice was not affected by injection of 1 mg/kg scopolamine compared to saline treatment. Pretreatment of WT mice with 10 mg/kg scopolamine caused fewer histamine-induced scratching bouts compared to WT mice pretreated with saline * $P < 0.05$.

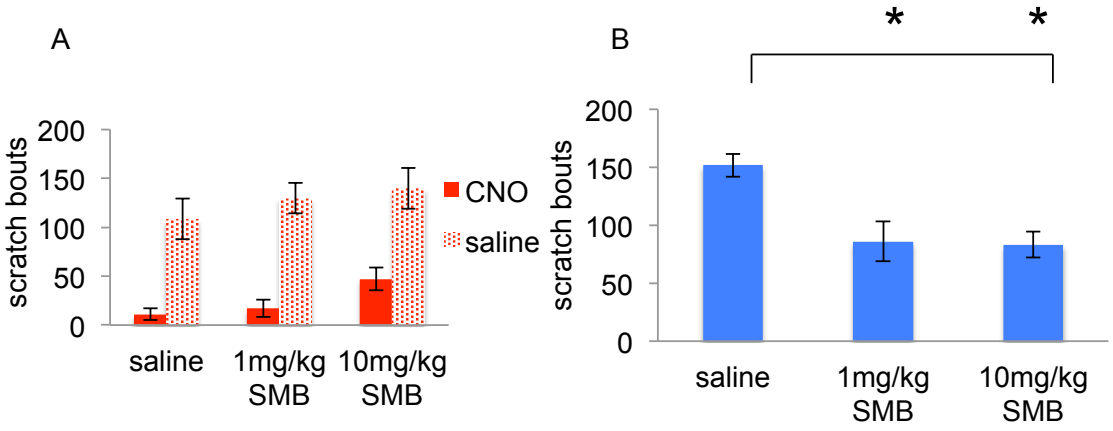

Figure 18. Inhibition of peripheral glial G_q-DREADD restores histamine-induced itch.

*(*A) G_q-DREADD mice exhibited normal histamine-induced itch when pretreated with saline or either dose of SMB followed by a saline injection. G_q-DREADD mice pretreated with saline and then injected with CNO did not exhibit histamine-induced itch. Pretreatment with 1 mg/kg SMB did not rescue histamine-induced itch in CNO-injected G_q-DREADD mice; pretreatment with 10 mg/kg SMB partially rescued histamine-induced itch in CNO-injected G_q-DREADD mice. (B) Pretreatment of WT mice with 1 mg/kg SMB or 10 mg/kg SMB caused fewer histamine-induced scratching bouts compared to WT mice pretreated with saline * $P < 0.05$.

REFERENCES

Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ (2003) Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. Proc Natl Acad Sci U S A (United States) 100:7947-7952.

Agulhon C, Fiacco TA, McCarthy KD (2010) Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca2+ signaling. Science (United States) 327:1250-1254.

Andrew D, Craig AD (2001) Spinothalamic lamina I neurons selectively sensitive to histamine: A central neural pathway for itch. Nat Neurosci (UNITED STATES) 4:72-77.

Apkarian AV, Baliki MN, Geha PY (2009) Towards a theory of chronic pain. Prog Neurobiol (England) 87:81-97.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: Glia, the unacknowledged partner. Trends Neurosci (ENGLAND) 22:208-215.

Aristotle (350 BCE) In: On the parts of animals.

Armbruster BN, Roth BL (2005) Mining the receptorome. J Biol Chem (United States) 280:5129-5132.

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A (United States) 104:5163-5168.

Atanassoff PG, Brull SJ, Zhang J, Greenquist K, Silverman DG, Lamotte RH (1999) Enhancement of experimental pruritus and mechanically evoked dysesthesiae with local anesthesia. Somatosens Mot Res (ENGLAND) 16:291-298.

Balasubramanyan S, Stemkowski PL, Stebbing MJ, Smith PA (2006) Sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons. J Neurophysiol (United States) 96:579-590.

Ballantyne JC, Loach AB, Carr DB (1988) Itching after epidural and spinal opiates. Pain (NETHERLANDS) 33:149-160.

Ballanyi K, Grafe P, ten Bruggencate G (1987) Ion activities and potassium uptake mechanisms of glial cells in guinea-pig olfactory cortex slices. J Physiol (ENGLAND) 382:159-174.

Barth AL, Gerkin RC, Dean KL (2004) Alteration of neuronal firing properties after in vivo experience in a FosGFP transgenic mouse. J Neurosci (United States) 24:6466-6475.

Basbaum AI (1999) Spinal mechanisms of acute and persistent pain. Reg Anesth Pain Med (UNITED STATES) 24:59-67.

Bashir ZI, Tam B, Collingridge GL (1990) Activation of the glycine site in the NMDA receptor is necessary for the induction of LTP. Neurosci Lett (NETHERLANDS) 108:261-266.

Bennett DL, Dmietrieva N, Priestley JV, Clary D, McMahon SB (1996) trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. Neurosci Lett (IRELAND) 206:33-36.

Besnard, J, Ruda, GF, Setola, V, Abecassis, K, Rodriguiz, RM, Huang, XP, Norval, S, Sassano, MF, Shin, AI, Webster, LA, Simeons FR, Stojanovski L, Prat A, Seidah NG, Constam DB, Bickerton GR, Read KD, Wetsel WC, Gilbert IH, Roth BL, Hopkins AL (2012) Automated design of ligands to polypharmacological profiles. Nature (ENGLAND) 492:215-220.

Besson JM, Conseiller C, Hamann KF, Maillard MC (1972) Modifications of dorsal horn cell activities in the spinal cord, after intra-arterial injection of bradykinin. J Physiol (ENGLAND) 221:189-205.

Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J, Volterra A (2001) CXCR4-activated astrocyte glutamate release via TNFalpha: Amplification by microglia triggers neurotoxicity. Nat Neurosci (United States) 4:702-710.

Bignami A, Eng LF, Dahl D, Uyeda CT (1972) Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res (NETHERLANDS) 43:429-435.

Blakeney P, Marvin J (2000) Itch man scale. Copyrighted by Shriners Hospitals for Children.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol (ENGLAND) 232:331-356.

Bohm-Starke N, Hilliges M, Falconer C, Rylander E (1998) Increased intraepithelial innervation in women with vulvar vestibulitis syndrome. Gynecol Obstet Invest (SWITZERLAND) 46:256-260.

Bowser DN, Khakh BS (2004) ATP excites interneurons and astrocytes to increase synaptic inhibition in neuronal networks. J Neurosci (United States) 24:8606-8620.

Brecha NC, Weigmann C (1994) Expression of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter in the rat retina. J Comp Neurol (UNITED STATES) 345:602-611.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. J Neurosci (UNITED STATES) 14:1030-1037.

Brew H, Attwell D (1987) Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature (ENGLAND) 327:707-709.

Brooks J, Tracey I (2005) From nociception to pain perception: Imaging the spinal and supraspinal pathways. J Anat (England) 207:19-33.

Burgess PR, Perl ER (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J Physiol (ENGLAND) 190:541-562.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci (United States) 22:183-192.

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE (2003) Insights into G protein structure, function, and regulation. Endocr Rev (United States) 24:765-781.

Campbell JN, Meyer RA (2006) Mechanisms of neuropathic pain. Neuron (United States) 52:77-92.

Carlsson AM (1983) Assessment of chronic pain. I. aspects of the reliability and validity of the visual analogue scale. Pain (NETHERLANDS) 16:87-101.

Carroll CL, Balkrishnan R, Feldman SR, Fleischer AB,Jr, Manuel JC (2005) The burden of atopic dermatitis: Impact on the patient, family, and society. Pediatr Dermatol (United States) 22:192-199.

Carstens E (2008) Scratching the brain to understand neuropathic itch. J Pain (United States) 9:973-974.

Carstens E (1997) Responses of rat spinal dorsal horn neurons to intracutaneous microinjection of histamine, capsaicin, and other irritants. J Neurophysiol (UNITED STATES) 77:2499-2514.

Carter GT, Galer BS (2001) Advances in the management of neuropathic pain. Phys Med Rehabil Clin N Am (United States) 12:447-459.

Caspar DL, Goodenough DA, Makowski L, Phillips WC (1977) Gap junction structures. I. correlated electron microscopy and x-ray diffraction. J Cell Biol (UNITED STATES) 74:605-628.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods (NETHERLANDS) 53:55-63.

Chen S, Rio C, Ji RR, Dikkes P, Coggeshall RE, Woolf CJ, Corfas G (2003) Disruption of ErbB receptor signaling in adult non-myelinating schwann cells causes progressive sensory loss. Nat Neurosci (United States) 6:1186-1193.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell (United States) 120:421-433.

Clapham DE, Neer EJ (1997) G protein beta gamma subunits. Annu Rev Pharmacol Toxicol (UNITED STATES) 37:167-203.

Colburn RW, Rickman AJ, DeLeo JA (1999) The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. Exp Neurol (UNITED STATES) 157:289-304.

COLLINS WR,Jr, NULSEN FE, RANDT CT (1960) Relation of peripheral nerve fiber size and sensation in man. Arch Neurol (Not Available) 3:381-385.

Cornell-Bell AH, Thomas PG, Smith SJ (1990a) The excitatory neurotransmitter glutamate causes filopodia formation in cultured hippocampal astrocytes. Glia (UNITED STATES) 3:322-334.

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990b) Glutamate induces calcium waves in cultured astrocytes: Long-range glial signaling. Science (UNITED STATES) 247:470-473.

Coward P, Wada HG, Falk MS, Chan SD, Meng F, Akil H, Conklin BR (1998) Controlling signaling with a specifically designed gi-coupled receptor. Proc Natl Acad Sci U S A (UNITED STATES) 95:352-357.

Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. Nature (England) 444:894-898.

D'Ambrosio R, Maris DO, Grady MS, Winn HR, Janigro D (1999) Impaired K(+) homeostasis and altered electrophysiological properties of post-traumatic hippocampal glia. J Neurosci (UNITED STATES) 19:8152-8162.

Davidson S, Zhang X, Khasabov SG, Simone DA, Giesler GJ,Jr (2009) Relief of itch by scratching: State-dependent inhibition of primate spinothalamic tract neurons. Nat Neurosci (United States) 12:544-546.

Davson H, Oldendorf WH (1967) Symposium on membrane transport. transport in the central nervous system. Proc R Soc Med (ENGLAND) 60:326-329.

DeLeo JA (2006) Basic science of pain. J Bone Joint Surg Am (United States) 88 Suppl 2:58-62.

Dermietzel R, Hertberg EL, Kessler JA, Spray DC (1991) Gap junctions between cultured astrocytes: Immunocytochemical, molecular, and electrophysiological analysis. J Neurosci (UNITED STATES) 11:1421-1432.

Descartes R (1972) Treatise of man. (Cambridge, MA).

Dib-Hajj SD, Black JA, Waxman SG (2009) Voltage-gated sodium channels: Therapeutic targets for pain. Pain Med (United States) 10:1260-1269.

Dickson DW, Farlo J, Davies P, Crystal H, Fuld P, Yen SH (1988) Alzheimer's disease. A double-labeling immunohistochemical study of senile plaques. Am J Pathol (UNITED STATES) 132:86-101.

Djouhri L, Bleazard L, Lawson SN (1998) Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J Physiol (ENGLAND) 513 (Pt 3):857-872.

Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD (2007) Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. J Neurosci (United States) 27:11354-11365.

Dong S, Allen JA, Farrell M, Roth BL (2010) A chemical-genetic approach for precise spatio-temporal control of cellular signaling. Mol Biosyst (England) 6:1376-1380.

Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ (2001) A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell (United States) 106:619-632.

Duffy S, MacVicar BA (1995) Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. J Neurosci (UNITED STATES) 15:5535-5550.

During MJ, Spencer DD (1993) Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. Lancet (ENGLAND) 341:1607-1610.

Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC, Ottersen OP, de Lanerolle NC (2004) Loss of glutamine synthetase in the human epileptogenic hippocampus: Possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. Lancet (England) 363:28-37.

Eisenach JC, Hood DD, Curry R, Shafer SL (2003) Cephalad movement of morphine and fentanyl in humans after intrathecal injection. Anesthesiology (United States) 99:166-173.

Elman S, Hynan LS, Gabriel V, Mayo MJ (2010) The 5-D itch scale: A new measure of pruritus. Br J Dermatol (England) 162:587-593.

Elson K, Simmons A, Speck P (2004) Satellite cell proliferation in murine sensory ganglia in response to scarification of the skin. Glia (United States) 45:105-109.

England JD, Gronseth GS, Franklin G, Carter GT, Kinsella LJ, Cohen JA, Asbury AK, Szigeti K, Lupski JR, Latov N, Lewis RA, Low PA, Fisher MA, Herrmann DN, Howard JF,Jr, Lauria G, Miller RG, Polydefkis M, Sumner AJ, American Academy of Neurology (2009) Practice parameter: Evaluation of distal symmetric polyneuropathy: Role of autonomic testing, nerve biopsy, and skin biopsy (an evidence-based review). report of the american academy of neurology, american association of neuromuscular and electrodiagnostic medicine, and american academy of physical medicine and rehabilitation. Neurology (United States) 72:177-184.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA (2009) Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell (United States) 139:380-392.

Eroglu C, Barres BA (2010) Regulation of synaptic connectivity by glia. Nature (England) 468:223-231.

Fairbanks CA (2003) Spinal delivery of analgesics in experimental models of pain and analgesia. Adv Drug Deliv Rev (Netherlands) 55:1007-1041.

Fiacco TA, Agulhon C, McCarthy KD (2009) Sorting out astrocyte physiology from pharmacology. Annu Rev Pharmacol Toxicol (United States) 49:151-174.

Fiacco TA, Agulhon C, Taves SR, Petravicz J, Casper KB, Dong X, Chen J, McCarthy KD (2007) Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity. Neuron (United States) 54:611-626.

Fields H (2004) State-dependent opioid control of pain. Nat Rev Neurosci (England) 5:565-575.

Fine SM, Angel RA, Perry SW, Epstein LG, Rothstein JD, Dewhurst S, Gelbard HA (1996) Tumor necrosis factor alpha inhibits glutamate uptake by primary human astrocytes. implications for pathogenesis of HIV-1 dementia. J Biol Chem (UNITED STATES) 271:15303-15306.

Fishman SM, Caneris OA, Stojanovic MP, Borsook D (1997) Intravenous lidocaine for treatment-resistant pruritus. Am J Med (UNITED STATES) 102:584-585.

Foley JC, McIver SR, Haydon PG (2011) Gliotransmission modulates baseline mechanical nociception. Mol Pain (England) 7:93.

Gao YJ, Ji RR (2010) Targeting astrocyte signaling for chronic pain. Neurotherapeutics (United States) 7:482-493.

Gao YJ, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu ZZ, Park JY, Lind AL, Ma Q, Ji RR (2009) JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. J Neurosci (United States) 29:4096-4108.

Garrison CJ, Dougherty PM, Carlton SM (1994) GFAP expression in lumbar spinal cord of naive and neuropathic rats treated with MK-801. Exp Neurol (UNITED STATES) 129:237-243.

Garrison CJ, Dougherty PM, Kajander KC, Carlton SM (1991) Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. Brain Res (NETHERLANDS) 565:1-7.

Giaume C, McCarthy KD (1996) Control of gap-junctional communication in astrocytic networks. Trends Neurosci (ENGLAND) 19:319-325.

Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N (2010) Astroglial networks: A step further in neuroglial and gliovascular interactions. Nat Rev Neurosci (England) 11:87-99.

Gilman AG (1995) Nobel lecture. G proteins and regulation of adenylyl cyclase. Biosci Rep (UNITED STATES) 15:65-97.

Gomes JA, Li X, Pan HL, Eisenach JC (1999) Intrathecal adenosine interacts with a spinal noradrenergic system to produce antinociception in nerve-injured rats. Anesthesiology (UNITED STATES) 91:1072-1079.

Gosselin RD, Varela C, Banisadr G, Mechighel P, Rostene W, Kitabgi P, Melik-Parsadaniantz S (2005) Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. J Neurochem (England) 95:1023-1034.

Green AD, Young KK, Lehto SG, Smith SB, Mogil JS (2006) Influence of genotype, dose and sex on pruritogen-induced scratching behavior in the mouse. Pain (Netherlands) 124:50-58.

Grudt TJ, Perl ER (2002) Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J Physiol (England) 540:189-207.

Guettier JM, Gautam D, Scarselli M, Ruiz de Azua I, Li JH, Rosemond E, Ma X, Gonzalez FJ, Armbruster BN, Lu H, Roth BL, Wess J (2009) A chemical-genetic approach to study G

protein regulation of beta cell function in vivo. Proc Natl Acad Sci U S A (United States) 106:19197-19202.

Guo W, Wang H, Watanabe M, Shimizu K, Zou S, LaGraize SC, Wei F, Dubner R, Ren K (2007) Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. J Neurosci (United States) 27:6006-6018.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci (UNITED STATES) 19:520-528.

Gutnick MJ, Connors BW, Ransom BR (1981) Dye-coupling between glial cells in the guinea pig neocortical slice. Brain Res (NETHERLANDS) 213:486-492.

Halassa MM, Fellin T, Takano H, Dong JH, Haydon PG (2007) Synaptic islands defined by the territory of a single astrocyte. J Neurosci (United States) 27:6473-6477.

Hall A (1998) G proteins and small GTPases: Distant relatives keep in touch. Science (UNITED STATES) 280:2074-2075.

Han ZS, Zhang ET, Craig AD (1998) Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. Nat Neurosci (UNITED STATES) 1:218-225.

Hansson E (2006) Could chronic pain and spread of pain sensation be induced and maintained by glial activation? Acta Physiol (Oxf) (England) 187:321-327.

Hantman AW, van den Pol AN, Perl ER (2004) Morphological and physiological features of a set of spinal substantia gelatinosa neurons defined by green fluorescent protein expression. J Neurosci (United States) 24:836-842.

Harden RN (2005) Chronic neuropathic pain. mechanisms, diagnosis, and treatment. Neurologist (United States) 11:111-122.

Harden TK, Waldo GL, Hicks SN, Sondek J (2011) Mechanism of activation and inactivation of Gq/phospholipase C-beta signaling nodes. Chem Rev (United States) 111:6120-6129.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain (NETHERLANDS) 32:77-88.

Harris W (1921) Persistent pain in lesions of the peripheral and central nervous system. Br Med J (England) 2:896-900.

Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science (UNITED STATES) 280:2112-2114.

Hartrick CT, Gatchel RJ, Conroy S (2012) Identification and management of pain medication abuse and misuse: Current state and future directions. Expert Rev Neurother (England) 12:601-610.

Hassel B, Paulsen RE, Johnsen A, Fonnum F (1992) Selective inhibition of glial cell metabolism in vivo by fluorocitrate. Brain Res (NETHERLANDS) 576:120-124.

Hawkins BT, Davis TP (2005) The blood-brain barrier/neurovascular unit in health and disease. Pharmacol Rev (United States) 57:173-185.

Hefti FF, Rosenthal A, Walicke PA, Wyatt S, Vergara G, Shelton DL, Davies AM (2006) Novel class of pain drugs based on antagonism of NGF. Trends Pharmacol Sci (England) 27:85-91.

Henneberger C, Papouin T, Oliet SH, Rusakov DA (2010) Long-term potentiation depends on release of D-serine from astrocytes. Nature (England) 463:232-236.

Hildebrandt JD, Sekura RD, Codina J, Iyengar R, Manclark CR, Birnbaumer L (1983) Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins. Nature (ENGLAND) 302:706-709.

Hirrlinger J, Hulsmann S, Kirchhoff F (2004) Astroglial processes show spontaneous motility at active synaptic terminals in situ. Eur J Neurosci (France) 20:2235-2239.

Hopkins AL, Groom CR (2002) The druggable genome. Nat Rev Drug Discov (England) 1:727-730.

Hsieh JC, Hagermark O, Stahle-Backdahl M, Ericson K, Eriksson L, Stone-Elander S, Ingvar M (1994) Urge to scratch represented in the human cerebral cortex during itch. J Neurophysiol (UNITED STATES) 72:3004-3008.

Huang YH, Bergles DE (2004) Glutamate transporters bring competition to the synapse. Curr Opin Neurobiol (England) 14:346-352.

Huang YH, Sinha SR, Tanaka K, Rothstein JD, Bergles DE (2004) Astrocyte glutamate transporters regulate metabotropic glutamate receptor-mediated excitation of hippocampal interneurons. J Neurosci (United States) 24:4551-4559.

Huck S, Grass F, Hortnagl H (1984) The glutamate analogue alpha-aminoadipic acid is taken up by astrocytes before exerting its gliotoxic effect in vitro. J Neurosci (UNITED STATES) 4:2650-2657.

Hunt SP, Pini A, Evan G (1987) Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature (ENGLAND) 328:632-634.

Hylden JL, Wilcox GL (1980) Intrathecal morphine in mice: A new technique. Eur J Pharmacol (NETHERLANDS) 67:313-316.

Ibuki T, Hama AT, Wang XT, Pappas GD, Sagen J (1997) Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. Neuroscience (UNITED STATES) 76:845-858.

Igarashi Y, Utsumi H, Chiba H, Yamada-Sasamori Y, Tobioka H, Kamimura Y, Furuuchi K, Kokai Y, Nakagawa T, Mori M, Sawada N (1999) Glial cell line-derived neurotrophic factor induces barrier function of endothelial cells forming the blood-brain barrier. Biochem Biophys Res Commun (UNITED STATES) 261:108-112.

Iiri T, Farfel Z, Bourne HR (1998) G-protein diseases furnish a model for the turn-on switch. Nature (ENGLAND) 394:35-38.

Ikoma A, Steinhoff M, Stander S, Yosipovitch G, Schmelz M (2006) The neurobiology of itch. Nat Rev Neurosci (England) 7:535-547.

Jaffe AB, Hall A (2005) Rho GTPases: Biochemistry and biology. Annu Rev Cell Dev Biol (United States) 21:247-269.

Janzer RC, Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. Nature (ENGLAND) 325:253-257.

Jasmin L, Vit JP, Bhargava A, Ohara PT (2010) Can satellite glial cells be therapeutic targets for pain control? Neuron Glia Biol (England) 6:63-71.

Jessen KR, Morgan L, Stewart HJ, Mirsky R (1990) Three markers of adult non-myelin-forming schwann cells, 217c(ran-1), A5E3 and GFAP: Development and regulation by neuron-schwann cell interactions. Development (ENGLAND) 109:91-103.

Ji RR, Strichartz G (2004) Cell signaling and the genesis of neuropathic pain. Sci STKE (United States) 2004:reE14.

Johannes CB, Le TK, Zhou X, Johnston JA, Dworkin RH (2010) The prevalence of chronic pain in united states adults: Results of an internet-based survey. J Pain (United States) 11:1230-1239.

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature (ENGLAND) 325:529-531.

Jones EA, Neuberger J, Bergasa NV (2002) Opiate antagonist therapy for the pruritus of cholestasis: The avoidance of opioid withdrawal-like reactions. QJM (England) 95:547-552.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature (England) 413:203-210.

Kacem K, Lacombe P, Seylaz J, Bonvento G (1998) Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: A confocal microscopy study. Glia (UNITED STATES) 23:1-10.

Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci (UNITED STATES) 1:683-692.

Kang N, Xu J, Xu Q, Nedergaard M, Kang J (2005) Astrocytic glutamate release-induced transient depolarization and epileptiform discharges in hippocampal CA1 pyramidal neurons. J Neurophysiol (United States) 94:4121-4130.

Karlsten R, Gordh T,Jr (1995) An A1-selective adenosine agonist abolishes allodynia elicited by vibration and touch after intrathecal injection. Anesth Analg (UNITED STATES) 80:844-847.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science (UNITED STATES) 274:1133-1138.

Kaufman DL, McGinnis JF, Krieger NR, Tobin AJ (1986) Brain glutamate decarboxylase cloned in lambda gt-11: Fusion protein produces gamma-aminobutyric acid. Science (UNITED STATES) 232:1138-1140.

Kawasaki Y, Zhang L, Cheng JK, Ji RR (2008a) Cytokine mechanisms of central sensitization: Distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J Neurosci (United States) 28:5189-5194.

Kawasaki Y, Xu ZZ, Wang X, Park JY, Zhuang ZY, Tan PH, Gao YJ, Roy K, Corfas G, Lo EH, Ji RR (2008b) Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. Nat Med (United States) 14:331-336.

Khurgel M, Koo AC, Ivy GO (1996) Selective ablation of astrocytes by intracerebral injections of alpha-aminoadipate. Glia (UNITED STATES) 16:351-358.

Kingery WS (1997) A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes. Pain (NETHERLANDS) 73:123-139.

Knall C, Johnson GL (1998) G-protein regulatory pathways: Rocketing into the twenty-first century. J Cell Biochem Suppl (UNITED STATES) 30-31:137-146.

Kozasa T, Hajicek N, Chow CR, Suzuki N (2011) Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. J Biochem (England) 150:357-369.

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: Molecular modeling and mutagenesis approaches to receptor structure and function. Pharmacol Ther (England) 103:21-80.

Krnjevic K, Schwartz S (1967) The action of gamma-aminobutyric acid on cortical neurones. Exp Brain Res (GERMANY, WEST) 3:320-336.

Kuffler SW, Nicholls JG (1966) The physiology of neuroglial cells. Ergeb Physiol (GERMANY, WEST) 57:1-90.

Kumazawa T, Perl ER (1978) Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: Indications of their place in dorsal horn functional organization. J Comp Neurol (UNITED STATES) 177:417-434.

Kunzelmann P, Schroder W, Traub O, Steinhauser C, Dermietzel R, Willecke K (1999) Late onset and increasing expression of the gap junction protein connexin30 in adult murine brain and long-term cultured astrocytes. Glia (UNITED STATES) 25:111-119.

Kwan CL, Crawley AP, Mikulis DJ, Davis KD (2000) An fMRI study of the anterior cingulate cortex and surrounding medial wall activations evoked by noxious cutaneous heat and cold stimuli. Pain (NETHERLANDS) 85:359-374.

Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS (2010) Coding of facial expressions of pain in the laboratory mouse. Nat Methods (United States) 7:447-449.

Latov N, Nilaver G, Zimmerman EA, Johnson WG, Silverman AJ, Defendini R, Cote L (1979) Fibrillary astrocytes proliferate in response to brain injury: A study combining immunoperoxidase technique for glial fibrillary acidic protein and radioautography of tritiated thymidine. Dev Biol (UNITED STATES) 72:381-384.

Lawrence TS, Beers WH, Gilula NB (1978) Transmission of hormonal stimulation by cell-to-cell communication. Nature (ENGLAND) 272:501-506.

Lawson SN, Crepps BA, Perl ER (1997) Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J Physiol (ENGLAND) 505 (Pt 1):177-191.

Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain (Netherlands) 115:71-83.

Lee B, Descalzi G, Baek J, Kim JI, Lee HR, Lee K, Kaang BK, Zhuo M (2011) Genetic enhancement of behavioral itch responses in mice lacking phosphoinositide 3-kinase-gamma (PI3Kgamma). Mol Pain (England) 7:96.

Lee SW, Kim WJ, Choi YK, Song HS, Son MJ, Gelman IH, Kim YJ, Kim KW (2003) SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. Nat Med (United States) 9:900-906.

Lewis DV, Mutsuga N, Schuette WH, Van Buren J (1977) Potassium clearance and reactive gliosis in the alumina gel lesion. Epilepsia (UNITED STATES) 18:499-506.

Li P, Zhuo M (1998) Silent glutamatergic synapses and nociception in mammalian spinal cord. Nature (ENGLAND) 393:695-698.

Li X, Zima AV, Sheikh F, Blatter LA, Chen J (2005) Endothelin-1-induced arrhythmogenic Ca2+ signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. Circ Res (United States) 96:1274-1281.

Light AR, Perl ER (1979a) Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J Comp Neurol (UNITED STATES) 186:133-150.

Light AR, Perl ER (1979b) Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. J Comp Neurol (UNITED STATES) 186:117-131.

Liu XY, Liu ZC, Sun YG, Ross M, Kim S, Tsai FF, Li QF, Jeffry J, Kim JY, Loh HH, Chen ZF (2011) Unidirectional cross-activation of GRPR by MOR1D uncouples itch and analgesia induced by opioids. Cell (United States) 147:447-458.

Lorenzo LE, Ramien M, St Louis M, De Koninck Y, Ribeiro-da-Silva A (2008) Postnatal changes in the rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat. J Comp Neurol (United States) 508:592-604.

Low VA, Sandroni P, Fealey RD, Low PA (2006) Detection of small-fiber neuropathy by sudomotor testing. Muscle Nerve (United States) 34:57-61.

Lu Y, Perl ER (2005) Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). J Neurosci (United States) 25:3900-3907.

Lu Y, Perl ER (2003) A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. J Neurosci (United States) 23:8752-8758.

Lund RD, Lund JS (1972) Development of synaptic patterns in the superior colliculus of the rat. Brain Res (NETHERLANDS) 42:1-20.

Lutz S, Freichel-Blomquist A, Rumenapp U, Schmidt M, Jakobs KH, Wieland T (2004) p63RhoGEF and GEFT are rho-specific guanine nucleotide exchange factors encoded by the same gene. Naunyn Schmiedebergs Arch Pharmacol (Germany) 369:540-546.

Lutz S, Freichel-Blomquist A, Yang Y, Rumenapp U, Jakobs KH, Schmidt M, Wieland T (2005) The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. J Biol Chem (United States) 280:11134-11139.

Ma W, Quirion R (2002) Partial sciatic nerve ligation induces increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) in astrocytes in the lumbar spinal dorsal horn and the gracile nucleus. Pain (Netherlands) 99:175-184.

MacFarlane SN, Sontheimer H (1997) Electrophysiological changes that accompany reactive gliosis in vitro. J Neurosci (UNITED STATES) 17:7316-7329.

Maeda S, Kawamoto A, Yatani Y, Shirakawa H, Nakagawa T, Kaneko S (2008) Gene transfer of GLT-1, a glial glutamate transporter, into the spinal cord by recombinant adenovirus attenuates inflammatory and neuropathic pain in rats. Mol Pain (England) 4:65.

Malan TP, Mata HP, Porreca F (2002) Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. Anesthesiology (United States) 96:1161-1167.

Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu JM, Gainetdinov RR, Caron MG (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. Proc Natl Acad Sci U S A (United States) 105:13656-13661.

Matterne U, Strassner T, Apfelbacher CJ, Diepgen TL, Weisshaar E (2009) Measuring the prevalence of chronic itch in the general population: Development and validation of a questionnaire for use in large-scale studies. Acta Derm Venereol (Sweden) 89:250-256.

Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW (2001) CNS synaptogenesis promoted by glia-derived cholesterol. Science (United States) 294:1354-1357.

Maxwell DS, Kruger L (1965) The fine structure of astrocytes in the cerebral cortex and their response to focal injury produced by heavy ionizing particles. J Cell Biol (United States) 25:141-157.

Mayer C, Quasthoff S, Grafe P (1998) Differences in the sensitivity to purinergic stimulation of myelinating and non-myelinating schwann cells in peripheral human and rat nerve. Glia (UNITED STATES) 23:374-382.

McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, Griffin JW, McArthur JC (1995) Cutaneous innervation in sensory neuropathies: Evaluation by skin biopsy. Neurology (UNITED STATES) 45:1848-1855.

McMahon SB, Malcangio M (2009) Current challenges in glia-pain biology. Neuron (United States) 64:46-54.

McMullen AB, Baidwan GS, McCarthy KD (2012) Morphological and behavioral changes in the pathogenesis of a novel mouse model of communicating hydrocephalus. PLoS One (United States) 7:e30159.

Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994) The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology (ENGLAND) 33:1471-1478.

Melzack R (1975) The McGill pain questionnaire: Major properties and scoring methods. Pain (NETHERLANDS) 1:277-299.

Melzack R, Wall PD (1965) Pain mechanisms: A new theory. Science (UNITED STATES) 150:971-979.

Mendell JR, Sahenk Z (2003) Clinical practice. painful sensory neuropathy. N Engl J Med (United States) 348:1243-1255.

Merskey H, Bogduk N (1994) Classification of chronic pain. Seattle, WA: IASP Press.

Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestley JV (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. J Neurosci (UNITED STATES) 17:8476-8490.

Michell RH (1975) Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta (NETHERLANDS) 415:81-47.

Miller RH, Raff MC (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. J Neurosci (UNITED STATES) 4:585-592.

Milligan ED, Watkins LR (2009) Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci (England) 10:23-36.

Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, Watkins LR (2003) Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J Neurosci (United States) 23:1026-1040.

Miraucourt LS, Peirs C, Dallel R, Voisin DL (2011) Glycine inhibitory dysfunction turns touch into pain through astrocyte-derived D-serine. Pain (Netherlands) 152:1340-1348.

Mogil JS, Crager SE (2004) What should we be measuring in behavioral studies of chronic pain in animals? Pain (Netherlands) 112:12-15.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci (United States) 22:6724-6731.

Moulton EA, Keaser ML, Gullapalli RP, Greenspan JD (2005) Regional intensive and temporal patterns of functional MRI activation distinguishing noxious and innocuous contact heat. J Neurophysiol (United States) 93:2183-2193.

Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew MV (2006) Essential protective roles of reactive astrocytes in traumatic brain injury. Brain (England) 129:2761-2772.

Nagasako EM, Oaklander AL, Dworkin RH (2003) Congenital insensitivity to pain: An update. Pain (Netherlands) 101:213-219.

Newman EA (1986) High potassium conductance in astrocyte endfeet. Science (UNITED STATES) 233:453-454.

Nicholls D, Attwell D (1990) The release and uptake of excitatory amino acids. Trends Pharmacol Sci (ENGLAND) 11:462-468.

Nichols CG, Lopatin AN (1997) Inward rectifier potassium channels. Annu Rev Physiol (UNITED STATES) 59:171-191.

Nilsson HJ, Levinsson A, Schouenborg J (1997) Cutaneous field stimulation (CFS): A new powerful method to combat itch. Pain (NETHERLANDS) 71:49-55.

North RA, Yoshimura M (1984) The actions of noradrenaline on neurones of the rat substantia gelatinosa in vitro. J Physiol (ENGLAND) 349:43-55.

Novakovic SD, Tzoumaka E, McGivern JG, Haraguchi M, Sangameswaran L, Gogas KR, Eglen RM, Hunter JC (1998) Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. J Neurosci (UNITED STATES) 18:2174-2187.

Oaklander AL (2011) Neuropathic itch. Semin Cutan Med Surg (United States) 30:87-92.

Oaklander AL, Cohen SP, Raju SV (2002) Intractable postherpetic itch and cutaneous deafferentation after facial shingles. Pain (Netherlands) 96:9-12.

Oaklander AL, Bowsher D, Galer B, Haanpaa M, Jensen MP (2003) Herpes zoster itch: Preliminary epidemiologic data. J Pain (United States) 4:338-343.

Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M (2009) Uniquely hominid features of adult human astrocytes. J Neurosci (United States) 29:3276-3287.

Ochoa J, Torebjork HE, Culp WJ, Schady W (1982) Abnormal spontaneous activity in single sensory nerve fibers in humans. Muscle Nerve (UNITED STATES) 5:S74-7.

Ohtori S, Takahashi K, Moriya H, Myers RR (2004) TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: Studies in murine DRG and spinal cord. Spine (United States) 29:1082-1088.

Orkand RK, Nicholls JG, Kuffler SW (1966) Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. J Neurophysiol (UNITED STATES) 29:788-806.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science (UNITED STATES) 289:739-745.

Pardo JV, Fox PT, Raichle ME (1991) Localization of a human system for sustained attention by positron emission tomography. Nature (ENGLAND) 349:61-64.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature (ENGLAND) 369:744-747.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science (United States) 310:113-116.

Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A (UNITED STATES) 91:10625-10629.

Pentreath VW, Kai-Kai MA (1982) Significance of the potassium signal from neurones to glial cells. Nature (ENGLAND) 295:59-61.

Perl ER (2007) Ideas about pain, a historical view. Nat Rev Neurosci (England) 8:71-80.

Perry MJ, Lawson SN (1998) Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. Neuroscience (UNITED STATES) 85:293-310.

Peters A, Palay SL, and Webster Hd (1976) The fine structure of the nervous system. Philadelphia1976: W.B. Saunders Company.

Petravicz J, Fiacco TA, McCarthy KD (2008) Loss of IP3 receptor-dependent Ca2+ increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity. J Neurosci (United States) 28:4967-4973.

Petzold GC, Murthy VN (2011) Role of astrocytes in neurovascular coupling. Neuron (United States) 71:782-797.

Pfrieger FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. Science (UNITED STATES) 277:1684-1687.

Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. Nat Rev Mol Cell Biol (England) 3:639-650.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature (ENGLAND) 360:464-467.

Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci (UNITED STATES) 16:5073-5081.

Price DD, Greenspan JD, Dubner R (2003) Neurons involved in the exteroceptive function of pain. Pain (Netherlands) 106:215-219.

Raghavendra V, Tanga FY, DeLeo JA (2004) Complete freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur J Neurosci (France) 20:467-473.

RALL TW, SUTHERLAND EW (1958) Formation of a cyclic adenine ribonucleotide by tissue particles. J Biol Chem (Not Available) 232:1065-1076.

Ransom CB, Sontheimer H (1995) Biophysical and pharmacological characterization of inwardly rectifying K+ currents in rat spinal cord astrocytes. J Neurophysiol (UNITED STATES) 73:333-346.

Rao VL, Baskaya MK, Dogan A, Rothstein JD, Dempsey RJ (1998) Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain. J Neurochem (UNITED STATES) 70:2020-2027.

Reeh PW (1986) Sensory receptors in mammalian skin in an in vitro preparation. Neurosci Lett (NETHERLANDS) 66:141-146.

Reese TS, Karnovsky MJ (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol (UNITED STATES) 34:207-217.

Regan MR, Huang YH, Kim YS, Dykes-Hoberg MI, Jin L, Watkins AM, Bergles DE, Rothstein JD (2007) Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. J Neurosci (United States) 27:6607-6619.

REXED B (1952) The cytoarchitectonic organization of the spinal cord in the cat. J Comp Neurol (Not Available) 96:414-495.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem (United States) 70:281-312.

Risau W, Wolburg H (1990) Development of the blood-brain barrier. Trends Neurosci (ENGLAND) 13:174-178.

Rodbell M (1995) Nobel lecture. signal transduction: Evolution of an idea. Biosci Rep (UNITED STATES) 15:117-133.

Rogan SC, Roth BL (2011) Remote control of neuronal signaling. Pharmacol Rev (United States) 63:291-315.

Rojas RJ, Yohe ME, Gershburg S, Kawano T, Kozasa T, Sondek J (2007) Galphaq directly activates p63RhoGEF and trio via a conserved extension of the dbl homology-associated pleckstrin homology domain. J Biol Chem (United States) 282:29201-29210.

Rolfe DF, Brown GC (1997) Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev (UNITED STATES) 77:731-758.

Ross SE (2011) Pain and itch: Insights into the neural circuits of aversive somatosensation in health and disease. Curr Opin Neurobiol (England) 21:880-887.

Ross SE et al (2010) Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron (United States) 65:886-898.

Rouach N, Koulakoff A, Abudara V, Willecke K, Giaume C (2008) Astroglial metabolic networks sustain hippocampal synaptic transmission. Science (United States) 322:1551-1555.

Roy CS, Sherrington CS (1890) On the regulation of the blood-supply of the brain. J Physiol (England) 11:85-158.17.

Sandkuhler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiol Rev (United States) 89:707-758.

Sandroni P, Martin DP, Bruce BK, Rome JD (2006) Congenital idiopathic inability to perceive pain: A new syndrome of insensitivity to pain and itch with preserved small fibers. Pain (Netherlands) 122:210-215.

Sawynok J, Sweeney MI, White TD (1986) Classification of adenosine receptors mediating antinociception in the rat spinal cord. Br J Pharmacol (ENGLAND) 88:923-930.

Scemes E, Giaume C (2006) Astrocyte calcium waves: What they are and what they do. Glia (United States) 54:716-725.

Schechter R, Yen SH, Terry RD (1981) Fibrous astrocytes in senile dementia of the alzheimer type. J Neuropathol (UNITED STATES) 40:95-101.

Schell MJ, Molliver ME, Snyder SH (1995) D-serine, an endogenous synaptic modulator: Localization to astrocytes and glutamate-stimulated release. Proc Natl Acad Sci U S A (UNITED STATES) 92:3948-3952.

Schmelz M, Schmidt R, Bickel A, Handwerker HO, Torebjork HE (1997) Specific C-receptors for itch in human skin. J Neurosci (UNITED STATES) 17:8003-8008.

Schmidt A, Hall A (2002) Guanine nucleotide exchange factors for rho GTPases: Turning on the switch. Genes Dev (United States) 16:1587-1609.

Schmidt A, Weber OF (2006) In memoriam of rudolf virchow: A historical retrospective including aspects of inflammation, infection and neoplasia. Contrib Microbiol (Switzerland) 13:1-15.

Scholz J, Woolf CJ (2007) The neuropathic pain triad: Neurons, immune cells and glia. Nat Neurosci (United States) 10:1361-1368.

Scholz J, Woolf CJ (2002) Can we conquer pain? Nat Neurosci (United States) 5 Suppl:1062-1067.

Schousboe A, Westergaard N, Waagepetersen HS, Larsson OM, Bakken IJ, Sonnewald U (1997) Trafficking between glia and neurons of TCA cycle intermediates and related metabolites. Glia (UNITED STATES) 21:99-105.

Scott J, Huskisson EC (1976) Graphic representation of pain. Pain (NETHERLANDS) 2:175-184.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from discosoma sp. red fluorescent protein. Nat Biotechnol (United States) 22:1567-1572.

Sharma L, Kapoor D, Issa S (2006) Epidemiology of osteoarthritis: An update. Curr Opin Rheumatol (United States) 18:147-156.

Sheppard CA, Simpson PB, Sharp AH, Nucifora FC, Ross CA, Lange GD, Russell JT (1997) Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca2+ release sites that support Ca2+ waves in cultured astrocytes. J Neurochem (UNITED STATES) 68:2317-2327.

Sherman SE, Loomis CW (1994) Morphine insensitive allodynia is produced by intrathecal strychnine in the lightly anesthetized rat. Pain (NETHERLANDS) 56:17-29.

Sherrington CS (1906) The integrative action of the nervous system. New York: Scribner.

Shields SD, Eckert WA,3rd, Basbaum AI (2003) Spared nerve injury model of neuropathic pain in the mouse: A behavioral and anatomic analysis. J Pain (United States) 4:465-470.

Shigetomi E, Tong X, Kwan KY, Corey DP, Khakh BS (2011) TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. Nat Neurosci (United States) 15:70-80.

Shimada SG, LaMotte RH (2008) Behavioral differentiation between itch and pain in mouse. Pain (Netherlands) 139:681-687.

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M (2003) Signaling at the gliovascular interface. J Neurosci (United States) 23:9254-9262.

Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. Science (UNITED STATES) 252:802-808.

Simonds WF (1999) G protein regulation of adenylate cyclase. Trends Pharmacol Sci (ENGLAND) 20:66-73.

Smrcka AV, Hepler JR, Brown KO, Sternweis PC (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. Science (UNITED STATES) 251:804-807.

Snider WD, McMahon SB (1998) Tackling pain at the source: New ideas about nociceptors. Neuron (UNITED STATES) 20:629-632.

Somjen GG (1988) Nervenkitt: Notes on the history of the concept of neuroglia. Glia (UNITED STATES) 1:2-9.

Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, Mapplebeck JC, Wei P, Zhan S, Zhang S, McDougall JJ, King OD, Mogil JS (2011) The rat grimace scale: A partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain (England) 7:55.

Souchet M, Portales-Casamar E, Mazurais D, Schmidt S, Leger I, Javre JL, Robert P, Berrebi-Bertrand I, Bril A, Gout B, Debant A, Calmels TP (2002) Human p63RhoGEF, a novel RhoA-specific guanine nucleotide exchange factor, is localized in cardiac sarcomere. J Cell Sci (England) 115:629-640.

Spacek J (1985) Three-dimensional analysis of dendritic spines. II. spine apparatus and other cytoplasmic components. Anat Embryol (Berl) (GERMANY, WEST) 171:235-243.

Spataro LE, Sloane EM, Milligan ED, Wieseler-Frank J, Schoeniger D, Jekich BM, Barrientos RM, Maier SF, Watkins LR (2004) Spinal gap junctions: Potential involvement in pain facilitation. J Pain (United States) 5:392-405.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA (2007) The classical complement cascade mediates CNS synapse elimination. Cell (United States) 131:1164-1178.

Stewart PA, Wiley MJ (1981) Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail--chick transplantation chimeras. Dev Biol (UNITED STATES) 84:183-192.

Stewart WF, Ricci JA, Chee E, Morganstein D (2003) Lost productive work time costs from health conditions in the united states: Results from the american productivity audit. J Occup Environ Med (United States) 45:1234-1246.

Stoddard BL, Biemann HP, Koshland DE,Jr (1992) Receptors and transmembrane signaling. Cold Spring Harb Symp Quant Biol (UNITED STATES) 57:1-15.

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a na(+)-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci U S A (UNITED STATES) 89:10955-10959.

Straub SV, Nelson MT (2007) Astrocytic calcium signaling: The information currency coupling neuronal activity to the cerebral microcirculation. Trends Cardiovasc Med (United States) 17:183-190.

Streb H, Irvine RF, Berridge MJ, Schulz I (1983) Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature (ENGLAND) 306:67-69.

Street SE, Walsh PL, Sowa NA, Taylor-Blake B, Guillot TS, Vihko P, Wightman RM, Zylka MJ (2011) PAP and NT5E inhibit nociceptive neurotransmission by rapidly hydrolyzing nucleotides to adenosine. Mol Pain (England) 7:80.

Sun YG, Chen ZF (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature (England) 448:700-703.

Sung B, Lim G, Mao J (2003) Altered expression and uptake activity of spinal glutamate transporters after nerve injury contribute to the pathogenesis of neuropathic pain in rats. J Neurosci (United States) 23:2899-2910.

SUTHERLAND EW, RALL TW (1958) Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J Biol Chem (Not Available) 232:1077-1091.

SUTHERLAND EW, RALL TW, MENON T (1962) Adenyl cylase. I. distribution, preparation, and properties. J Biol Chem (Not Available) 237:1220-1227.

Swain MG, Rothman RB, Xu H, Vergalla J, Bergasa NV, Jones EA (1992) Endogenous opioids accumulate in plasma in a rat model of acute cholestasis. Gastroenterology (UNITED STATES) 103:630-635.

Sweger EJ, Casper KB, Scearce-Levie K, Conklin BR, McCarthy KD (2007) Development of hydrocephalus in mice expressing the G(i)-coupled GPCR Ro1 RASSL receptor in astrocytes. J Neurosci (United States) 27:2309-2317.

Sweitzer SM, Schubert P, DeLeo JA (2001) Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. J Pharmacol Exp Ther (United States) 297:1210-1217.

Takata N, Mishima T, Hisatsune C, Nagai T, Ebisui E, Mikoshiba K, Hirase H (2011) Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo. J Neurosci (United States) 31:18155-18165.

Takumi T, Ishii T, Horio Y, Morishige K, Takahashi N, Yamada M, Yamashita T, Kiyama H, Sohmiya K, Nakanishi S (1995) A novel ATP-dependent inward rectifier potassium channel expressed predominantly in glial cells. J Biol Chem (UNITED STATES) 270:16339-16346.

Tanga FY, Raghavendra V, DeLeo JA (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int (England) 45:397-407.

Taussig R, Zimmermann G (1998) Type-specific regulation of mammalian adenylyl cyclases by G protein pathways. Adv Second Messenger Phosphoprotein Res (UNITED STATES) 32:81-98.

Taylor AM, Peleshok JC, Ribeiro-da-Silva A (2009) Distribution of P2X(3)-immunoreactive fibers in hairy and glabrous skin of the rat. J Comp Neurol (United States) 514:555-566.

Taylor SJ, Chae HZ, Rhee SG, Exton JH (1991) Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature (ENGLAND) 350:516-518.

Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K (1992) The formalin test: An evaluation of the method. Pain (NETHERLANDS) 51:5-17.

Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci (England) 11:823-836.

Tracey I, Dickenson A (2012) SnapShot: Pain perception. Cell (United States) 148:1308-1308.e2.

Tran ND, Correale J, Schreiber SS, Fisher M (1999) Transforming growth factor-beta mediates astrocyte-specific regulation of brain endothelial anticoagulant factors. Stroke (UNITED STATES) 30:1671-1678.

Tsacopoulos M, Magistretti PJ (1996) Metabolic coupling between glia and neurons. J Neurosci (UNITED STATES) 16:877-885.

Tsien RY (1988) Fluorescence measurement and photochemical manipulation of cytosolic free calcium. Trends Neurosci (ENGLAND) 11:419-424.

Twycross R, Greaves MW, Handwerker H, Jones EA, Libretto SE, Szepietowski JC, Zylicz Z (2003) Itch: Scratching more than the surface. QJM (England) 96:7-26.

Ullian EM, Christopherson KS, Barres BA (2004) Role for glia in synaptogenesis. Glia (United States) 47:209-216.

Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. Science (United States) 291:657-661.

Urashima R, Mihara M (1998) Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. Virchows Arch (GERMANY) 432:363-370.

Urban MO, Gebhart GF (1999) Supraspinal contributions to hyperalgesia. Proc Natl Acad Sci U S A (UNITED STATES) 96:7687-7692.

Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci (UNITED STATES) 19:6897-6906.

Verhaak PF, Kerssens JJ, Dekker J, Sorbi MJ, Bensing JM (1998) Prevalence of chronic benign pain disorder among adults: A review of the literature. Pain (NETHERLANDS) 77:231-239.

Victor TW, Jensen MP, Gammaitoni AR, Gould EM, White RE, Galer BS (2008) The dimensions of pain quality: Factor analysis of the pain quality assessment scale. Clin J Pain (United States) 24:550-555.

Vit JP, Jasmin L, Bhargava A, Ohara PT (2006) Satellite glial cells in the trigeminal ganglion as a determinant of orofacial neuropathic pain. Neuron Glia Biol (England) 2:247-257.

Vit JP, Ohara PT, Bhargava A, Kelley K, Jasmin L (2008) Silencing the Kir4.1 potassium channel subunit in satellite glial cells of the rat trigeminal ganglion results in pain-like behavior in the absence of nerve injury. J Neurosci (United States) 28:4161-4171.

Vogt BA (2005) Pain and emotion interactions in subregions of the cingulate gyrus. Nat Rev Neurosci (England) 6:533-544.

Wall PD (1967) The laminar organization of dorsal horn and effects of descending impulses. J Physiol (ENGLAND) 188:403-423.

Wang DD, Bordey A (2008) The astrocyte odyssey. Prog Neurobiol (England) 86:342-367.

Wang F, Smith NA, Xu Q, Fujita T, Baba A, Matsuda T, Takano T, Bekar L, Nedergaard M (2012) Astrocytes modulate neural network activity by ca(2)(+)-dependent uptake of extracellular K(+). Sci Signal (United States) 5:ra26.

Wang H, Zylka MJ (2009) Mrgprd-expressing polymodal nociceptive neurons innervate most known classes of substantia gelatinosa neurons. J Neurosci (United States) 29:13202-13209.

Watkins LR, Maier SF (2005) Immune regulation of central nervous system functions: From sickness responses to pathological pain. J Intern Med (England) 257:139-155.

Weerth SH, Holtzclaw LA, Russell JT (2007) Signaling proteins in raft-like microdomains are essential for Ca2+ wave propagation in glial cells. Cell Calcium (Scotland) 41:155-167.

Wei F, Guo W, Zou S, Ren K, Dubner R (2008) Supraspinal glial-neuronal interactions contribute to descending pain facilitation. J Neurosci (United States) 28:10482-10495.

Wetherington J, Serrano G, Dingledine R (2008) Astrocytes in the epileptic brain. Neuron (United States) 58:168-178.

White FA, Sun J, Waters SM, Ma C, Ren D, Ripsch M, Steflik J, Cortright DN, Lamotte RH, Miller RJ (2005) Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. Proc Natl Acad Sci U S A (United States) 102:14092-14097.

Willcockson WS, Chung JM, Hori Y, Lee KH, Willis WD (1984) Effects of iontophoretically released amino acids and amines on primate spinothalamic tract cells. J Neurosci (UNITED STATES) 4:732-740.

Willis W (1985) The pain system. Basel: Karger.

Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM (2011) TRPA1 is required for histamine-independent, mas-related G protein-coupled receptor-mediated itch. Nat Neurosci (United States) 14:595-602.

Wong SK (2003) G protein selectivity is regulated by multiple intracellular regions of GPCRs. Neurosignals (Switzerland) 12:1-12.

Woolf CJ, Mannion RJ (1999) Neuropathic pain: Aetiology, symptoms, mechanisms, and management. Lancet (ENGLAND) 353:1959-1964.

Vetter IR, Wittinghofer A (2001) The guanine nucleotide-binding switch in three dimensions. Science (United States) 294:1299-1304.

Xin WJ, Weng HR, Dougherty PM (2009) Plasticity in expression of the glutamate transporters GLT-1 and GLAST in spinal dorsal horn glial cells following partial sciatic nerve ligation. Mol Pain (England) 5:15.

Yaksh TL (1989) Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: Effects of modulatory receptor systems and excitatory amino acid antagonists. Pain (NETHERLANDS) 37:111-123.

Yamamoto T, Yaksh TL (1993) Effects of intrathecal strychnine and bicuculline on nerve compression-induced thermal hyperalgesia and selective antagonism by MK-801. Pain (NETHERLANDS) 54:79-84.

Yan XX, Ribak CE (1998) Developmental expression of gamma-aminobutyric acid transporters (GAT-1 and GAT-3) in the rat cerebellum: Evidence for a transient presence of GAT-1 in purkinje cells. Brain Res Dev Brain Res (NETHERLANDS) 111:253-269.

Yosipovitch G, Greaves MW, Schmelz M (2003) Itch. Lancet (England) 361:690-694.

Zhang H, Mei X, Zhang P, Ma C, White FA, Donnelly DF, Lamotte RH (2009) Altered functional properties of satellite glial cells in compressed spinal ganglia. Glia (United States) 57:1588-1599.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron (United States) 40:971-982.

Zhang RX, Liu B, Wang L, Ren K, Qiao JT, Berman BM, Lao L (2005) Spinal glial activation in a new rat model of bone cancer pain produced by prostate cancer cell inoculation of the tibia. Pain (Netherlands) 118:125-136.

Zhang RX, Li A, Liu B, Wang L, Ren K, Zhang H, Berman BM, Lao L (2008) IL-1ra alleviates inflammatory hyperalgesia through preventing phosphorylation of NMDA receptor NR-1 subunit in rats. Pain (Netherlands) 135:232-239.

Zhuang ZY, Gerner P, Woolf CJ, Ji RR (2005) ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain (Netherlands) 114:149-159.

Zhuang ZY, Wen YR, Zhang DR, Borsello T, Bonny C, Strichartz GR, Decosterd I, Ji RR (2006) A peptide c-jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: Respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. J Neurosci (United States) 26:3551-3560.

Zhuo L, Sun B, Zhang CL, Fine A, Chiu SY, Messing A (1997) Live astrocytes visualized by green fluorescent protein in transgenic mice. Dev Biol (UNITED STATES) 187:36-42.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci (United States) 6:43-50.