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Intracranial Pharmacotherapy and Pain Assays in Rodents

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TITLE:**Intracranial Pharmacotherapy and Pain Assays in Rodents****AUTHORS & AFFILIATIONS:**

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Stereotaxic Implant, Intracranial Injection, Analgesia, Pain Assessment, *In vivo* Pharmacology, AMPAkines

SUMMARY

Here we present a protocol to perform intracranial pharmacological experiments followed by pain behavior assays in rodents. This protocol allows researchers to deliver molecular and cellular targets in the brain, for pharmacologic agents in the treatment of pain.

LONG ABSTRACT:

Pain is a salient sensory experience with affective and cognitive dimensions. However, central mechanisms for pain remain poorly understood, hindering the development of effective therapeutics. Intracranial pharmacology presents an important tool for understanding the molecular and cellular mechanisms of pain in the brain, as well as for novel treatments. Here we present a protocol that integrates intracranial pharmacology with pain behavior testing. Specifically, we show how to infuse analgesic drugs into a select brain region, which may be responsible for pain modulation. Furthermore, to determine the effect of the candidate drug in the central nerve system, pain assays are performed after intracranial treatment. Our results demonstrate that intracranial administration of analgesic drugs in a targeted region can provide relief of pain in rodents. Thus, our protocol successfully demonstrates that intracranial pharmacology, combined with pain behavior testing, can be a powerful tool for the study of pain mechanisms in the brain.

INTRODUCTION:

The central nervous system is known to play a key role in pain regulation. For example, glutamate signaling in the brain has a regulatory role in the context of pain^{1,2}. Hence, there is a need to

study cellular and molecular signaling pathways in the brain with respect to pain. In addition, there is a need to understand if molecular targets in specific brain regions can be modified to treat pain. Current studies of pain in the brain rely on *in vitro* studies of electrophysiology in combination with systemic (intraperitoneal) delivery of pharmacological agents. *In vitro* studies have obvious deficits in revealing *in vivo* pain mechanisms. Meanwhile, systemic drug delivery does not delineate the precise cellular targets. *In vivo* intracranial injections of chemical and biologic agents have become a powerful tool to study neurological and molecular pathways in the brain. In recent years, other fields have used *in vivo* intracranial injections to successfully study addiction and reward behaviors and circuit pathways in rodents^{3,4}. However, in the context of pain, the use of *in vivo* intracranial pharmacology is lacking.

Intracranial injections allow for precise injection of a drug into a specific area of the brain. Furthermore, specific pathways and receptors can be targeted using highly selective drugs. The combination of an intracranial delivery system with precision drugs allows us to target molecular and cellular targets for pain. After intracranial delivery of these drugs, researchers can observe the immediate effects in the behavior of rodents. From well conducted experiments, rodents' behaviors can be linked with pharmacology.

In this protocol, we used the example of AMPAkinase infusion in the prefrontal cortex (PFC) to demonstrate the mechanism of cortical glutamate signaling in pain regulation. AMPAkinases are synthetic compounds that are known allosteric modulators. They have shown the ability to relieve acute and chronic pain in animal models^{5,6}. Previous studies suggest that the likely sites of action of AMPAkinases are in the brain^{5,6}. The PFC is a region in the brain that exhibits top down control to subcortical areas to regulate mood and behavior. Some of these output projections have been shown to be key in pain regulation^{1,2,7}. More specifically, glutamate signaling in the PFC has been shown to regulate pain. Thus, the PFC was chosen as a targeted brain area for the study of AMPAkinases in pain states.

PROTOCOL:

All procedures in this study were approved by the New York University School of Medicine Institutional Animal Care and Use Committee (IACUC) as consistent with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

1. Stereotaxic Cannula Implantation

1.1. Use 10-12 week old male Sprague-Dawley rats.

1.2. As previously described, anesthetize animals with 1.5-2% isoflurane^{1,3,4}. Once the animal becomes unresponsive to a strong pinch with sharp forceps, perform the surgery. Be sure to autoclave all instruments and use sterile surgical gloves.

1.3. Stereotactically implant bilateral 26-gauge guide cannulas into the PFC at a 12.5 degree angle with coordinates AP: +2.9 mm; ML: +/-1.6 mm; DV: -2.1 mm. In order to insert the cannulas, drill holes in the skull at the desired coordinates, with hole diameter according to the size of the

cannulas used.

Note: Here, the PFC was studied as the potential target for intracranial implants because of its important role in pain processing, which has been demonstrated in a number of previous studies^{1,2,7}. To study the function of other specific brain areas, researchers can use different coordinates according to the brain atlas.

1.4. Allow rats to recover from surgery for at least 1 week. Following surgery, inject subcutaneous fluids prior to recovery to help support the metabolic requirements, and apply topical bupivacaine in the freshly closed incision. Place the animal on a warm pad until they wake up, and monitor the animal post operation for 3 days to ensure good health and a proper recovery.

1.5. Once the animals have fully recovered from surgery, begin injections (next step).

2. Intracranial and Intraperitoneal Injections

2.1. For intracranial injections, use PE-50 tubing attached at one end to 10- μ L Hamilton syringes with 33-gauge injector cannula that extend 1.0 mm beyond the implanted guides.

2.2. Inject 0.5 μ L (or less if desired) of either the study drug or saline in the PFC of these rats. Because the PFC is a larger region in rats, this amount will not be spread to other regions. However, for smaller brain regions or for mice, use a smaller volume. The amount injected will depend on the region of the brain and species of animal.

Note: Please note that saline should be used as a control instead of DMSO because DMSO is neurotoxic. However, a very small amount of DMSO may be safe for infusion, as studies have shown that less than 50% DMSO (*i.e.*, less than a total volume of 0.3 μ L, as in this case) may not interfere with behavior studies^{8,9}.

2.3. Inject volume bilaterally over a period of 100 s and keep injector cannulas in place for an additional 60 s prior to removal to allow slow diffusion of this solution.

2.4. For studies of synergistic pharmacological effects, co-administer another drug through systemic methods. In this case, inject the desired drug or control intracranially and administer an additional drug intraperitoneally immediately thereafter. As an example, in this study we studied the synergistic analgesic effects of morphine and Ampakines to test for an additive effect. We infused an AMPAkinine intracranially, in combination with intraperitoneal delivery of 1 mg/kg morphine (a safe systemic dose)¹⁰.

Note: It is recommended that intracranial injections are done first, as they are more difficult to perform than intraperitoneal injections.

3. Analgesia Assays and Assessment

3.1. To study the effect of intracranial injections on acute pain behavior in rats, use the plantar test (Hargreaves' tests) to calculate the withdrawal latency in response to thermal stimuli. The Hargreaves' apparatus focuses an infrared beam through a glass plane onto the rat's foot; the rat is standing and freely moving above the glass plane. When performing Hargreaves' test, focus the infrared beam under the plantar area of the rat's foot.

3.1.1. Begin by performing baseline Hargreaves' tests prior to injections, to establish a baseline value for comparison.

3.2. Do not inject any drug of any kind prior to establishing a baseline value. Conduct 5 good trials, 5 min apart. A good trial is indicated by a clear withdrawal, *i.e.*, when the rat bends its knee and lifts its foot upward and into the body.

Note: Make sure the trials are 5 min apart to prevent sensitization of the rat. The Hargreaves' apparatus automatically records the time of withdrawal, once the infrared beam is broken. As a result, be sure to discount trials from locomotion, shifting weight, *etc.* If a discounted trial occurs, wait 5 min and repeat the trial.

3.3. Calculate withdrawal thresholds by taking the average of 5 trials.

3.4. After obtaining a baseline average, begin the experiment to get withdrawal times after infusion of drugs. Hargreaves' tests can be performed 20-30 min after intracranial injections, although the precise timing may be dependent on the pharmacokinetics of the specific agents. This is to ensure that the rat has absorbed the drug and is experiencing its effects. Conduct this experiment in the same manner as step 3.1.

3.5. Calculate withdrawal thresholds as mentioned in step 3.2.

REPRESENTATIVE RESULTS:

As an example, we infused an AMPAkin into the PFC via cannulas (**Figure 1**). We also infused morphine systemically to assess the synergistic analgesic effects between AMPAkin and morphine. These results show that AMPAkin and morphine have an additive analgesic effect. It also shows that intracranial injections have the power to discover, at least in part, a mechanism for drug activation in the context of pain.

FIGURE AND TABLE LEGENDS:

Figure 1. Intracranial injection of AMPAkin and systemic injection of morphine provide complementary analgesia. A graph comparing withdrawal latencies from Hargreaves' test after intracranial injections of either saline, AMPAkin, or AMPAkin with systemic injection of morphine. Error bars represent mean with SEM. $n = 8$; **** $p < 0.0001$, ** $p = 0.0099$, * $p = 0.0124$, unpaired Student's *t*-test. This figure has been adapted from Sun *et al.* (2017)¹⁰, with permission from Elsevier.

DISCUSSION:

In this study, we have demonstrated that intracranial pharmacology is a powerful tool to study pain mechanisms and has potential as a therapeutic delivery system. In our protocol, we delivered AMPAkinases directly into the PFC and found that by enhancing glutamate signaling in the PFC, AMPAkinases provided pain relief. We were able to demonstrate this through the use of intracranial injections combined with intraperitoneal injections, with subsequent pain assays. Based on the evidence of pain-relieving effects, when AMPAkinases are delivered to the PFC, the current study suggests that the PFC may be involved as a target of AMPAkinases. This is an important advantage of the intracranial pharmacological approach, when combined with behavior testing. In addition, the ability to combine intracranial with systemic delivery of drugs allows us to understand the therapeutic relationship between two different drugs and the potential pharmacologic interactions. In the example shown in this study, administering an AMPAkinase into the PFC in combination with morphine shows an expected additive effect, which indicates that AMPAkinases and morphine operate through different molecular mechanisms.

Although *in vivo* pharmacology is a powerful tool to study pain, it does have limitations. First, it is possible that, in inexperienced hands, the infused drug can diffuse to neighboring brain regions. This is a particular problem with mice. This could be overcome with the use of light-activated drugs and implantation of optic fibers, by measurement of drug levels at different distances from the injection site, or by injecting the drug at close anatomical sites. Second, intracranial injections can be short lasting, but the time period of effect isn't entirely known due to the pharmacokinetics of the drug. Other techniques, such as optogenetics, allow for instantaneous activation or inactivation of a desired region of the brain; this technique is more direct and has a known period of effect. On the other hand, *in vivo* pharmacology can target specific receptors or signaling pathways, and thus provide an additional level of molecular specificity. Thus, in the future, it will be necessary to explore combining *in vivo* pharmacology with additional techniques, such as *in vivo* physiology and optogenetics. With the combination of these tools, new pathways and receptor specific pathways can be discovered and used to treat pain.

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DISCLOSURES:

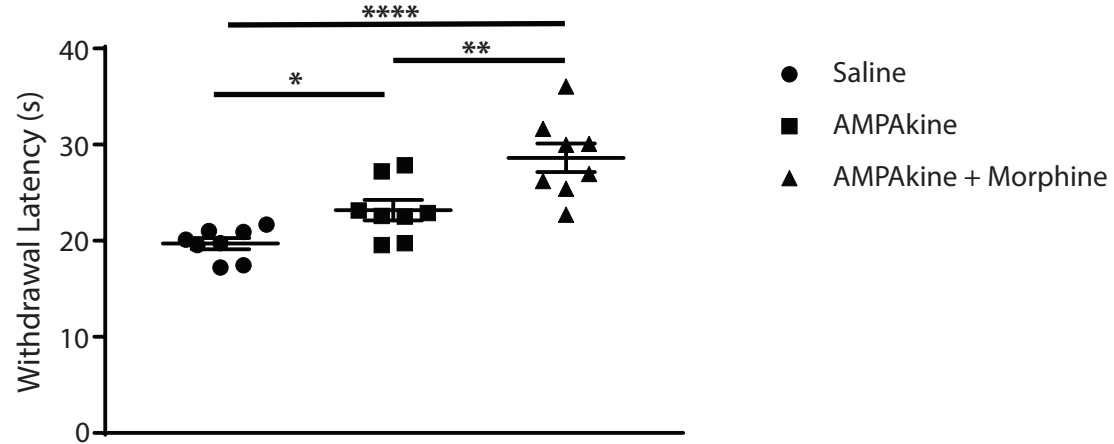
The authors have nothing to disclose.

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Hargreaves' Test



Name of Material/ Equipment	Company
Serotaxic Cannula	PlasticsOne
Digital Syringe	Hamilton
AMPAkine	Sigma Aldrich
Dimethyl Sulfoxide	Sigma Aldrich
Hargreaves Apparatus	Ugo Basile
Male Sprague-Dawley rats	Taconic Farms
Sterile Surgical gloves	Dynarex

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2. Please number references in the order they are cited in the paper; currently, the first works cited are references 5-7.

We apologize for this oversight and have renumbered the references based on the order in which they were introduced in the text.

3. Please include at least 2 more keywords or phrases, for a total of 6-12.

We have added 2 additional key words to the manuscript. We have highlighted them in red.

4. 1.2: Please include more information about the operation; e.g., is this done with a drill? How are instruments sterilized, and how is a sterilized environment maintained? Please also split this long step up into substeps, and include any necessary material in the Table of Materials.

A drill is used to make holes in the skull so that cannulas can be inserted. The instruments are sterilized via autoclaving them beforehand. Sterile surgical gloves are used during the surgery to maintain a sterile environment. We have added the requested details and created additional steps and highlighted the changes in red.

5. 1.3/2: So, are injections therefor done 1 week after surgery?

Injections are done after the animals are fully recovered. The experimenter should wait at least 1 week after surgery before injecting the animals. Thus, if the animal has fully recovered, the injections may be done one week after surgery. We have added details to the protocol and have highlighted them in red.

6. 2.2: The volume used here is unclear-how much less than 0.5 μ L, or do you mean to use up to 0.5 μ L? How much lower volume for mice and smaller brain regions? Please also split this step up to substeps and notes.

For the PFC, .5 microliters were used. Less can be injected if desired. For mice and smaller brain regions, the amount injected depends on the size of the region and the species of animal. As this is an example, we only included the PFC in this protocol. We have added details and notes to the manuscript, as well as additional steps. We highlighted the changes in red.

7. 2.4: Should intracranial and i.p. injections be done simultaneously?

It would be extremely difficult to do them at the exact same time. However, they are performed one immediately after the other. We have added these details to the manuscript and have highlighted them in red.

8. 3.1: This is unclear (and should be split up into substeps as well). What is a “good trial”? How exactly is the withdrawal latency determined? Is there always a “clear withdrawal”, and what if there isn’t?

We apologize for the lack of clarity. A good trial is a clear withdrawal not due to locomotion, shifting weight, etc. A clear withdrawal involves the lifting of the foot from the ground up and into the body, accompanied by the bending of the knee. We have added additional details, notes, and steps to the protocol. Also, we have highlighted the changes in red.

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