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Chapter 18 In Vitro and in Vivo Assays for the Discovery of Analgesic Drugs Targeting TRP Channels

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18.1. INTRODUCTION

Pain is the most common symptom of disease and the most frequent complaint presented to physicians. Current pain therapies, mostly nonsteroidal, anti-inflammatory drugs (NSAIDs) and opioids, often have limited efficacy and produce severe gastrointestinal and cardiovascular side effects. A major recent advancement in pain research is the identification of novel analgesic targets including several TRP channels. These channels (TRPV1-4, TRPM8, and TRPA1) are expressed in primary sensory neurons and involved in sensing temperature, mechanical, and chemical stimuli.[1] Among them, TRPV1 is the most extensively studied and has been the subject of extensive reviews.[1–3] As a polymodal receptor, TRPV1 responds to various noxious stimuli, including noxious heat, (>43°C), acidic pH, noxious chemicals (e.g., capsaicin) and endogenous lipid products.[2] Tissue injury and inflammation increase TRPV1 expression and function, causing heightened pain sensitivity. Reduction of TRPV1 function, as either a result of gene knockout or pharmacological blockade, affects heat sensation and produces analgesia in various animal models. It is estimated that >55 pharmaceutical companies have pursued TRPV1 modulators as therapeutics, with a combined investment of >$1 billion.[4] These efforts have identified numerous TRPV1 modulators, with several compounds currently in clinical trials.

TRPA1 is another validated target for pain. It colocalizes with TRPV1 and markers of peptidergic nociceptors.[5] The expression of TRPA1 is increased in inflammatory and neuropathic pain models, as well as in avulsion-injured human dorsal root ganglion (DRG) neurons.[6] TRPA1 is activated by noxious cold, intracellular Ca^{2+}, hypertonic solutions, and, most prominently, by numerous electrophilic compounds, including active ingredients of pungent natural products (e.g., allyl isothiocyanate or AITC), environmental irritants (acrolein), and endogenous molecules involved in pain, oxidative stress, and inflammation (e.g., 4-hydroxynonenal or 4-HNE, 15-d- PGJ2, H_2O_2).[7,8] TRPA1 knockout mice exhibit deficits in pain hypersensitivity to agonists and bradykinin; also, treatment with antisense oligodeoxynucleotides and small molecular antagonists attenuate pain in inflamed and neuropathic rats.[9–11] Collectively, these studies support the role of TRPA1 in sensory function and its utility as a pain target.

Although capsaicin, the active ingredient in chili pepper, has been used for thousands of years for treating ulcers, backache, cough, and other maladies, modern drug discovery targeting TRP channels started about a decade ago. Drug discovery is a lengthy and expensive process (>10 years and $1.5 billion on average for each new drug) and typically consists of a sequence of events including target identification, assay development, compound screening, optimization, preclinical characterization, and human clinical trials. Here we focus on several aspects of analgesic drug discovery, including expression systems to express TRP channels, in vitro assays to identify modulators, and in vivo assays to characterize pain efficacy and side effects in preclinical animal models. TRPA1 and TRPV1 are used as examples in the chapter. However, the methods can be adapted to other TRP channels and other therapeutic targets as well.

18.2. EXPRESSION SYSTEMS

In theory, in vitro assays can be based on endogenous expression in native cell lines and heterologous expression in bacteria, yeast, *Xenopus* oocytes, insect, and mammalian cells. In practice, expression in

mammalian cells is the default platform, because it offers abundant gene-specific expression, an environment similar to native tissues, and opportunity for correct posttranslational modification. Furthermore, because most TRP studies are conducted using mammalian expression, this platform allows meaningful reference and comparison to literature data.[5] Expression in mammalian cells can be achieved either through stable cell lines or by transient expression. Baculovirus expression systems (e.g., BacMam) can also be used for in vitro assays, although extra steps and optimization are required.

18.2.1. Stable Cell Lines

The generation of stable cell lines has become routine, and detailed protocols can be found in textbooks, manuals, and the Internet (see Chapter 1). In general, the gene of interest is cloned in a vector (e.g., pcDNA3) that contains a promoter to allow highlevel expression in mammalian cells (e.g., CMV) and a resistance gene for selection (e.g., neomycin phosphotransferase). After being transfected into a mammalian cell line such as human embryonic kidney 293 (HEK293) or Chinese hamster ovary (CHO) cells, the target gene gets into the nucleus and integrates into the chromosomal DNA. Because chromosomal integration is a relatively rare event, cells have to be cultivated in a medium containing selective agents (e.g., Geneticin or G418) for rigorous selection. Only cells with integrated plasmid survive. In most scenarios, drug-resistant cell lines also carry the gene of interest. A procedure for establishing TRPA1 stable cell lines is described below.[12]

HEK293 cells are cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The pcDNA3.1/V5-His/ human TRPA1 plasmid is transfected using LipofectAMINE 2000 (Invitrogen). At 48 hours after transfection, cells are split to 1:4, 1:20 and cultured in the same medium as described above but supplemented with 1 mg/mL Geneticin. After 2 weeks, single colonies (1–2 mm in diameter) are picked by cloning cylinders, transferred to 24- or 48-well plates and cultured in medium supplemented with 300 μg/mL Geneticin. Clones are first screened for protein expression and AITC responses in a Ca^{2+} assay and then characterized in patch clamp recordings. Stable clones with robust and homogeneous (>90% positive) functional activities are chosen.

The process of generating, characterizing, and maintaining stable cell lines is labor-intensive and time-consuming (requiring at least 3 months). Constitutive expression of TRP channels often leads to cellular toxicity and degrading signals. Although inducible expression can be used,[5] it still requires generation and maintenance of stable cell lines. Moreover, induction of gene expression in a large scale can be tedious, expensive, and inconsistent.

18.2.2. Transiently Transfected Cells

Traditionally, transient transfection is conducted in a small scale (e.g., 10^7 cells) using adherent cells; therefore, its application is generally limited to characterization of the gene product, assay development, and pilot screens. Recently, large-scale transfection using suspension growth cell lines has become available, allowing transfection of $>10^9$ cells within a short time frame. The FreeStyle™ 293 Expression System (Invitrogen) uses suspension HEK293 cells (HEK293-F) and suspension CHO cells. In our hands, this system has been proven to be extremely useful in advancing our TRP channel efforts. Described below is a routinely used protocol.[12]

Briefly, HEK293-F cells are grown in suspension in flasks (cell volume 30 mL to 1 L) or in a Wave Bioreactor (Wave Biotech, Somerset, NJ, USA) (6 L). To support high-density, suspension culture and transfection, cells are grown in FreeStyle™ 293 media, an optimized and serum-free formulation. Transfection is carried out using 293fectin™ (Invitrogen). To transfect 3×10^7 cells (30 mL volume), 30 μg of plasmid DNA and 40 μL 293fectin are used. For larger volumes, each reagent is scaled up

proportionally. At 2 days posttransfection, cells (at a density of 2.5–3 × 10^6 cells/mL) are harvested by centrifugation (1000 × g, 5 min) and resuspended to a density of 1.5 × 10^7 cells/mL in freezing medium (Freestyle media/10% fetal bovine serum/10% DMSO). Cells are transferred in 2-mL aliquots into cryovials, which are then placed in Nalgene Mr. Frosty slow-freeze devices (Sigma-Aldrich, St. Louis, MO, USA) at –80°C. As needed, vials are removed from –80°C and quickly thawed in a 37°C water bath. Cells are aseptically transferred into conical tubes containing FreeStyle media (10 mL/vial). After centrifugation at 1000 × g for 3–5 min, the medium is removed by aspiration, and cells are re-suspended in FreeStyle medium again. Cells revived from frozen stock have a viability of ~90% as assessed by using VioCell (Beckman Coulter, Brea, CA, USA). Routinely, we generate 1.5–2.0 × 10^{10} cells from a 6-L transfection, which is sufficient to prepare 1950-2600 384-well plates (2 × 10^4 cells/well).

18.2.3. Transient Transfectlon versus Stable Cell Lines

Compared to stable cell lines, large-scale transient transfection offers multiple advantages. It can generate samples in days instead of months required to establish and select stable cell lines. It circumvents instability and cellular toxicity associated with constitutive gene expression, especially during long-term passage of stable cell lines. It can generate a large quantity (e.g., >10^{10}) of cells, therefore reducing labor and reagent costs dramatically. It also offers substantial flexibility because the cells can be stored at –80°C and thawed on demand for screening efforts. The relative low transfection rate (~60 versus ~90% positive in a high-quality stable cell line) could be a concern for some of the automated electrophysiology formats. However, this problem has been circumvented by the introduction of a population patch clamp, wherein multiple cells are simultaneously tested in a single well.[13]

18.3. IN VITRO ASSAYS FOR TRP CHANNELS

18.3.1. An Overview

One of the major challenges in drug discovery is identification of lead compounds with optimal chemical and pharmacological properties. This is often achieved by high-throughput screening (HTS) and lead optimization (secondary screening). At the HTS stage, 100,000 to several millions of compounds from chemical libraries are screened against the target of interest. The major considerations are assay robustness, throughput, reagent supply, and cost. At the lead optimization or secondary screening stages, HTS hits and lead compounds (often in the thousands) are characterized, with data quality as the major consideration. Their activities are interrogated in greater detail (e.g., IC_{50} or EC_{50} determination), in different assay formats, against various orthologs (e.g., human, rat, and mouse) and against related family members. Therefore, a panel of in vitro assays is required.

Many assays previously used for other ion channel targets have been adapted to TRP channels.[14] Ligand binding assays identify compounds that recognize the same binding site as a known radio-labeled ligand. This method is amenable to HTS, but it does not distinguish agonism, antagonism, and allosteric effects. Therefore, cell-based functional assays, which monitor channel conductance to Ca^{2+}, Na^+, and K^+ ions, are most widely used. These assays differ in measurement parameters, information content, throughput, and cost and are suited for different stages of drug discovery (Table 18.1). The patch-clamp technique directly measures current flow through the channel and is considered as the gold standard. It is very useful for mechanistic studies and detailed characterization of advanced compounds, but its application in compound screen is limited by the requirement for high technical skills and notoriously low throughput. The recently introduced automated electrophysiology platforms (e.g., PatchXpress, Q-Patch, IonWorks) are amenable to higher throughput and are increasingly used for evaluation of some voltage-gated channels and ligandgated channels.[15] Their application to TRP channels has been quite limited with only rare examples cited in the literature.[16] This is possibly due to several factors including requirement for fast

perfusion, and current run-up and run-down commonly observed for TRP channels. Ion flux assays using radioactive isotopes (e.g., $^{45}Ca^{2+}$, $^{86}Rb^{+}$, and [$^{14}C^{+}$]-guanidine) have been successfully used to identify TRP ligands (e.g., capsaizepine).[17] Recently, a nonradioactive ion flux assay using Rb^{+} and atomic absorption spectroscopy technology (ICR8000, Aurora Biomed, Vancouver, Canada) has been applied to TRP channels.[18] Compared to electrophysiology, the flux assay has increased throughput, although it offers no kinetic information and requires radioactivity or specialized equipment. In our opinion, the fluorescence-based Ca^{2+} assay and membrane potential assays are currently the predominant formats for TRP channel screening.

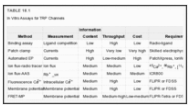

TABLE 18.1

In Vitro Assays for TRP Channels

18.3.2. FLUORESCENCE CA^{2+} ASSAYS

Fluo-4 and its derivatives are the most commonly used intracellular Ca^{2+} indicators. When excited at a wavelength of 480 nm, these dyes give an emission at 525 nm, with intensities corresponding to free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In cells expressing TRP channels, addition of exogenous agonists activates TRP channels and significantly increases $[Ca^{2+}]_i$ (e.g., >10-fold). The rise in $[Ca^{2+}]_i$ causes a strong increase in fluorescence signals of Fluo-4, which can be detected by the CCD camera equipped in a FLIPR (Fluorescence Image Plate Reader, MDS Analytical Technologies, Sunnyvale, CA, USA) or FDSS instrument (Functional Drug Screening System, Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). As shown in Figure 18.1, capsaicin (Cap) elicits a robust, dose-dependent increase in fluorescence signals in TRPV1-expressing cells. When 30 nM capsaicin is used, the signal-to-background ratio is 21 and the Z factor is 0.79 ($n = 24$) (see Chapter 1 for definition of Z factor). Routinely, 20-30 microplates (96-, 384-, or 1535-well format) can be screened on a daily basis, amassing a large number of data points. Described below is a detailed protocol describing use of 96-well plates, which can be adapted easily to accommodate 384- or 1536-well formats.[19]

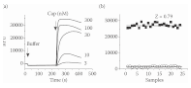

FIGURE 18.1

A Ca^{2+} assay for rTRPV1. (a) Representative traces of fluorescence changes evoked by different capsaicin (Cap) concentrations in FLIPR-based Ca^{2+} assay. Arrows indicate addition of buffer and Cap. Change in fluorescence is represented in relative fluorescence (more...)

Frozen aliquots of transiently transfected cells are quickly thawed in a 37°C water bath, transferred to 50-mL conical polypropylene centrifuge tubes containing the 293 medium (10 mL/vial), and pelleted by low-speed centrifugation (1000 × g for 5 min). Supernatants are removed, and cells are resuspended in the 293 medium at a density of 10^6 cells/mL. Resuspended cells are seeded into black-walled clear-bottom Biocoat™ poly-D-lysine assay plates (10^5 cells/well for 96-well plate, or 2×10^4 cells/well for 384-well plate; BD Biosciences, Bedford, MA, USA) and incubated overnight at 37°C under a humidified 5% CO_2 atmosphere.

Ca^{2+} influx is measured using a FLIPR calcium assay kit (R8033; MDS Analytic Technology). The Ca^{2+} indicator dye is dissolved in Hanks' balanced salt solution supplemented with 20 mM HEPES buffer (HBSS/HEPES) according to manufacturer's instructions. Before initiating the assay, the medium is removed by aspiration, and cells are loaded with 100 µL Ca^{2+} dye for 2 to 3 hours at room temperature. For TRPV1, TRPM8, and TRPA1, capsaicin, menthol, or AITC is used to activate each channel,

respectively. Solutions of the test compounds (4×) are prepared in HBSS/ HEPES, and 50 µL is added to the cells at a delivery rate of 10 µL/s. Changes in fluorescence are measured over time in FLIPR. Two additions are made over the course of an experimental run. For agonist experiments, assay buffer is added at the 10 s time point, followed by addition of agonist at the 3 min 10-s time point. For antagonist experiments, the antagonist is added at the 10 s time point, followed by addition of the agonist 3 min later. Final assay volume for both the agonist and antagonist experiments is 200 µL. Total length of an experimental run is 6.5 min. For TRPV4, hypotonic solutions of 212 mOsm are used to activate the channel and evoke Ca^{2+} influx. For filter setting in FLIPR, an excitation wavelength of 485 nm and emission wavelength of 525 nm are used.

18.3.3. Fluorescence Membrane Potential Assays

Opening of TRP channels elicit changes in membrane potentials, which can be detected by a combination of a membrane potential-sensitive dye and a fluorescence quencher. At hyperpolarized potentials, the negatively charged oxonol dye is localized at the outer layer of the lipid bilayer; upon excitation, the emission is absorbed by the quencher near the membrane. At depolarized potentials (i.e., opening of TRP channels), the oxonol dye moves to the inner layer of the lipid bilayer, and a strong fluorescent emission occurs. The fluorescence signals can be detected by the CCD camera in FLIPR or FDSS instruments. This assay has been applied to identify TRP modulators, with a procedure nearly identical to the Ca^{2+} assay.[19] In brief, we use a FLIPR membrane potential assay kit (R8034, MDS Analytic Technology), an excitation wavelength of 488 nm, and an emission wavelength of 550 nm. A more detailed protocol is covered in Chapter 1.

In addition to the fluorescence membrane potential assay, a fluorescence energy transfer-based membrane potential (FRET-MP) assay is also available. This assay is based on the fact that energy transfer between coumarin dye (donor) and oxonol dye (acceptor) increases at hyperpolarized potentials and deceases at depolarized potentials; therefore, the ratio of emission (460/580 nm) reflects membrane potential. This assay has been adapted to several TRP channels (J. Chen, R. M. Reilly, P. R. Kym, and S. Joshi, unpublished data). Although execution and data analysis for FRET-MP assays are more complex, its performance is generally superior to that of the fluorescence membrane potential assay in reducing well-to-well variation and incurrence of false-positive hits.

18.3.4. Considerations

Fluorescent Ca^{2+} and membrane potential assays are neither direct measurements nor linear correlates of channel activities. These assays can be prone to various artifacts, including those associated with compounds that are fluorescent, toxic, or those with the propensity to interact with dye or cellular pathways. Rigorous counterscreens should be performed to confirm that the compounds are specific to the intended target. For example, to confirm agonist activity, compounds should not have effects in mock-transfected cells, and signals should be sensitive to specific benchmark blockers. Lead compounds should be characterized, particularly for potency and selectivity in orthogonal assays. Compounds with adequate potency, selectivity, and pharmacokinetic profiles are subsequently evaluated in animal models.

18.4. PRECLINICAL PAIN AND SIDE EFFECT MODELS

The use of valid predictive preclinical animal models is critical for the identification and development of novel pharmacotherapies for treating human chronic pain syndromes. Here we describe some commonly used acute and pathological pain models in rats, which have proven to be valuable in understanding pain mechanisms and identifying novel drugs targeting TRP channels, particularly TRPV1 and TRPA1. Acute pain is assessed in normal animals, as measured by nocifensive withdrawal or escape behavior in response to painful stimuli including noxious temperatures, chemical reagents, and mechanical insult. Chronic pain

models are used to study pathological pain transmission, validate pain targets, and characterize the potential analgesic profile of novel compounds. Historically, these models were developed to replicate inflammatory, osteoarthritic, and neuropathic pain in humans.[20,21] One of the major challenges in pain drug discovery is identification of agents that alleviate pathological pain without eliciting severe side effects. Therefore, several commonly used models for assessing motoric, CNS side effects, and core body temperature are also described.

18.4.1. Models of Pharmacological Antagonism (Capsaicin-or AITC-Induced Nocifensive Response)

TRPV1 and TRPA1 agonists (e.g., capsaicin and AITC/mustard oil, respectively) are known to produce pain behavior upon intrapaw injection by activating their respective receptors on primary afferents.[22,23] These acute pain models can therefore be used to assess whether a compound behaves as a TRPV1 or TRPA1 antagonist upon systemic or local administration in rats. For testing TRPV1 antagonists in the capsaicin-induced flinching model, rats are placed in individual observation cages. Following an acclimation period of 30 min, the test compound is administered. At the appropriate time following compound administration, 2.5 µg of capsaicin in a 10-µL solution of 10% ethanol/90% hydroxypropyl-ß-cyclodextrin is injected subcutaneously into the dorsal aspect of the right hind paw. The observation cage is then suspended above mirrors in order to facilitate observation of the rat. Rats are observed for a continuous period of 5 min. The number of flinching behaviors of the injured paw is recorded during the 5 min observation period. Similarly, for testing TRPA1 antagonists, 5% mustard oil in 50-µL ethanol is injected subcutaneously into the dorsal aspect of the right hind paw. A TRPV1 or TRPA1 antagonist with adequate drug exposure should decrease the number of flinching behaviors induced by capsaicin or mustard oil, respectively. An example of a dose-related effect of a TRPV1 antagonist in attenuating capsaicin-induced flinching response is shown in Figure 18.2a.

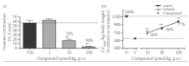

FIGURE 18.2

Antinociceptive effects of a TRPV1 antagonist. (a) In the capsaicin-induced nocifensive response model, a TRPV1 antagonist (compound) dose dependently attenuates the nocifensive flinching evoked by capsaicin. 2.5 µg of capsaicin is injected subcutaneously (more...)

18.4.2. Carrageenan Inflammatory Pain Model

Various models have been developed to induce a localized inflammatory reaction by injecting substances such as formalin, carrageenan, or complete Freund's adjuvant (CFA) into the paw or joints of rats. Following the initial injection, the inflammogens render the paw/joint very sensitive to thermal and mechanical stimuli; pain can be measured minutes to days later, either at the site or away from the site of injury.[24] Carrageenan produces a more subacute edema and inflammatory pain compared to CFA. In the carrageenan model, unilateral inflammation is induced by injecting 100 µL of a 0.5% solution of λ-carrageenan (Sigma, St. Louis, MO, USA) in 0.9% sterile saline into the plantar surface of the right hind paw. Thermal hyperalgesia is assessed 2 hours later. The compound of interest is administered, and its effects are determined at the appropriate time depending on its pharmacokinetic attributes. Response to thermal stimulation is determined using a commercially available paw "hotbox" thermal stimulator (UARDG, Department of Anesthesiology, University of California, San Diego, CA, USA) modeled after that described previously.[24] Rats are placed in individually partitioned Plexiglas cubicles that are on a glass surface maintained at 30°C. A thermal stimulus, in the form of radiant heat emitted by a focused projection bulb, is applied to the plantar surface of each hind paw. The stimulus current is 4.5 amperes, and the maximum exposure time is limited to 20 s in order to avoid tissue damage from the stimulus. In

each test session, both hind paws of each rat are tested in three sequential trials at approximately 5 min intervals. Paw withdrawal latencies are calculated as the mean of the two shortest latencies. A compound that alleviates thermal hyperalgesia in this model restores the paw withdrawal latency of the ipsilateral injured paw to that of the contralateral/uninjured paw.

18.4.3. Monoiodoacetate-Induced Osteoarthritic Pain Model

The development of preclinical osteoarthritic pain models in rodents has been particularly challenging owing to an inadequate understanding of the etiology of human osteoarthritic pain.[25] While it is clear that no single animal model can precisely replicate human osteoarthritis, a monosodium iodoacetate (MIA)-induced osteoarthritic model has been extensively used, based on the fact that robust pain behaviors in this model are amenable to pharmacological intervention.[26]

The procedures mentioned here for the induction and testing of osteoarthritic pain have been described previously.[27] Unilateral knee joint osteoarthritis is induced in rats by a single intra-articular (i.a.) injection of MIA (Sigma, St. Louis, MO, USA) (3 mg in 0.05 mL sterile isotonic saline) into the joint cavity using a 26G needle under light (2–4%) isoflurane anesthesia. Following injection, the animals are allowed to recover from the effects of anesthesia (5–10 min) before being returned to their home cages. After approximately 21 days, testing is performed. Grip strength is assessed in osteoarthritic rats as a measure of activity-induced pain. Measurements of hind limb grip force are conducted by recording the maximum compressive force (CF_{max}) exerted on the hind limb strain gauge, in a commercially available grip force measurement system (Columbus Instruments, Columbus, OH, USA). Each rat is gently restrained and allowed to grasp the wire mesh frame attached to the strain gauge. The experimenter then moves the animal in a rostral-to-caudal direction until the grip is broken. Each rat is sequentially tested twice at an approximately 2–3 min interval to obtain a raw mean grip force (CF_{max} in gram force units). In order to account for the body weight differences among the rats, this raw mean grip force is normalized by the body weight of the rat (in kilograms) to generate a maximum hind limb compressive force for each animal [(CF_{max} in gram force)/kg body weight]. A group of age-matched naïve animals is included in each experiment. Data obtained from various dose groups for the test compound are compared with data from the naïve group. The vehicle control group is assigned a value of 0%, whereas the naïve group is assigned a value of 100%. The percent effects for each dose group are expressed as percent return to normal grip force as found in the naïve group, and calculated using the formula: (percent return to normalcy = [(Treatment CF_{max} – Vehicle CF_{max}) / (Naïve CF_{max} – vehicle CF_{max})] × 100%). An example of a dose-response curve with this protocol is shown in Figure 18.2b.

18.4.4. Chronic Constriction Injury Model of Neuropathic Pain

Neuropathic pain can result from inflammation around peripheral nerves and peripheral nerve compression. The chronic constriction injury (CCI, Bennett model) of the sciatic nerve model has been developed to mimic this clinical situation.[28] In this model, the right common sciatic nerve is isolated at the midthigh level and loosely ligated by four chromic gut ties separated by an interval of approximately 1 mm. All animals are allowed to recover for at least two weeks and no more than four weeks prior to testing of cold or mechanical allodynia. Cold allodynia is assessed following acetone application.[29] Rats are acclimated in a transparent plastic box for about 20 min before behavioral testing. Acetone (100 μL) is gently sprayed onto the plantar surface of the hind paw. For baseline recordings, acetone is sprayed three times on the right ipsilateral nerve-injured hind paw at 5 min intervals to identify allodynic animals. Animals included in the study are also tested on the contralateral paw. Following compound administration, acetone is sprayed 5 times at 5 min intervals. The paw flinching response after the acetone spray is considered as a positive response, and frequency of response to five acetone sprays is noted. The response frequency is calculated using the formula: ([number of positive responses following treatment/5] × 100%).

Mechanical allodynia testing is performed using calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA). Paw withdrawal threshold is determined by increasing and decreasing stimulus intensity, and estimated using the Dixon's up-down method.[30] Rats are placed into inverted individual plastic containers (20 × 12.5 × 20 cm) on top of a suspended wire mesh with a 1-cm^2 grid to provide access to the ventral side of the hind paws and acclimated to the test chambers for 20 min. The von Frey filaments are then presented perpendicularly to the plantar surface of the selected hind paw, and held in this position for approximately 8 s with enough force to cause a slight bend in the filament. Positive responses include an abrupt withdrawal of the hind paw from the stimulus or flinching behavior immediately following removal of the stimulus. A 50% withdrawal threshold is determined using an up-down procedure.[30] The strength of the maximum filament used for von Frey testing is 15.0 g. Only rats with a baseline threshold score of less than 5 g are used in this study, and animals demonstrating motor deficit are excluded.

18.4.5. Models of Acute Thermal Nociception

It is important that a clinically used analgesic does not significantly impair the protective ability to sense acute noxious thermal or mechanical sensation. This is a particularly important issue for the modulators of TRPV1 and TRPA1, because these channels have been shown to play a role in sensing noxious heat and cold, respectively.[1] A tail immersion assay is used to assess effects on noxious heat sensation. In this assay, a circulating water bath is heated to 55°C. At an appropriate time post-dosing, the rats are handled for a few seconds to calm them down and then cupped with their back against the testers hand at a slight angle with the head facing away from the tester. With the rat in one hand and a 0.01 s stopwatch in the other hand, the tail is quickly immersed 6–8 cm in a water bath or to a distance leaving 2–3 cm of tail out of water. The timer is started simultaneously. When the rat flinches or attempts withdrawal, the timer is immediately stopped and the tail is quickly removed from the water bath. The response latency (in seconds) is recorded and repeated 3 times with 3–4 min between readings. A percent increase in the average response latency for tail withdrawal relative to a vehicle control is determined. Effects of compounds on noxious cold sensation can be assessed using cold plate apparatus (Columbus Instruments, Columbus, OH, USA) in rats or mice. The latency to first jump is measured after the animal is placed on the cold plate (0°C) with a cutoff time of 180 s.

18.4.6. Locomotor Activity and Rotarod CNS Side Effect Assays

It is very important to evaluate compound effects on the locomotor activity or the motor system (rotarod assay) for at least two reasons. First, such side effects are undesirable from a clinical standpoint. Second, they could confound interpretation of observed efficacy in pain models. Locomotor activity is measured in an open field using photo beam activity monitors (AccuScan Instruments, Columbus, OH, USA). Rats are acclimated to the test room for 30 min. Following compound administration, the AccuScan activity boxes measure horizontal activity as photo beam breaks in the horizontal plane. Rotarod performance is measured using an accelerating Rotarod apparatus (Omnitech Electronics, Inc., Dartmouth, NS, Canada). Rats are placed on a 9 cm diameter rod with an increasing rotating speed from 0 to 20 rpm over a 6 s period. Each rat is given three training sessions. The Rotarod performance is determined by the total amount of time within 60 s that rats stayed on the rod without falling off (the maximum score is 60 s).

18.4.7. Telemetry for Assessment of Core Body Temperature

TRPV1 is involved in regulating body temperature, and blockade of TRPV1 by some antagonists increases core body temperature.[4] Such effect can be assessed in rats implanted with telemetry catheters (TL11M2-C50PXT, Data Sciences International, St. Paul, MN, USA), which accurately measure core-body temperature. Anesthetized rats are placed on a heating pad and covered with a sterile surgical drape. A ventral midline abdominal incision is made, and sterile cotton tip applicators are used to gently move

internal tissue and expose the abdominal aorta for implantation of the telemetry catheter. Blood flow is temporarily stopped to the lower extremities (5–7 min) with Diffenbach clamps to allow the insertion of the telemetry catheter into the abdominal aorta. A sterile cellulose patch is placed over the catheter/aorta and secured using a small amount of tissue adhesive (Vetbond, 3M, St. Paul, MN, USA). Once catheter placement is complete, the clamps are removed and blood flow is restored to the lower extremities. The transmitter is placed in the intraperitoneal cavity. The transmitter suture rib is sewn into the abdominal sutures to secure it in place. The skin is closed using sterile wound clips and the animal removed from Sevoflurane. Buprenex (0.01 mg/mL s.c.; Reckitt Benckiser Pharmaceuticals, Inc., Bristol, UK) is administered for post-operative analgesia. Animals are maintained on a heating pad until ambulatory and then individually housed with food and water *ad libitum.* Surgical staples are removed after 7–10 days postimplantation. Rats are allowed a two-week postsurgical recovery period before compound treatment.

Rats are randomly divided into study groups and administered either vehicle or test compound by oral dosing at 8:00 AM. Further entry into the telemetry room is minimal. Data collected during the time when staff is present are not included in the analysis. Figure 18.3 shows that the effect of a TRPV1 antagonist on core body temperature. Clearly, at 3 and 10 μmol/kg dose, this compound increases body temperature (approximately 1°C). In addition to core body temperature, the telemetry device also measures cardiovascular functions such as mean arterial pressure and heart rate. All parameters are continuously recorded and analyzed using commercial software and a signal processing workstation (Dataquest Art v4.0, Data Sciences International).

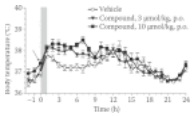

FIGURE 18.3

Effect of a TRPV1 antagonist on core body temperature in conscious-telemetry rats. After oral dosing at 3 and 10 μmol/kg, a TRPV1 antagonist increases core body temperature (approximately 1°C; lasting for ~7 hours) compared with vehicle. (more...)

18.5. CONCLUSION

The tremendous progress in TRP sensory biology has been greatly facilitated by the availability of potent and selective pharmacological tools. These agents are identified in in vitro assays and characterized in preclinical models to reveal the physiological function and therapeutic utility of the channel. As a recent example, evaluation of TRPV1 antagonists in telemetrized rodents uncovered the role of TRPV1 in body temperature regulation.[4] Currently, a variety of cell-based functional assays are available, each with its own utility in drug discovery. For example, the fluorescence Ca^{2+} assay and membrane potential assays are best suited for HTS, while manual patch clamp electrophysiology is best suited for detailed characterization of compounds. Emerging technologies, such as automated electrophysiology, will likely have an increasing impact. Preclinical behavior models have been, and will continue to be, essential for assessing analgesic efficacy and side effects, and their predictive values will be revealed when more compounds enter human clinical trials in the coming years. Through emerging technologies and concerted efforts across industry and academia, there is reason to believe novel TRP drugs are on the horizon.

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