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Constitutive Activity at the Cannabinoid CB₁ Receptor and Behavioral Responses

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Abstract

The cannabinoid receptor type 1, found mainly on cells of the central and peripheral nervous system, is a major component of the endogenous cannabinoid system. Constitutive and endogenous activity at cannabinoid receptor type 1 regulates a diverse subset of biological processes including appetite, mood, motor function, learning and memory, and pain. The complexity of cannabinoid receptor type 1 activity is not limited to the constitutive activity of the receptor: promiscuity of ligands associated with and the capability of this receptor to instigate G protein sequestration also complicates the activity of cannabinoid receptor type 1. The therapeutic use of cannabinoid receptor type 1 agonists is still a heavily debated topic, making research on the mechanisms underlying the potential benefits and risks of cannabinoid use more vital than ever. Elucidation of these mechanisms and the quest for agonists and antagonists with greater specificity will allow a greater control of the side effects and risks involved in utilizing cannabinoids as therapeutic agents. In this chapter, we review a small subset of techniques used in the pharmacological application of and the behavioral effects of molecules acting at the paradoxical cannabinoid receptor type 1.

1. Introduction

The endocannabinoid system (ECS) is a complex and far reaching network comprised of the endogenous cannabinoids *N*-arachidonylethanolamine (anandamide or AEA) and 2-arachidonyl glycerol (2-AG), cannabinoid hydrolyzing enzymes fatty acid amide hydrolase (FAAH), and monoacylglyceride lipase (MAGL), the endogenous cannabinoid receptors: type 1 (CB₁) and type 2 (CB₂); and the recently characterized G-protein-coupled receptor (GPCR) GPR55 (Alpini and DeMorrow, 2009; Pertwee, 2001a). Endogenous cannabinoid ligands and many synthetic cannabimimetic compounds also display activity at a number of other receptor classes, including transient receptor potential cation channel, subfamily V, member 1 (TRPV1) channels, and the peroxisome proliferator-activated receptors (PPARs) of the nucleus (De Petrocellis and Di Marzo, 2010; Di Marzo *et al.*, 1998; Hermann *et al.*, 2003; Patwardhan *et al.*, 2006; Zygmunt *et al.*, 1999). The promiscuity of cannabinoids is due in part to their lipophilic properties and contributes greatly to the complexity of the ECS.

Another major factor complicating the ECS is the abnormal activity of the CB₁ receptor. The CB₁ receptor is not only constitutively active, but it has the ability to sequester G_{i/o} proteins (Vasquez and Lewis, 1999)—either preventing other receptors from signaling at all, or increasing the signaling of other receptors through G_s or G_q, ultimately forcing a nonnative

response. Review of the literature reveals some degree of discrepancy with the claim of constitutive activity since it can be difficult to distinguish between endogenous ligand activity and constitutive activity of a receptor in an intact biological system (Seifert and Wenzel-Seifert, 2002; Turu and Hunyady, 2010; Turu *et al.*, 2007); however, it is generally accepted that the CB₁ GPCR does behave constitutively in most systems (Bouaboula *et al.*, 1997; Canals and Milligan, 2008; Fioravanti *et al.*, 2008; Nie and Lewis, 2001; Pertwee, 2005). Howlett *et al.* (1998) described a peptide sequence in the juxtamembrane region of the proximal C-terminal tail of the CB₁ receptor that can activate G proteins. Nie and Lewis (2001) further enumerated the underlying mechanism of CB₁ constitutive activity by defining another region of the C-terminal tail, distal to the G-protein-activating region, acting as a regulatory sequence to prevent activation of G proteins. Truncation of this distal portion of the CB₁ C-terminus increases the constitutive activity of the receptor (Nie and Lewis, 2001). A third site within the CB₁ receptor also contributes to constitutive activity—a sequence in the second transmembrane domain stabilizes the receptor in one of two states: the inactive R_{GDP} or constitutively active R*_{GTP} conformation. Interestingly, alteration of this stabilization region also inhibits the CB₁ receptor's ability to sequester G_{i/o} proteins (Howlett *et al.*, 1998; Mukhopadhyay *et al.*, 1999).

Pharmacological manipulation of the CB₁ receptor is significantly impacted by the constitutive activity of the receptor. Long after the introduction of the so-called selective antagonists of CB₁, including AM251 and SR141716A (Rimonabant[®]), it was revealed that many if not all are actually inverse agonists (MacLennan *et al.*, 1998; Pertwee, 2005). The search for a neutral antagonist of CB₁ has been extensive, and only recently have compounds begun to surface claiming to be neutral antagonists—most notably NESS 0327 (Ruiu *et al.*, 2003). Further characterization is still needed to determine the true activity of these compounds.

CB₁ receptors are widely distributed in the CNS and to a lesser extent in the periphery (Pertwee, 1999). CB₁ receptors are found in the gastrointestinal tract, mainly on cells of the enteric nervous system, as well as on neurons of the peripheral nervous system (Pertwee, 2001b). CB₁ receptors display a limited expression on immune cells as well (Maresz *et al.*, 2007). The CB₁ receptor couples primarily to G_{i/o} and is found in the highest density in GABAergic neurons, though CB₁ has also been shown to act via G_{i/o} in excitatory neurons (Howlett *et al.*, 2002). This results in inhibition of neurotransmitter release via a blockage of voltage-dependent Ca²⁺ channels and activation of inwardly rectifying K⁺ channels. In addition, CB₁ affects the intracellular focal adhesion kinase cascade, the mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol 3-kinase (PI3K) pathway, and modulates production of nitric oxide through nNOS (Bouaboula *et al.*, 1997; Carney *et al.*, 2009; Howlett *et al.*, 2002; Turu and Hunyady, 2010). CB₁ has been implicated in a number of different biological processes including, but not limited to, appetite/satiation, mood, learning and memory, pain processing, and inflammation (Abush and Akirav, 2009; Herkenham *et al.*, 1990; Howlett *et al.*, 2002; Pertwee, 2001b; Richardson *et al.*, 1998a,b; Zhou and Shearman, 2004).

One of the most famous characteristics of exogenous cannabinoids is their ability to stimulate appetite. In 1992, Marinol[®] (dronabinol), a product composed of purified (–)-

trans-D⁹-tetrahydrocannabinol (THC), was approved as an appetite stimulant in AIDS patients; for review on cannabinoids and patents see Galal *et al.* (2009). Cannabinoid agonists also make excellent antiemetics. Marinol[®] (dronabinol) was approved by the FDA in 1985 for nausea and vomiting caused by chemotherapeutic agents (Galal *et al.*, 2009). Other studies have shown that cannabinoid agonists are effective in the relief of opioid-induced emesis (Simoneau *et al.*, 2001), as well as emesis induced by radiotherapy (Stewart, 1990). The antiemetic effect of CB₁ agonists can be attributed to the CB₁ receptors present in the dorsal–vagal complex of the brain stem (Van Sickle *et al.*, 2001), as well as CB₁ receptors present within the gastrointestinal tract; CB₁ activation also reduces electrically stimulated contractions of the small intestine by inhibiting evoked acetylcholine release (Gifford and Ashby, 1996; Izzo and Coutts, 2005).

Gastrointestinal inflammation may also be regulated by the ECS. Inflammatory bowel disease is a persistent state of inflammation within the gastrointestinal tract that comprises two main diseases—ulcerative colitis and Crohn’s disease. Both conditions are treated as autoimmune disorders, though the status of ulcerative colitis as such is still debated (Kunos and Pacher, 2004). CB₁ activation within the enteric nervous system reduces acid production and reduces production of the proinflammatory cytokines TNF α and IL-1 β (Engel *et al.*, 2008). Further, CB₁^{−/−} mice have increased susceptibility to the well-established *in vivo* model of IBD-like symptoms—trinitrobenzene sulfonic acid (TNBS)-induced colitis (Engel *et al.*, 2010).

Endogenous CB₁ involvement in learning and memory processes has been well established (for review see Moreira and Lutz, 2008). Several studies implicate CB₁ agonists as inhibitors of memory, plasticity, and long-term potentiation while CB₁ antagonists improve these activities in hippocampus-mediated tasks (Abush and Akirav, 2009). Amygdala-dependent processes, including emotional memory, however, do not tend to follow the same etiology (Chhatwal and Ressler, 2007), further complicating the physiological role of CB₁.

Finally, one of the most notable central roles of the ECS lies in pain processing. CB₁ receptors are heavily colocalized with μ -opioid receptors (MOPs) (Pickel *et al.*, 2004; Rodriguez *et al.*, 2001; Salio *et al.*, 2001) and the two receptor classes have been shown to heterodimerize (Rios *et al.*, 2006; Schoffelmeer *et al.*, 2006). Several groups have shown cross regulation by ligands at these two receptors (Fioravanti *et al.*, 2008); Canals and Milligan (2008) demonstrated that the function of MOPs is regulated by constitutive activity at the CB₁ receptor. In addition, a substantial number of studies have shown the analgesic properties of CB₁ agonists (for review see Pertwee, 2001a) along with the upregulation of CB₁ receptors at the spinal cord level in models of neuropathic pain (Walczak *et al.*, 2006; Wang *et al.*, 2007). While the involvement of CB₁ in ascending pain pathways has been well established, the CB₁ role in descending pain modulation pathways is still being elucidated (Wilson *et al.*, 2008). One is hard pressed to find a more diverse and interesting endogenous system than the ECS. In this chapter, we explore methods employed in the study of the CB₁ receptor and CB₁ selective compounds.

2. Modifying CB₁ Activity

While a great number of viable genetic manipulations exist and aid in either the silencing or the overexpression of a given receptor or protein, in this section we will focus on pharmacological manipulations. Pharmacological approaches are much simpler to carry out *in vivo* than gene targeted approaches due to their time- and dose-relationships without long-term compensation found in knockout animals. It should be mentioned that CB₁ knockout mice and rats are commercially available and have been instrumental in the study of endogenous CB₁ activity.

2.1. Pharmacological manipulation

When selecting a drug delivery method a number of factors must be considered, including animal size, solubility of the drug being administered, volume being administered, half-life of the drug, and the drug's ability to cross the blood–brain barrier (Scheld, 1989). Given the lipophilicity of compounds acting at the cannabinoid receptors, solubility is an issue that demands attention in the design of an effective vehicle; see Table 1.1 for a brief summary. In this section, we will focus on drug delivery methods in mice and rats, though many of these practices can be applied to a number of different species.

2.1.1. Vehicle preparation—While dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) seem to be the universal solvents as far as cannabinoid agonists and inverse agonists are concerned, they are by no means infallible. DMSO is less toxic than DMF (El, 1996); however, preparations of 100% DMSO are not recommended as it is neurotoxic at levels as low as 0.3 mL/kg (Junior *et al.*, 2008). To avoid neurotoxicity the organic solvent must be diluted; this can be done relatively easily with a 0.9% saline solution. Typical vehicle dilutions are 10% DMSO with a volume of Tween 80 that ranges from 3% of the final volume for spinal preparations to 10% of the final volume for parenteral injection; the solution is then brought to volume with saline. The addition of Tween 80 is critical—diluting the concentration of DMSO will cause the compound to fall out of solution: adding a small volume of Tween 80 helps homogenize the mixture.

2.1.2. Routes of drug delivery

2.1.2.1. Intrathecal catheter: The placement of an intrathecal catheter in order to deliver drugs by the spinal route both acutely and chronically was first reported by (Yaksh and Rudy, 1976). Rats should be anesthetized by either injecting a mixture of ketamine/xylazine (100 mg/kg, i.p.), or isoflurane (inhaled, 5% in O₂ for induction and maintained at 2.5% in O₂ at 2 L/min). Shave from the dorsal nape of the neck to between the ears and clean the area with a povidone–iodine topical antiseptic followed by 70% ethanol in diH₂O. Anesthetized rats should be placed in a stereotaxic head holder by lightly gripping the skull between thumb and forefinger (thumb on top of the skull and forefinger beneath chin); support the rat's body weight with the remaining fingers and palm wrapping lightly around the animal's ribcage. The stereotaxic head holder should have one arm fixed in place. Which arm remains fixed is up to the user; in general a right handed person holding the animal with the right hand will find it most comfortable to tighten the left arm of the stereotaxic device and vice versa. Gently maneuver the rat onto the fixed arm by inserting the arm into the rat's

ear. Still holding the rat steadily, slide the free arm into the rat's other ear and tighten. Slide the nose piece back until the rat's head is bent downward such that the top of the nose is perpendicular to the bench. Using a number 10 blade, make a skin incision approximately 2 cm in length down the midline beginning slightly higher than ear level. Using the same number 10 blade and holding the scalpel nearly horizontal, make an incision through the exposed muscle down the midline by resting the blade on the midline and applying a gentle rocking pressure. Expose the cisterna magna by inserting a retractor and opening the muscle incision just enough to place a cotton swab inside of the opening. Using a number 11 blade at a 90° angle, very gently make a shallow 2 mm incision in the cisterna magna. It is extremely important to only allow the blade to puncture the membrane; a deeper incision will damage the brain stem and cause paralysis. Drain the cerebrospinal fluid using cotton swabs and gently massaging the spine. In general a 300 g rat will require two cotton swabs. Widen the opening in the cisterna magna slightly using the number 11 blade again at a 90° angle but this time perpendicular to the original incision to yield a diamond shaped opening. Insert the catheter (PE: 10, 8 mm) down the length of the spinal column such that it terminates in the lumbar region of the spinal cord. At this stage it is important to avoid nicking the spinal cord with the catheter to avoid paralysis: holding the catheter horizontally, insert the tubing into the opening while holding the tail of the animal with the opposite hand to maintain a straight and rigid spinal column. While inserting the catheter, attempt to keep the catheter at the dorsal side of the column away from the spinal cord, which should now be lying on the ventral surface of the column. If any resistance is felt during insertion of the catheter, stop, retract the catheter, turn slightly and try again. Once the catheter has been inserted it can be stitched into place deep in the muscle with 3-0 silk and the skin closed above it via 3-0 silk suture or staples. Allow the animals to recover 5–7 days postsurgery before any pharmacological manipulations are made.

For intrathecal drug administration to the lumbar section of the spinal cord, all drugs should be injected in a 5 µL volume followed by a 9 µL saline flush using a 25 mL Hamilton syringe fitted with tubing connected to an injection cannula (C3131I) to allow the animals to move freely within their home cages during injection. Saline (9 µL) followed by a 1 µL of air then 5 µL of drug should be drawn up into the tubing. The 1 µL of air is important to observe while injecting to assure that the intrathecal catheter is not blocked. If the catheter is blocked, the 1 µL of air will compress alarming the experimenter that the drug may not be moving to the site of action. The 9 µL of saline is important to push the drug entirely through the intrathecal catheter and into the site of action. If drug is to be administered to other areas of the spinal cord the volume of saline should be adjusted (i.e., less saline to thoracic and more to sacral).

2.1.2.2. Lumbar puncture: While the intrathecal catheter is an excellent method of spinal delivery in rats, it is a cumbersome procedure in mice. Alternatively a simple spinal administration can be made via lumbar puncture in the oft significantly smaller mouse without requiring anesthesia. For lumbar puncture, inject a volume of 5 µL using a 30 gauge 0.5 in. needle fitted to a 10 µL Hamilton syringe. Holding the mouse firmly by the pelvic girdle, insert the needle at a 20° angle into one side of the L5 or L6 region in between the spinous and transverse processes. Decrease the angle to approximately 10° and insert 0.5 cm

into the vertebral column. Expel the volume slowly (over a period of at least 5 s) and rotate the needle on withdrawal to ensure delivery of the entire volume. No recovery period is required.

2.1.2.3. Oral gavage: Oral gavage can be performed in mice or rats when oral dosing is required and the researcher is unable to dissolve the compound into the animal's food or water. Additionally, oral gavage allows for a greater control of the dosing schedule and volume. A maximum of 10 mL/kg may be administered via oral gavage and may be repeated up to three times daily. Round tip feeding tubes or ball tip feeding needles may be used: 14–18 gauge for rats or 20–24 gauge for mice; length should be determined by measuring from mouth to most caudal rib. Anesthesia is not required for oral gavage; however, the animal will need to be restrained. This condition makes oral gavage administration easiest to perform on mice, as they can be held by the scruff, pinching the skin over the shoulders such that the forelimbs extend to either side of the body. Holding the animal horizontally parallel to the work surface, straighten head so that no bends in the esophagus occur. Insert the gavage tube or needle over the tongue through the pharynx in one motion; if any resistance is felt withdraw and try again, do not attempt to push through the obstruction, as this is a clear sign of entering into the lungs. The researcher should observe a natural gag and swallow reflex so that the animal swallows the feeding tube or needle. After administering the compound, observe the animal for 5 min, watching for difficulty in breathing or distress.

2.1.2.4. Parenteral injections in mice: The most effective parenteral injections into a mouse include, subcutaneous, intraperitoneal, and intravenous. Concentrations should be based upon a 10 mL/kg injection volume. Intramuscular injections are discouraged due to the small amount of muscle mass compared to injection volume and the potential for muscle destruction.

Subcutaneous (s.c.): insert a 30 gauge, 0.5 in. needle fitted to a 1 cc syringe parallel to the body of the animal into the base of the fold formed when pinching the skin constituting the scruff and gently pulling away from the mouse's body. In general, no more than 1 mL may be administered subcutaneously to an adult mouse. If a greater volume is required, repeat the procedure at multiple sites sufficiently separated such that the sites are not overlapping.

Intraperitoneal (i.p.): insert a 30 gauge 0.5 in. needle fitted to a 1 cc syringe at a 45° angle, positioned so that the tip points towards the midline. The mouse should be lightly restrained by hand, pinching the skin at the scruff of the neck between thumb and forefinger and securing the tail to the researchers palm with the pinky finger. Invert the animal prior to injecting. An adult mouse can withstand up to a 2 mL total volume of injection with i.p. administration.

Intravenous (i.v.): place the animal into a restraining device (available through VWR) such that the tail protrudes from the device. Hold the tail under warm water for 10 s (do not allow the water temperature to exceed 55 °C) to dilate the tail vein. Insert a 30 gauge 0.5 in. needle attached to a 1 mL syringe into the tail vein at an angle of approximately 20°, confirm placement by observation of blood in the tip of the syringe prior to injection. Intravenous injection volume in an adult mouse should not exceed 0.5 mL, and should be administered

slowly (over a period of at least 10 s). If an out-pocketing of the tail skin is observed at the injection site the researcher should stop, remove the needle and try again.

2.1.2.5. Parenteral injections in rats: Parenteral injections in rats are similar to those in mice with the exception of concentration and volume. Parenteral injections in rats should have drug concentrations based on a 1 mL/kg volume. In addition, intramuscular (i.m.) injections in rats can be extremely useful: i.m. injections are not as time consuming as i.v. and the compound reaches the circulatory system more quickly than s.c. or i.p. injections.

Subcutaneous (s.c.): insert a 25 or 30 gauge 0.5 in. needle fitted to a 1 cc syringe parallel to the body of the animal into the base of the fold formed when pinching skin on the dorsal thoracic area and gently pulling the skin away from the rat's body. In general, no more than 1 mL may be administered subcutaneously to an adult rat. If a greater volume is required, repeat the procedure at multiple sites sufficiently separated such that the sites are not overlapping.

Intramuscular (i.m.): insert a 25 or 30 gauge 0.5 in. needle fitted to a 1 cc syringe at a 20–30° angle into either the hamstring on the ventral side of the femur or the quadriceps on the dorsal side of the femur, aligning the needle length-wise to the direction of muscle fiber extension. A maximum volume of 0.25 mL should be injected i.m. in a rat.

Intraperitoneal (i.p.): insert a 25 or 30 gauge 0.5 in. needle fitted to a 1 cc syringe at a 45° angle, positioned so that the tip points towards the midline. The rat should be restrained by hand, wrapping the thumb and forefinger around the shoulder area (aligning the rat's spine to the soft area of the researchers hand where the forefinger and thumb meet) such that the animal's forelimbs are crossed in front and the weight of the animal is supported by the remaining fingers and palm surrounding the ribcage firmly, but gently. Invert the animal prior to injecting. An adult rat can withstand up to a 5 mL total volume of injection with i.p. administration.

Intravenous (i.v.): place the animal into a restraining device (available through VWR) such that the tail protrudes from the device. Hold the tail under warm water for 10 s (do not allow the water temperature to exceed 55 °C) to dilate the tail vein. Insert a 30 gauge 0.5 in. needle attached to a 1 mL syringe into the tail vein at an angle of approximately 20°, confirm placement by observation of blood in the tip of the syringe prior to injection. Intravenous injection volume in an adult rat should not exceed 1 mL, and should be administered slowly (over a period of at least 10 s). If an out-pocketing of the tail skin is observed at the injection site the researcher should stop, remove the needle and try again.

3. Behavioral Models

Because the scope of the authors experience lies primarily in the pain field, this section will be heavily focused on pain models and the associated behavioral testing. Other models relevant to the CB₁ receptor are also briefly described, including multiple sclerosis, emesis, and some models used in learning and memory studies. The CB₁ receptor has been described as the most abundant receptor in the brain (Howlett, 2005); its study is an ever

expanding field and as such the scope of pharmacological manipulation is not limited to the methods described here.

3.1. Pain models

3.1.1. Neuropathic pain models—During the late twentieth century several pain models were developed that enabled the study of neuropathic pain. While the models use primarily mice and rats, the symptoms closely mimic those of humans with conditions such as causalgia, diabetic neuropathies, and trauma-induced neuropathies (Bennett and Xie, 1988). Initially, the models primarily focused on the transection of a peripheral nerve; often used was the sciatic nerve due to its size, location, and relative ease of manipulation. The constraints of these models lie in the inability to measure pain caused by a stimulus, and instead relied on spontaneous pain behaviors since all sensory information distal to the transection site was no longer transmitted (Decosterd and Woolf, 2000). Until the realization of the advantage that constriction models, either partial or full, offered, it was not possible to distinguish between allodynia and hyperalgesia, let alone study these two phenomena separately.

Described below are three widely used methods for inducing neuropathic pain via nerve ligation. In general, these models are usually employed in rats bearing intrathecal catheters; however, these can be modified for use in a mouse population as described below with exception to the spinal nerve ligation (SNL). It is recommended that rats be given a recovery period of at least 5–7 days between procedures; either the intrathecal catheter implantation or the nerve ligation may be performed first, however, due to the higher risk of paralysis with the catheter implantation it is in general recommended to first implant the intrathecal catheter.

3.1.1.1. Chronic constriction injury of the sciatic nerve (CCI): In 1988, Bennett and Xie (Bennett and Xie, 1988) described the groundbreaking method of constricting the sciatic nerve. The model was initially described for use in rats; however, it can be modified and applied to mice by altering the length of incision and reducing the number of loose ligations. The procedure is relatively simple and provides a means of measuring both allodynia and hyperalgesia while simultaneously retaining the ability to study spontaneous pain. Spontaneous symptoms normally present in the form of self mutilation to the toes or foot of the affected limb. Because of this, the timeline in which this model is used may be limited to include acute and short chronic studies.

Induce anesthesia in a 250–300 g rat with 5% isoflurane in O₂, maintain the animal at 2.5% isoflurane for the duration of the procedure (or induce anesthesia in a 20 g mouse with 2.5% isoflurane in O₂ and maintain the mouse at 1.5% isoflurane). Shave or use depilatory cream to remove fur from the thigh of the animal, cleaning the area with a povidone–iodine topical antiseptic followed by 70% ethanol in diH₂O. Make an incision through the biceps femoris to expose the sciatic nerve. Separate a portion (7 mm in rats, 3 mm in mice) of the sciatic from surrounding tissue proximal to the trifurcation. Loop 5-0 suture silk around the freed section of nerve and loosely ligate such that the nerve appears to be barely constricted. Insert four ligations 1 mm apart in a rat or two ligations 1 mm apart in a mouse. Close the muscle

incision with 3-0 suture silk; the skin may be closed with either surgical staples or 3-0 suture silk. Allow the animal a minimum of 5–7 days recovery prior to pharmacological manipulation. Appropriate controls for this model should include a sham surgery group in which the same surgery is performed without ligation of the aforementioned nerve.

3.1.1.2. L5/L6 spinal nerve ligation (SNL): Full ligation of the L₅ and L₆ spinal nerves provides an excellent model of neuropathic pain. Onset is quick, the model induces both hyperalgesia and allodynia as well as spontaneous pain in the affected limb, and the model is ideal for both acute and chronic studies as the effects last upward of 60 days after the procedure. The procedure described here is based on that developed by Kim and Chung (1992).

Prior to beginning the SNL an operating board should be purchased or constructed. Constructing a Chung board can be done easily with a piece of 1/4 in. pressboard cut to approximately 18 in. lengthwise and 6 in. wide. Cut three strips of heavy grade tubing (at least a 1/4 in. thick for strength, but still malleable by hand) to approximately 15 in. and tightly tie widthwise around the board to form three taught loops. Cut the excess tubing after a tight knot has been fashioned. Affix thick (~1/4 in.) 1 in. diameter rubber bands to the tubing (one band per tubing loop) by looping the band around the tubing and pulling through itself. Arrange the board such that the long side sits parallel to the edge of the work surface and arrange the bands so that the two outer bands sit at the bottom (closest to the researcher's torso) and the middle band is at the top. The bands should be approximately 1 in. apart. Fold a bench underpad and wrap around the board so that the entire board is covered but the rubber bands protrude either side. Cut and sterilize three pieces of 18-gauge wire to approximately 3 in. in length. Bend each wire into a hook on one end and a loop on the other that can be affixed to each of the rubber bands on the board (see Fig. 1.1 for a visual guide). The purpose of the Chung board is to provide a firm stable surface that will double as nonimposing retractors to hold back muscle surrounding the incision site without blocking the operators view. Alternatively, instead of purchasing or constructing a board, long handle retractors can be used during the SNL. We do not recommend this, however, because the use of retractors without the Chung board does not immobilize the animal. The small movements induced by the operator's manipulation of the rat make locating, isolating, and ligating the L₅/L₆ spinal nerves very difficult. Use of the Chung board prevents the rat's body from shifting even slightly. The surgical procedure described below induces injury to the left hind paw. The procedure can be modified to affect the right hind paw as well.

Induce anesthesia in a 250–300 g rat with 5% isoflurane in O₂, maintain the animal at 2.5% isoflurane for the duration of the procedure. Shave or use depilatory cream to remove fur in a 1 in. wide and 2 in. long section up the midline centering on the lumbar region of the spinal cord. Clean the area with a povidone–iodine topical antiseptic followed by 70% ethanol in diH₂O. Lay the anesthetized rat in a prone position and make an incision slightly (about 5 mm) to the left of the spinal column from L₄ to S₂. Position the hooks protruding from the Chung board to hold the incision open wide enough to insert a cotton swab. Remove excess blood from the incision with cotton swabs and carefully remove the muscle from the dorsal aspect of the exposed pelvic bone with forceps. Using a small rongeur, remove the transverse process of the L₆ vertebra. Ensure that no bone fragments remain by

checking the surrounding area with forceps. This is an extremely important step required to avoid damaging the spinal cord during recovery. Slide a ball tipped (preferably glass) probe under the anterior edge of the S₁ transverse process. Lightly scraping the ventral surface of the process with the ball of the probe, slowly draw the probe back out until it catches on a slightly elastic thread extending diagonally out from the midline across the S₁ vertebra. This is the L₆ spinal nerve. Carefully hook the nerve across the ball of the probe and draw it out into the space created by removing the transverse process of L₆. Carefully slide the loop of a piece of 5-0 suture silk 3 in. long and fitted with a slipknot around the ball of the probe. Loop the suture ligand around the nerve by grabbing one of the loose ends with a pair of forceps and pulling through the slipknot to completely encircle the nerve. Tighten until half to two-thirds of the diameter has been constricted. Repeat the same ligation to the L₅ spinal nerve which is easily locatable by sliding the probe with the handle extending vertically and the hook end perpendicular to the spinal column under the L₆ spinous process and pulling out the L₄/L₅ bundle. Do not injure L₄ as this will significantly impair motor function, causing paralysis in the left hind limb. L₅ is distinguishable from L₄ by recognizing that L₅ will appear to sit on top of L₄ and will have a slightly smaller diameter. Separate L₄ and L₅ by sliding the process between the two spinal nerves. Once both ligation sutures are in place, remove the wire hooks and close the muscle using 1 or 2 sutures with 3-0 silk. Staple or suture the skin incision. Allow the ligated animal to recover for a minimum of 7 days prior to pharmacological manipulation. Appropriate controls for this model should include a sham surgery group in which the same surgery is performed without ligation of the aforementioned nerves.

3.1.1.3. Spared nerve injury (SNI): In order to minimize the degree of variability of results between animals and researchers that exists with the CCI as well as eliminate the possibility of inflammation affecting the L₄ spinal nerve introduced with the L₅/L₆ SNL, the spared nerve injury (SNI) was introduced in 2000 by Decosterd and Woolf (Decosterd and Woolf, 2000). The SNI is similar to the CCI except that the ligations are made distal to the trifurcation of the sciatic nerve in the common peroneal and tibial nerves (not affecting the sural nerve), only one ligation per nerve is performed, and an axotomy 2–4 mm above the distal stump is also performed on the common peroneal and tibial nerves. Decosterd and Woolf report symptoms of allodynia, hyperalgesia, and spontaneous pain that persist for greater than 100 days.

Induce anesthesia in a 200–250 g rat with 5% isoflurane in O₂, maintain the animal at 2.5% isoflurane for the duration of the procedure (or induce anesthesia in a 20 g mouse with 2.5% isoflurane in O₂ and maintain the mouse at 1.5% isoflurane). Shave or use depilatory cream to remove fur from the thigh of the animal, cleaning the area with a povidone–iodine topical antiseptic followed by 70% ethanol in diH₂O. Make an incision through the biceps femoris to expose the sciatic nerve and trifurcation. Separate a portion of the trifurcated common peroneal and tibial nerves from surrounding tissue. Distinction can be made between the sural, common peroneal, and tibial nerves by denoting the branching location from the sciatic nerve. The first (most proximal) to branch off will be the sural nerve, followed by the simultaneous bifurcation into the common peroneal and tibial nerves. Loop 5-0 suture silk around the freed section of nerve and ligate tightly such that the nerve appears to be

constricted by about 1/2 to 2/3 of the diameter. Distal to the ligation, 2–4 mm proximal to the nerve stump, section the nerve. Close the muscle incision with 3-0 suture silk; the skin may be closed with either surgical staples or 3-0 suture silk. Allow the animal a minimum of 5–7 days recovery prior to pharmacological manipulation. Appropriate controls for this model should include a sham surgery group in which the same surgery is performed without ligation or axotomy of the aforementioned nerves.

3.1.2. Inflammatory pain models—Inflammatory pain models are a highly diverse set of models, ranging from models mimicking human conditions such as multiple sclerosis or arthritis to paw/intra-articular injections of irritant solutions aimed at isolating the mechanism of inflammatory pain. In this section, we describe some commonly used models targeting inflammatory pain mechanism.

3.1.2.1. Formalin: A major breakthrough in analgesic study occurred in 1977 when Dubuisson and Dennis introduced the formalin test (Dubuisson and Dennis, 1977): subcutaneous injection of dilute formalin induced continuous rather than transient pain, and no restraint of the animal is required, avoiding potential confounding factors that may arise from restraint.

Allow a 250–300 g rat to acclimate to the testing environment for a period of 30 min for rats and 90 min for mice. In general, with the formalin test flinching and guarding are measured and should be carried out in a plastic or plexiglass enclosure of 30 cm³. Dilute sterile formalin to 2–5% by volume in 0.9% saline (5% formalin will produce robust flinching and should be the maximum concentration used to avoid serious tissue damage). Using a 1 mL syringe equipped with a 30 gauge 0.5 in. needle, inject 0.05 mL subcutaneously into the dorsal aspect of either hind-paw. For ease of injection a leather workers glove can be used to gently restrain the rat by placing the glove on the work surface and allowing the rat to climb inside of the glove until the most caudal rib area is no longer visible. Gently hold the rat inside of the glove such that the animal cannot back out of the glove but being careful not to restrict breathing. By applying gentle pressure with the palm of the hand, the back limbs should be visible and planted securely on the work surface. Gently insert the needle subcutaneously to the dorsal aspect and discharge the formalin solution. Effects should be noticeable immediately and allow testing periods of at least 1 h; appropriate controls for this type of study should include a group injected with the vehicle saline.

3.1.2.2. Complete freund's adjuvant (CFA): CFA is a suspension of desiccated mycobacterium in paraffin oil and mannide monooleate that induces inflammation, tissue necrosis, and ulceration. It can be used subcutaneously in the paw, or intraperitoneally in mice and rats. A period of 24 h is required for onset after injection. Due to the rate of tissue necrosis, it is recommended to euthanize CFA injected animals within 1 week.

Intraperitoneal injections of CFA in rats should have a maximum volume of 0.5 mL or 0.25 mL in mice. For s.c. injections in the plantar aspect of a hind paw, use a CFA volume of 0.2 mL in rats and 0.1 mL in mice. Use a 30 gauge 0.5 in. needle on a 1 mL syringe. For ease of injection, a leather workers glove can be used to gently restrain the animal by placing the glove on the work surface and allowing the animal to climb inside of the glove until the most

caudal rib area is no longer visible. Gently hold the animal inside of the glove such that the animal cannot back out of the glove but being careful not to restrict breathing. By applying gentle pressure with the palm of the hand the back limbs should be visible and planted securely on the work surface. Gently extend one of the hind legs until the plantar aspect of the paw is facing upwards away from the work surface. Insert the needle subcutaneously to the plantar aspect and discharge the CFA solution. Gentle pressure should be applied to the injection site for 10 s to prevent leakage. Appropriate controls for this model should include a group injected with incomplete Freund's adjuvant (mannide monooleate in paraffin oil). *Note:* the use of CFA will result in necrosis of the surrounding tissue over time. These areas of necrosis should be avoided for behavioral testing as they will give false positives of analgesic drugs.

3.1.2.3. Carrageenan: Carrageenan is a seaweed extract that induces local inflammation in approximately 4 h when injected s.c. to the dorsal aspect of a hind paw in rats or mice. The inflammatory pain may persist for upwards of 12 hours. Use a 3% lambda-carrageenan in diH₂O. Lambda-carrageenan is recommended due to its solubility in cool water. An injection volume of 0.2 mL in rats and 0.1 mL in mice is recommended and should be done with a 30 gauge 0.5 in. needle on a 1 mL syringe. For ease of injection, a leather workers glove can be used to gently restrain the animal by placing the glove on the work surface and allowing the animal to climb inside of the glove until the most caudal rib area is no longer visible. Gently hold the animal inside of the glove such that the animal cannot back out of the glove but being careful not to restrict breathing. By applying gentle pressure with the palm of the hand the back limbs should be visible and planted securely on the work surface. Insert the needle subcutaneously to the dorsal aspect and discharge the lambda-carrageenan solution.

3.1.3. Cancer pain—The prototypical model of cancer pain was developed by the Mantyh group in 1999 (Schwei *et al.*, 1999). It is a highly relevant model—it is estimated that up to 50% of cancer patients report pain as the first symptom of the disease. It is particularly relevant to cancers that have a high rate of metastasis to bone, and as such can be used with bone cancer cells, breast cancer cells, prostate cancer cells, lung cancer cells, or any other cancer line with a high rate of metastasis to bone. This model requires the use of a small drill (dental or dremel drill) and a faxitron imaging system to ensure proper placement, and consists of the intramedullary implantation of tumor cells into a mouse femur. Aggressiveness of the tumor cell line used will determine the time-line of the experiment and must be determined empirically.

Mice should be anesthetized by injecting a mixture of ketamine/xylazine (100 mg/kg, i.p.); concentration of the solution should be based on a final volume of 10 mL/kg. Shave or use depilatory cream to remove fur from the right thigh of the animal, cleaning the area with a povidone-iodine topical antiseptic followed by 70% ethanol in diH₂O. Place the anesthetized mouse in a supine position and using a number 10 blade make a skin incision to expose the right distal femur and proximal tibia. Holding the scalpel at a 5° angle, section the patellar tendon just distal to the patella and gently move the patella proximal by about 3 mm to expose the condyles of the distal femur. Between the condyles, drill a hole with the

bit of the drill extending in the same direction as the cortical shaft of the femur. Insert a cannulation needle (C3131I) (Fig 1.2) through the hole into the intramedullary space and obtain a faxitron image to ensure that the needle lies completely within the intramedullary space without any other bone or tissue damage. Remove the cannulation needle and with a 10 μ L Hamilton syringe inject a volume of 5 μ L of either control culture medium or a population of tumor cells suspended in culture medium. Seal the hole with bone cement and apply 1 suture from the section of patellar tendon still attached to the patella to the portion attached to the tibia to replace the patella in the original location. Ensure that the full range of motion is retained. The skin incision may be closed with either 3-0 suture silk or surgical staples. Allow the animal a recovery period of at least 7 days, this period will also allow the tumor to establish.

3.1.4. Quantification of pain-related behaviors

3.1.4.1. Rotarod: The rotarod test is not a measure of pain behavior, but rather a measure of the amount of sedation or motor impairment imposed upon an animal undergoing an experimental therapy. Prior to inducing a particular pain state, uninjured animals should be subjected to the rotarod test with sufficient variety in dosages to construct a dose-response curve. The rotarod device (Columbus Instruments International, Columbus, OH) has interchangeable rods in order to accommodate rodents of differing size. Naïve animals should be trained to ambulate on the device for a period of 180 s by simply placing a group of up to four animals on the rod and turning the device on after a few minutes. If a rat falls to the lower chamber prior to 180 s or a mouse prior to 60 s, pick them up and put them back on the rod. In order to avoid fatigue, training should occur in increments no greater than 10 min in length for rats and 5 min in length for mice. It should take one training session to train a rat or mouse to ambulate on the device. The timecourse and testing intervals after drug administration will have to be empirically determined depending on the composition of the drug and the site of administration. A general rule of thumb for spinal administration is testing intervals of 15 min over a timecourse of 60 min. Measure the time a treated animal is able to ambulate on the device and compare between treatment groups with a cut off time of 180 s.

3.1.4.2. Tail flick: The tail flick model is a measure of acute nociception and does not require use of an injury model. A water bath maintained at 52 °C is used to immerse the distal 2/3 of a rat or mouse's tail. The latency until withdrawal (rapid flick) should be measured prior to drug treatment (baseline) and compared among treatment groups. Typically a 10 s cut off time is utilized in order to avoid damage to the tail. Temperatures can be increased or decreased along with cut off times; however, temperatures should not increase over 55 °C as this temperature will cause tissue damage if tails remain in the water over 10 s.

$$\% \text{ Activity} = 100 \times (\text{tail withdrawal latency post drug} - \text{baseline tail withdrawal latency}) / (\text{cut off tail withdrawal latency} - \text{baseline tail withdrawal latency})$$

(1)

3.1.4.3. Von Frey Filament Up-Down Method: The Von Frey up-down method (Dixon, 1980) is a measure of tactile allodynia and can be used with the injury models above with exception to the carrageenan model of inflammatory pain. This is because the inflammation induced by the carrageenan model is so significant on the plantar surface of the hind paw that mechanical nociceptors may not properly identify stimulus. Prior to inducing an injury model, all animals should be subjected to a pre-injury baseline test of paw withdrawal threshold. After induction of injury and the appropriate recovery period, the animal should be subjected to a postinjury baseline test. Within group comparisons will be made to this value with the Dixon nonparametric statistical test. The Von Frey up-down method can be employed in mice and rats, though the caliber of filaments used in mice is generally lower.

The calibration range used in adult mice is 2.44–4.56 (corresponding to a logarithmic range of force from 0.03 to 2.34 g); adult rats are subjected to a filament range from 3.61 to 5.18 (corresponding to a range of force from 0.41 to 15 g).

Place the animals in suspended wire mesh cages and allow to acclimate for an appropriate period of time (30 min for rats and 90 min for mice). Beginning with the middle calibration fiber, for example in a mouse this would be the 3.61 fiber, apply the tip of the filament to the plantar aspect of the affected paw for a duration of 3 s. If no response is received (the paw is not withdrawn) apply the next higher weight filament. If a response is observed, apply the next lower weight filament. Proceed until either the highest or lowest caliber filament has been exhausted or observing three “switches” in the direction of calibration. A switch in direction can be summarized as follows: if a mouse gives a positive response at the 3.61 filament and a positive response at the 3.08 filament then the direction of calibration is currently downward since the researcher is proceeding downward in filament stiffness. If the mouse then gives no response to the 2.44 filament then the direction of calibration has “switched,” causing the researcher to then proceed upward until a response is received. The Dixon statistic should be calculated for each pattern of calibration using Flashcalc (University of Arizona) or a similar program. The Dixon statistic can then be averaged for each group and plotted against time points, or % activity can be calculated as follows for comparison between groups:

$$\% \text{ Activity} = 100 \times (\text{withdrawal threshold postdrug} - \text{postinjury baseline threshold}) / (\text{preinjury baseline threshold} - \text{postinjury baseline threshold}) \quad (2)$$

Since it is not plausible to induce mechanical allodynia in an uninjured animal (it would require a caliber filament that would support the weight of the paw), no calculation exists for sham animals with the Von Frey up-down method.

3.1.4.4. Hargreaves method: The Hargreaves method (Hargreaves *et al.*, 1988) measures thermal hypersensitivity, a form of hyperalgesia. This method can be used with all of the pain models described above. A mobile radiant heat source (Ugo Basile, Italy) is required for use of this method. Preinjury and postinjury baselines should be taken for comparison between and within groups.

Allow animals to acclimate to a plexiglass enclosure on a glass plate. Place the radiant heat source beneath the glass plate at a location corresponding to the plantar surface of the affected hind limb. During the preinjury baseline test, a baseline heat intensity must be established for each individual animal; this baseline intensity will be maintained for the duration of the experiment. The baseline intensity is identified as the heat intensity at which the animal is able to withstand the heat source for a given latency (i.e., the heat source can be adjusted for baselines within 10–15 s or 20–25 s depending on the desired outcome). Establishing baseline intensities that comply with a range will minimize standard error and standard deviations within groups. Once a baseline range is established, the intensity should be recorded for later application. When applying different heat intensities to identify the baseline intensity, it is essential to give the animal a rest period of at least 1 min between each intensity level to avoid tissue damage. A cut off latency for thermal testing will be 32 s. A thermometer or calibrating probe should be available to detect ramp heating and confirm temperature values for publication. For comparison to baseline or between groups, percent activity of the experimental animal groups can be calculated using Eq. (2) above, and sham, or control activities can be calculated as follows:

$$\% \text{ Activity} = 100 \times (\text{withdrawal latency postdrug} - \text{postsham baseline latency}) / (\text{cut off latency (32 s)} - \text{postsham baseline latency}) \quad (3)$$

3.1.4.5. Spontaneous pain: Spontaneous pain is commonly measured by observing flinching and guarding. Allow animal to acclimate to a plastic or plexiglass enclosure. Establish a postinjury baseline (prior to treatment) by counting the number of flinches of the affected limb in a 2-min period and count the number of seconds the animal spends guarding the affected limb in a 2-min period. A flinch is defined as a fast jerking motion of the affected paw and the number of flinches will be recorded on a five-channel counter for each minute and totaled. Guarding stance is that in which the animal retracts the affected limb up against the torso and does not allow the limb to bear weight with recording performed using a stop watch. Comparisons can be made between groups (Lozano-Ondoua *et al.*, 2010).

3.1.4.6. Movement-evoked pain: Movement-evoked pain is measured in the same manner as spontaneous pain; however, it is only measured at a time in which the animal is moving throughout the enclosure and is most often associated with a bone cancer model of pain. Movement evoked pains evaluate the severity of pain the mouse experienced during normal ambulation. Animals will be placed in an empty housing pan and limping and guarding

behavior of the right leg (injured or inoculated with cancer cells) will be observed for 2 min. As each animal walks across the empty pan, the use of the afflicted hind limb will be rated with the following scale: 0, no use of hind limb at all; 1, partial nonuse; 2, limp and guard; 3, limp; and 4, normal use (Lozano-Ondoua *et al.*, 2010).

3.2. Other behavioral models

3.2.1. Emesis—Briefly, ferrets of approximately 1.5 kg should be acclimated in plexiglass enclosures for a period of 30 min. Anesthesia should be induced with 4% halothane in O₂ only until loss of righting occurs (less than 1 min). The dorsal nape of the neck should be shaven and cleaned with a povidone–iodine topical antiseptic followed by 70% ethanol in diH₂O. Make a subcutaneous injection of either control vehicle or experimental compound; volume of injection should not exceed 0.5 mL. The injection should be performed with a 1 cc syringe equipped with a 30 gauge 0.5 in. disposable needle. Observe the animal for a period of approximately 30 min, recording retching and vomiting. A retch is defined as any contraction of the abdomen without expulsion, while vomiting is any oral expulsion episode (Simoneau *et al.*, 2001). Ferrets may be tested in this manner multiple times; however, a washout period is required. The length of the washout period is dependent on the half life of the compound administered; however, with opiates and cannabinoids, a washout period of 3 days is generally acceptable.

3.2.2. Multiple sclerosis—Multiple sclerosis (MS) is a debilitating disease of the central nervous system (CNS) that involves inflammation and demyelination (Rossi *et al.*, 2010). To date there are two methods of inducing MS like symptoms *in vivo*: experimental autoimmune encephalomyelitis (EAE) and viral-induced demyelination models (Kubajewska and Constantinescu, 2010). Here, we will briefly describe EAE since it is an extensively studied and widely accepted model (Mokhtarian and Griffin, 1984). EAE can be induced by s.c. injection on the plantar surface of a hindlimb of either rats or mice. Hundred micrograms of myelin basic protein should be dissolved in 50 µL of 0.9% saline emulsified in CFA. The injection should be performed in the same manner described above for the CFA model. After the inoculation, animals should be scored for symptoms of EAE based on the following scale: 0, no neurological symptoms; 1, limp tail [stage E1, usually observed on day 10 postinjection (PI)]; 2, hindlimb weakness (stage E2, day 11 PI); 3, hindlimb paralysis (stage E3, day 12–13 PI); 4, quadriplegia (stage E4; day 14–15 PI); 5, moribund (past day 16 PI).

3.2.3. Learning and memory—There are a multitude of models for the study of learning and memory (for review see Ouerstreet and Russell (2010)); these models can be classified as hippocampus dependent and include tasks such as spatial learning and social recognition; or as amygdala-dependent- encompassing emotional memory. Traditionally emotional learning and memory tasks have been based on fear conditioning—due to a disputed range of emotion in animals and inability to positively identify “good” or “happy” emotional response in rodents, it has proven quite difficult to design positive emotional memory models. Here we briefly describe a few well developed models that can utilize cannulation of either the hippocampus or the basolateral amygdala, or systemic administration as methods of drug delivery.

3.2.3.1. Radial maze: Developed by Iwasaki *et al.* (Iwasaki *et al.*, 1992), the radial maze comprises of a central platform and eight radial arms extending from the platform. The maze may either be elevated and open sided, or enclosed. An enclosed format will specify evaluation to spatial cognition and provide less stress to the animal. Food should be placed at the terminus of each arm prior to beginning. Whether naive animal training should be done prior to administration of the test compound will be determined by the scope and aims of the study. Place a rat (age and weight of the animal will also be determined by the aims of the experiment) on the central platform. Count the number of correct and incorrect choices and the time required for the rat to complete the task. A cut-off of 10 min should be established regardless of whether the animal has completed the task. A correct choice is defined as traversing an arm that has not previously been visited, yielding the food pellet; an incorrect choice is defined as traversing an arm that has already been visited. If the researcher chooses a format in which maze training occurs prior to compound administration, then animals that make at least seven correct choices and either 1 or 0 errors in the first eight decisions of three consecutive tests should be considered as having acquired spatial cognition.

3.2.3.2. Social recognition: The social recognition model developed by Thor and Holloway (Thor *et al.*, 1982) and modified by Dantzer *et al.* (Dantzer *et al.*, 1987) deals with an adult male rat's ability to recognize a juvenile rat based on the amount of time the adult male spends investigating the juvenile on subsequent meetings. The model consists of two phases: a training phase and an experimental phase. The experimental phase should be comprised of four conditions (facilitation, nonfacilitation, interference, and noninterference), which each animal will experience in a random order on the testing day. The facilitation condition is described as placement of the juvenile in the adult's home cage for 5 min, withdrawal of the juvenile for 5 min, replacement of the juvenile into the adult's cage for an additional 5 min, withdrawal of the juvenile for 120 min, and final replacement of the juvenile into the adult's cage for 5 min. The nonfacilitation condition is the same as the facilitation condition but without the second 5-min "facilitatory" visit. The interference condition is similar to the facilitation condition; however, the second 5-min visit is done with a second, different juvenile and time of withdrawal between the interference juvenile and the final visit of the original juvenile is reduced to 30 min. The noninterference condition is the same as the nonfacilitatory condition but the withdrawal time is reduced to 30 min.

Prior to conducting the experimental phase, the training phase should be carried out by allowing the rats to acclimate for a period of 2 h in a dark room with a background of white noise. The room should be equipped with an infrared spotlight (only on during testing) and a video camera or other visual recording device. Introduce a juvenile into an adult cage for a period of 5 min, then withdraw the juvenile for varying lengths of time. A second 5-min visit with either the same or a different juvenile should be made after the withdrawal period. On the 1st and 2nd training days, the withdrawal period should be 5 min; on the 3rd and 4th days the period should be increased to 30 min; and finally to 120 min on the 5th and 6th days. On testing days, record the final visit and score—time positive for investigation includes nosing, sniffing, and grooming of the juvenile as well as pawing and close following. Adult males that display aggressive behavior towards the juveniles during the training phase should not be advanced to the experimental phase. Comparisons between

treatment groups can be made based on the average time spent investigating a juvenile; this model assumes that a lower amount of time spent investigating indicates greater social recognition of the juvenile.

3.2.3.3. Morris water maze: A pool should be constructed (1–2 m in diameter), and filled with 26 °C water. Opaque water can be created by the addition of a small amount of either powdered or liquid milk. An escape platform can be constructed by filing large diameter (~9 cm) tubing cut to a height that would render the upper platform just beneath water level. Fill with rocks and seal both ends to create a base and platform. The escape platform should be placed in the pool. Four points around the perimeter of the pool should be designated as N, S, E, W; allowing the pool to be divided into four quadrants. Placing the hidden platform in a set location allows the researcher to train rats to escape from the water onto the hidden platform in a relatively short period of time. The model can be modified simply by moving or adding additional platforms, or changing water visibility or the color of the platform (for complete review see Morris (1984)).

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Figure 1.1.

Setting up an SNL operating board. (A) Wrap tubing around short edge of board and tie snugly to form three loops. (B–D) Tie one rubber band around each tubing loop. (E) When wrapping board with bench pad, fold so that rubber bands are still reachable. (F) Form hooks from sterile wire with closed loop ends that can be attached to each of the rubber bands.

Figure 1.2.

Needle placement (A) into the intramedullary space equidistant from the ends of the femur was verified prior to injection of 66.1 cells (10^5 in 5 μ l). Dental amalgam (B) is used to seal the cells inside the bone (arrow).

Table 1.1

A brief summary of cannabinoid solubility

Commercial name	Activity	K _i at human receptor			Solubility			
		CB ₁	CB ₂		Ethanol	DMSO	DMF	
AM251 ^a	Inverse agonist	7.5 nM	2290 nM		×	×	×	
AM630 ^b	Inverse agonist	5.2 μM	31.2 nM			×	×	
CAY10508 ^c	Inverse agonist	243 nM	Unavailable			×	×	
NESS 0327 ^d	Neutral antagonist	0.35 pM	21 nM		×	×	×	
(S)-SLV 319 ^e	Inverse agonist	7.8 nM	>1000 nM		×	×	×	
SR141716 ^f	Inverse agonist	1.8 nM	>1000 nM		×	×	×	
SR144528 ^g	Inverse agonist	400 nM	0.6 nM		×	×	×	
2-Arachidonyl Glycerol ^h	Agonist	472 nM	1400 nM		×	×	×	
AM1241 ⁱ	Agonist	280 nM	2 nM		×	×	×	
Anandamide (AEA) ^j	Agonist	52 nM	90 nM		×	×	×	
(±)-CP 47,497 ^k	Agonist	2.2 nM	Unavailable		×	×	×	
(±)-CP 55,940 ^l	Agonist	0.58 nM	0.69 nM		×	×	×	
HU-210 ^m	Agonist	0.06 nM	.52 nM		×	×	×	
HU-308 ⁿ	Agonist	>1000 nM	22.7 nM		×	×	×	
JWH 015 ^o	Agonist	383 nM	13.8 nM		×	×		
JWH 018 ^p	Agonist	9.0 nM	2.94 nM		×	×	×	
O-2545 ^q	Agonist	1.5 nM	0.32 nM		×	×	×	
WIN 55,212-2 ^r	Agonist	3.3 nM	62.3 nM		×	×	×	

^aLan *et al.* (1999).^bRoss *et al.* (1999).^cMuccioli *et al.* (2006).^dRuiu *et al.* (2003).

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