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The application of conditioning paradigms in the measurement of pain

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Abstract

Pain is a private experience that involves both sensory and emotional components. Animal studies of pain can only be inferred by their responses, and therefore the measurement of reflexive responses dominate the pain literature for nearly a century. It has been argued that although reflexive responses are important to unveil the sensory nature of pain in organisms, pain affect is equally important but largely ignored in pain studies primarily due to the lack of validated animal models. One strategy to begin to understand pain affect is to use conditioning principles to indirectly reveal the affective condition of pain. This review critically analyzed several procedures that are thought to measure affective learning of pain. The procedures regarding the current knowledge, the applications, and their advantages and disadvantages in pain research are discussed. It is proposed that these procedures should be combined with traditional reflex-based pain measurements in future studies of pain, which could greatly benefit both the understanding of neural underpinnings of pain and preclinical assessment of novel analgesics.

Keywords

Pavlovian conditioning; Operant conditioning; Animal model; Pain; Pain affect

1. Introduction

Chronic pain is a highly prevalent symptom and disease, and poses a significant global public health challenge to society, affecting 20–30% of the adult population in the developed countries. In the United States alone, chronic pain affects at least 116 million adults, reduces quality of life, and costs society at least $ 636 billion annually (Institute of Medicine, 2010). Consequently, the quest for more efficacious and safer analgesics has been a primary goal of the pain researchers in both academia and industry. Unfortunately, despite the decades of active research, the goal of developing breakthrough novel analgesics has been far from achieved. This frustrating reality has been highlighted by a recent analysis of the marketed analgesics in the past half a century and concluded the lack of breakthrough analgesics (Kissin, 2010).

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This review attempted to analyze the disadvantages of traditional meausres of pain in preclinical pain studies and critically reviewed the emerging trend of incorporating conditioning based procedures into pain studies. It is argued that the combination of reflex-based pain-like behaviors and conditioning based pain measures will expedite the understanding of pain processing at an integral level and faciliate the development of novel analgesics for pain treatment.

2. Traditional pain measures and their limitations

In the dismay of analgesic drug development, some put the blame on the less-than-ideal animal models and measurements of pain (Le Bars et al., 2001; Mogil, 2009; Mogil and Crager, 2004; Negus et al., 2006). Indeed, until recently, preclinical pain researchers have been focusing predominantly on reflex-based withdrawal responses or other nocifensive behaviors of animals (primarily mammals) provoked by the presentation of an acute noxious stimulus (Le Bars et al., 2001; Taber, 1974). For example, five of the most widely used acute pain models in the literature are tail-flick test, hot-plate test, paw pressure test, writhing test and formalin test (Le Bars et al., 2001). However, the most challenging problem of these pain assays is their poor predictive validity. As put by Peter Dews in his elegant thesis *What is analgesia?*, "People working with animal subjects have come to take their analgesic assays seriously as indicators of specific analgesia and so have reported as analgesic drugs a whole variety of substances…. Fortunately, most such claims are either ignored or soon forgotten" (Dew, 1974). There have been increasing attempts to develop preparations to better model clinical pain (particularly chronic pain) conditions and incorporate measurements of different dimensions of pain to improve the predictive validity. These efforts generally fall into the following three categories: create pain conditions that presumably model clinical persistent pain (e.g., various inflammatory and neuropathic pain models); create painful disease conditions that mimic human diseases (e.g., cancer pain, diabetes neuropathy); adapt a battery of endpoints to measure the "quality of life" of animals (e.g., walking, weight-bearing, social interaction, sleeping) (Berge, 2011; Ferreira-Gomes et al., 2008; Jaggi et al., 2011; Millecamps et al., 2005; Mogil, 2009; Pacharinsak and Beitz, 2008). However, in most studies, models of the first two categories still have to rely upon the stimulus-evoked withdrawal responses as the indicator of "pain". Efforts of the third categories seem to be more close to the clinical reality because, after all, those behaviors are largely consistent with many pain patients' complaints. However, recent findings cast doubt on the correlation between the presumed pain responses (mostly reflexive responses) and the "quality of life". In three of the widely used chronic pain models (spared nerve injury, chronic constriction injury, intraplantar complete Freund's adjuvant), the "quality of life" of the injured mice seems to be quite normal as judged by their daily locomotion, feeding, drinking, circadian rhythm and affect states (Urban et al., 2011). Thus, it clearly indicates that such a relationship requires further investigation. So, it appears that after nearly one century's quest, the pain researchers are still looking for the perfect preclinical model of pain, and such a model may not exist at all.

Bearing on the above limitations of animal models of pain, some argue that since pain is by nature multidimensional, the better measurement of painful conditions in animals should also be multidimensional (Berge, 2011; Mogil, 2009; Vierck, 2006a). Pain has two qualitatively different and dissociable components: the sensory-discriminative component and affective-motivational component (Auvray et al., 2010; Melzack and Casey, 1968; Uhelski et al., 2012). The withdrawal- or reflex-based measurements are thought to represent the sensory-discriminative component of pain, which mostly are innate behaviors that are limited to spinal or brainstem levels of processing, without the necessity of cerebral processing (Mauderli et al., 2000; Vierck, 2006a). Thus, measuring the learned behaviors related to pain and nociception may represent an improvement as compared to traditional

reflex-based behaviors (Mauderli et al., 2000). Indeed, pain can serve as an aversive teaching signal in both animals and humans (Baliki et al., 2010; Johansen and Fields, 2004) and the learning of pain-related stimuli is a fundamental strategy for subjects to cope with pain.

In addition, the reflex-based measurements focus on a noxious stimulus-evoked pain, while in clinical settings the ongoing tonic pain and/or paroxysmal spontaneous pain are very common in chronic pain patients (Backonja and Stacey, 2004). Thus, the measurement of affective-motivational component (pain affect) of pain and ongoing tonic pain in animals represent another significant challenge and, until recently, no validated procedures have been used to measure affective pain. Recently, several conditioning procedures have gained popularity for the measurement of affective-motivational component of pain and ongoing pain, all of which move beyond the traditional reflex responses and entail learned behavioral responses that require cerebral processing (Colpaert et al., 1980; Colpaert et al., 2001; Gutierrez et al., 2011; King et al., 2009; LaBuda and Fuchs, 2000; Mauderli et al., 2000; Nijsen et al., 2003; Sufka, 1994). However, although these new developments may represent an important progress in the toolkit of pain studies and analgesic drug discovery, they have not been synthetically discussed albeit briefly mentioned in several commentary and review articles (Barrot, 2012; Berge, 2011; Li and Zhang, 2012; Mogil, 2009; Sufka, 2011). This review attempts to fill the void in the literature by summarizing these new procedures and discussing their potential applications in future pain studies. A summary of several aspects of these procedures is given in Table 1.

3. Classical conditioning and operant conditioning

Modern behavioral theory has described three different conditioning (learning) processes: habituation, classical (Pavlovian) conditioning and operant (instrumental) conditioning. Habituation explains the single event learning process, classical conditioning deals with event-event learning process while operant conditioning is related to behavior-event learning process (Schwartz and Robbins, 1995). Only the latter two processes are discussed in this thesis as currently available procedures for pain measurement are primarily related to them.

For classical conditioning, there is an environmental trigger called unconditioned stimulus and there is a response itself called unconditioned response such that the application of unconditioned stimulus reliably induces unconditioned response (e.g., food in the mouth induces salivation). When another stimulus called conditioned stimulus is repeatedly presented temporally close to the presentation of unconditioned stimulus, the conditioned stimulus will gradually gain the ability to induce a response that is qualitatively similar to the unconditioned response (called conditioned response) (Schwartz and Robbins, 1995). One successful application of classical conditioning theory in biobehavioral science is the place conditioning procedures, and one version of the place conditioning procedure, conditioned place preference paradigm, is widely used in the field of reward research (Bardo and Bevins, 2000; Tzschentke, 1998; 2007). For a typical conditioned place preference paradigm, experimental subjects (usually rats or mice) are alternately constrained into two contextually distinct environments with or without pairing of the stimulus of interest (e.g. drug, food) which serves as the unconditioned stimulus. The unconditioned stimulus elicits an unconditioned response which in this case is presumed as a subjective gratification (although it may be impossible to know the exact nature of the unconditioned response in animals). Conditioning occurs during the repeated pairing of the unconditioned stimulus (e.g., drug treatment) with the conditioned stimulus (drug-paired environment) together with the contrast pairing of non-unconditioned stimulus (e.g., vehicle treatment) with the non-conditioned stimulus (distinct contextual environment from the drug-paired environment). Following conditioning animals are exposed to a choice test in which animals have free

access to explore the two distinct environments. An increase in the time the animals spend in the unconditioned stimulus-paired environment as compared to a preconditioning baseline value is interpreted that the unconditioned stimulus is rewarding.

For operant conditioning, the major focus is the understanding of the relationship between behavior and its consequences or between responses and outcomes (Schwartz and Robbins, 1995). One of these relations is known as positive reinforcement, in which the outcomes of the behavior increase the probability that the same behavior will reoccur in the future. If this relation holds, the outcome is called a reinforcer. Many environmental events have been identified as reinforcers under certain conditions, such as food, drugs, water, electric shock and termination of an electric shock (Findley and Ames, 1965; Hodos, 1961; Morse and Kelleher, 1966; Seevers and Schuster, 1967). One application of operant conditioning theory is the drug self-administration procedure, which has been extensively used in the studies of reinforcing effects of drugs and drug addiction in both nonhumans and humans (Comer et al., 2008; Haney and Spealman, 2008). In a typical drug self-administration paradigm, experimental subjects are given the opportunity to operate an operandum (e.g., a lever press or a nose poke in animals, or a verbal choice in humans) to get access to drug intake. If the subject's response meets the pre-set criteria, the drug is administered to the subject; and if the drug-taking behavior (measured by response rate or frequency) is above the level that is maintained by saline or a placebo, then this drug is interpreted as being reinforcing.

Several procedures based on the classical conditioning and operant conditioning principles have been developed to study pain and evaluate the effects of analgesics. These procedures represent important addition to the traditional reflex-based pain measurements. Procedures that are based primarily on classical conditioning principle include conditioned place preference and conditioned place aversion (Sufka, 1994; Johansen et al., 2001). Procedures that involve both classical and operant conditioning processes include place escape/avoidance paradigm, passive avoidance test (step-down test), self-administration procedure, operant escape paradigm, electric shock titration procedure and intracranial self-stimulation procedure (Colpaert et al., 1980; Dykstra and McMillan, 1977; LaBuda and Fuchs, 2000; Mauderli et al., 2000; Nijsen et al., 2003; Pereira Do Carmo et al., 2009). It should be noted that this classification is arbitrary and many of these procedures involve both classical and operant conditioning processes that are difficult to separate. For example, in the self-administration procedure, a procedure that is developed on the basis of operant theory, an environmental cue (light or tone) is usually paired with the delivery of drug/food reinforcers. Repeated pairing (conditioning) of the cue with the reinforcers renders the cue gaining the ability to maintain the operant behavior and the cue becomes a discriminative stimulus and conditioned reinforcer. This process clearly involves classical conditioning. The following sections will review the applications and findings of the different conditioning procedures in the study of pain and analgesics. Electric shock titration procedure is not discussed in this text. Although this procedure has historical importance (Dykstra and McMillan, 1977; Smith and McKearney, 1977; Weiss and Laties, 1958), its shortcomings have been well recognized and extensively discussed (Franklin and Abbott, 1989; Vierck C. J. et al., 1983; Vierck, 2006b). In effect, this procedure is no longer a popular procedure for pain studies in the context that more valid procedures such as those discussed in this text are increasingly used.

4. Procedures used in pain studies that involve conditioning

4.1 Conditioned place preference

The first observation that pain may alter the subjective property of drugs was conducted with rats using a drug discrimination procedure (Weissman, 1976). For this procedure, the rats were required to differentiate (discriminate) two conditions: the presence or absence of an analgesic aspirin. Interestingly, although the control rats can recognize aspirin as a

discriminative stimulus over extensive training, the presence of pain (arthritic) significantly enhances the discriminability of aspirin. Thus, the discriminability of an analgesic is greater in subjects with pain than those without pain, suggesting that the subjective effects of an analgesic may vary depending on the pathological status of the subjects (Weissman, 1976). In one of the earlier studies measuring the rewarding effects of analgesics in animals with pain using conditioned place preference paradigm, it was found that unlike in control animals in which a kappa opioid receptor agonist and analgesic, U-69593, produced robust conditioned place aversion (animals spent less time in drug-paired side), animals with pain (right hind paw treated with complete Freund's adjuvant, a widely used animal model of inflammatory pain) did not demonstrate place aversion when treated with U-69593 (Shippenberg et al., 1988). One interpretation of these findings is that although U-69593 produces place aversion in control animals, its analgesic effects are rewarding in animals with pain and thus counteracts its aversive effects. This interpretation is supported by a more recent study (King et al., 2009). In rats with spinal nerve ligation, a commonly used animal model of peripheral neuropathic pain, several clinically proven analgesics (e.g., clonidine, conotoxin, lidocaine) consistently produce place preference at the doses that markedly decrease mechanical stimulus-induced hyperalgesia, although they demonstrate no preference in sham-operated animals (King et al., 2009). This finding is interpreted as that because nerve lesion accompanies ongoing pain and the attenuation of ongoing pain with clinically effective analgesics is rewarding. Subsequent studies have confirmed the validity of using conditioned place preference as a measure to reveal the ongoing pain in several different animal models of chronic pain both in rats and in mice (Davoody et al., 2011; He et al., 2012; Liu et al., 2011; Okun et al., 2011; Okun et al., 2012; Qu et al., 2011).

The relatively reliable results, the readiness of execution and the apparently straightforward interpretation of the data make the conditioned place preference procedure very attractive to study ongoing pain and paroxysmal spontaneous pain, a field that has long been constrained by the lack of a valid behavioral procedure (King et al., 2009; Shippenberg et al., 1988; Sufka, 1994). However, this procedure and the interpretation are not without challenge. For example, the predictive validity of this procedure to non-steroid anti-inflammatory drugs remains unclear, which represents one of the most widely used classes of analgesics. Aspirin and indomethacin failed to produce either conditioned place prefence or aversion in both control rats and complete Freund's adjuvant-treated rats (Shippenberg et al., 1988; Sufka, 1994; Suzuki et al., 1996). Although in these studies, indomethacin also failed to alter thermal nociception at the doses studied (Sufka, 1994), the aspirin doses used were well above the reported doses that produce antinociception (Shippenberg et al., 1988). Future studies are needed to examine this issue in more detail. Another challenge is the effects of opioids in this procedure. Opioids as one pharmacological class stand in sharp contrast to other studied analgesics such as clonidine, lidocaine and conotoxin. There are inconsistencies in the literature regarding opioids-induced conditioned place preference in animals with pain. For example, morphine-induced conditioned place preference reportedly either did not change or was enhanced in complete Freund's adjuvant-treated rats as compared to control rats in earlier reports (Shippenberg et al., 1988; Sufka, 1994). However, later studies have generally shown that morphine- or other opioid receptor agonists-induced conditioned place preference was decreased in animals with formalin-, carrageenan- or complete Freund's adjuvant-induced inflammatory pain, sciatic nerve ligation-induced neuropathic pain or cancer pain in both rats and mice (Betourne et al., 2008; Nakamura et al., 2008; Narita et al., 2005; Niikura et al., 2008; Suzuki et al., 1996). More importantly, these behavioral results were supported by extensive pharmacological, neurochemical and biochemical studies (Niikura et al., 2010). Thus, in formalin-induced inflammatory pain, the κ opioid receptor system is activated, which attenuates morphine-evoked dopamine release in the brain and consequently decreases morphine-induced rewarding effects (Narita et al., 2005; Suzuki et al., 1999). Another potential mechanism could be that the sustained release

of the endogenous opioid peptide beta-endorphin leads to the dysfunction of μ opioid receptors in rats and mice with sciatic nerve ligation-induced neuropathic pain as both the knockout of beta-endorphin gene and the application of a specific beta-endorphin antibody eliminate pain-induced suppression of morphine conditioned place preference (Niikura et al., 2008). A third potential mechanism could be that the pain status enhances the central anti-opioid neuropeptides such as neuropeptide FF2 activity which subsequently suppresses morphine-induced place preference in animals with cancer pain or inflammatory pain (Betourne et al., 2008). Although it is unclear whether the different mechanisms are specific to the different animal models of pain and species, it is likely that more than one mechanism contributes to the decreased rewarding effects of morphine in these pain models. Nonetheless, most studies in the literature consistently show that the magnitude of morphine-induced conditioned place preference is decreased in animals with pain.

Given the undisputed analgesic activity of opioids in various painful conditions, the decreased rewarding effects of morphine in animals with pain is somewhat surprising. Increasing evidence using non-opioid analgesics have consistently shown that alleviation of the painful conditions is rewarding in animals (King et al., 2009; Okun et al., 2012), thus opioid-induced pain-relieving effects *per se* must also be rewarding, which is independent of the rewarding properties of opioids. It should, therefore, be expected that the rewarding effects of opioids are enhanced in animals with pain and the dose-effect curve of opioids for inducing conditioned place preference should be shifted leftward and/or upward as compared to control animals, as suggested by an earlier study (Sufka, 1994). Thus this apparent paradox needs a careful interpretation. One reasonable interpretation is that the presence of pain markedly decreases the efficacy of μ opioid receptor agonists in activating μ opioid receptors (Niikura et al., 2010), thus as compared to the control (pain-free) animals the dose-effect curve of morphine for producing conditioned place preference is shifted rightward and/or downward. Although morphine remains rewarding when it attenuates pain, the morphine-induced conditioned place preference test in animal models of pain represents the net effect between the two opposing actions and in most cases this summation effect is in fact decreased.

Although conditioned place preference as a well-validated behavioral procedure to measure the rewarding effects is suitable to measure the neuropharmacology of ongoing pain, it does have a disadvantage (Table 1). Because the conditioned place preference paradigm typically requires repeated pairing of both contexts with a drug and its vehicle, this type of studies could be cumbersome, particularly when considering the test of complete dose-effect functions of study drugs. This may also become an issue when certain investigational drugs are difficult to procure and only limited amount is available. In addition, the magnitude of pain may fluctuate over a period of several days and the training with a fixed dose of analgesic may functionally be different across different training sessions. This may implicate the interpretation of the data which exclusively rely on results from the test day. However, less training sessions is possible (e.g., single-trial conditioning), which could greatly decrease the workload and increase the consistency of such experiments (King et al., 2009).

What aspect of pain does conditioned place preference measure? While it is usually thought that conditioned place preference measures the ongoing pain in different animal models of chronic pain (King et al., 2009; Liu et al., 2011), this is worth further analysis. Ongoing pain usually refers to the pain when the subject is at rest, that is, pain with no apparent stimulators. While it is true that during conditioned place preference training and testing, the experimenter does not apply peripheral stimuli to the injured part of the animal (e.g., complete Freund's adjuvant-treated paw or the side of paw that receives nerve ligation surgery) as reflex-based pain measurements usually do, the animals do commit a significant

portion of the session time to active locomotion and exploration, and voluntary limb movement certainly provokes pain. Thus, conditioned place preference may not only measure the ongoing pain, but also measure the movement evoked (sensory and affective components of) pain.

4.2 Conditioned place aversion

In the early study of place conditioning effects of drugs, it was found that sometimes animals spent less time in the drug-paired side, suggesting an aversive learning attributable to the drug effect (Mucha and Herz, 1985; Mucha et al., 1985; van der Kooy et al., 1983). This conditioned place aversion (or avoidance) paradigm was first adapted to study the affective component of pain in 2001 and soon gained its popularity in understanding the neurobiology of affective pain (Johansen and Fields, 2004; Johansen et al., 2001; Tanimoto et al., 2003; van der Kam et al., 2008). Unlike the conditioned place preference procedure, in this test, experimenters directly administer a painful stimulus (algogenic chemicals) to the animals and it is the demonstration of associative learning between the environment and the algogenic stimulus that is of research interest.

Although the apparatus of the conditioned place aversion paradigm is identical to the conditioned place preference apparatus, the principles used by the two paradigms are fundamentally different. Specifically, for this paradigm, usually a painful stimulus is conditioned with one contextual environment and a non-painful stimulus is conditioned with another distinct environment. Thus, the presence of pain is associated with the conditioning environment in the conditioned place aversion paradigm (aversion, the other side associated with pain-free condition) whereas the amelioration of the pre-existing pain is associated with the conditioning environment in the conditioned place preference paradigm (preference, the other side associated with pre-existing pain condition). This is not a semantic matter but rather a matter of research question, that is, pain-induced conditioned place aversion asks whether the presence of pain is aversive whereas analgesics-induced conditioned place preference asks whether there exists pre-existing (ongoing) pain. Thus, one important difference between conditioned place preference and place aversion studies of pain is that for place aversion studies, only short-lasting painful manipulations can be used because the presence and absence of pain are alternately conditioned with the two distinct contextual environments. Because of this constraint, almost all chronic pain models cannot be used in this type of studies. Thus far, diluted formalin (intraplantar injection), acetic acid (i.p. injection) and carrageenan (intraplantar injection) have been used as algogenic agents to induce acute visceral or inflammatory pain for the study of pain-induced conditioned place aversion (Johansen et al., 2001; Tanimoto et al., 2003; van der Kam et al., 2008).

This procedure has contributed significantly to the understanding of several aspects of pain and analgesics. Because pain *per se* is aversive which is the basis of using conditioned place aversion to study the affective component of pain (e.g., pain-related aversion and unpleasantness), effective analgesics should be able to reverse or attenuate the affective pain and thus pain-induced conditioned place aversion. Indeed, clinically used analgesics such as morphine, tramadol, oxycodone, ibuprofen and pregabalin all can attenuate carrageenan-induced conditioned place aversion in rats (Rutten et al., 2011; van der Kam et al., 2008). Thus, this procedure can be used to evaluate potential analgesics that are effective against affective pain. The application of this procedure has also provided direct evidence that the sensory-discriminative and affective-motivational components of pain are functionally and anatomically separable (Johansen et al., 2001). This study opens the window of mapping the neural underpinnings of affective pain. Indeed, increasing evidence has been emerging from the use of pain-induced conditioned place aversion that begin to map the neural circuitry of affective pain including several critical brain regions (e.g., anterior cingulate cortex, amygdala and bed nucleus of the stria terminalis) (Deyama et al., 2007; Johansen et al.,

2001; Minami, 2009; Tanimoto et al., 2003). This procedure has also been proposed to differentiate the *bona fide* rewarding effects and the anti-aversive (presumably affective pain-reducing) effects of analgesics (Rutten et al., 2011). Opioid analgesics at smaller doses decrease pain-induced conditioned place aversion, but increasing the dose completely reverses the CPA and produces CPP, which is in striking contrast to drugs like ibuprofen which reverses the conditioned place aversion but does not produce conditioned place preference (Rutten et al., 2011).

One variant of the conditioned place aversion procedure was reported, which combines experimenter-provoked mechanical hyperalgesia and place conditioning (Hummel et al., 2008). In this setup, a modified conditioned place preference apparatus was used which includes a grid floor accessible by von Frey filaments from the underneath. This paradigm can measure the affective pain related to mechanical stimulation in both acute pain (e.g., carrageenan injection) and chronic neuropathic pain (e.g., spinal nerve ligation) models. During the place conditioning training, repeated mechanical stimulations that are above and below the threshold to elicit a paw withdrawal response are applied to the injured paw, which are paired with two distinct contextual environments, respectively. During the post-conditioning test, animals reliably spend less time in the chamber that is previously paired with above-threshold mechanical stimulation, suggesting the aversive nature of that environment (and presumably the mechanical stimulation). By using this procedure, it was found that both acute and chronic pain have an aversive affective state that is long lasting and surpasses the course of tissue injury (Hummel et al., 2008).

The advantages and disadvantages of conditioned place aversion paradigm are similar to those of conditioned place preference paradigm, as described above (Table 1). Pain-induced conditioned place aversion has good face validity to model the negative affective status of pain, and limited data also support its predictive validity, as clinically used analgesics (e.g., opioids) are able to prevent conditioned place aversion (Rutten et al., 2011; van der Kam et al., 2008). Some evidence also supports the construct validity of conditioned place aversion to measure affective pain. For example, the role of anterior cingulate cortex in affective pain processing is well elaborated. The finding that chemical ablation of anterior cingulate cortex region also abolishes pain-induced conditioned place aversion in rats corroborates the importance of anterior cingulate cortex in negative affective status of pain (Johansen et al., 2001). Because the negative affective status of pain also contributes importantly to the overall pain experience in humans, this procedure may be useful in future studies of delineating the neural mechanism of affective pain.

4.3 Place escape/avoidance paradigm

LaBuda and Fuchs described a place escape/avoidance paradigm to measure the aversive state of pain (LaBuda and Fuchs, 2000). Patients with chronic pain are often reluctant to commit movements that may exacerbate their ongoing pain and demonstrate various guarding behaviors. Such a desire to avoid pain is clearly part of the affective response comprising the whole pain experience (Price et al., 1980). This procedure was designed to test the hypothesis that animals (e.g., rats) will avoid the environment that is associated with mechanical stimulation to the hyperalgesic area (LaBuda and Fuchs, 2000). For this procedure, two distinct environments (e.g., white vs. black walls) are located on a mesh floor which is readily accessible by a von Frey filament from the underneath. Rats with chronic pain (e.g., L5 spinal nerve ligation) are exposed simultaneously to and have free access to both environments. Training typically follows the following strategy: a mechanical stimulus (von Frey filament) that is adequate to elicit a withdrawal response on the ipsilateral paw (e.g., the side that receives nerve ligation surgery) is applied every 15 sec to the plantar surface of one of the hind paws of the animal, depending on the location of the animal during that time, for a period of 30 min. As a rule, the ipsilateral paw is stimulated

whenever the animal is located at one of the environments and the contralateral paw is stimulated when the animal is located at the alternate environment (LaBuda and Fuchs, 2000). A consistent finding of this procedure is that at the beginning of the session, animals allocate roughly the similar amount of time at both environments if animals do not show natural preference or aversion to one of the two environments. However, over time the animals progressively stay longer in the environment when the contralateral paw is stimulated, thus demonstrating a temporal trend to avoid the environment where the ipsilateral paw is provoked (LaBuda and Fuchs, 2000; LaGraize et al., 2006; LaGraize et al., 2004; Pedersen and Blackburn-Munro, 2006; Pedersen et al., 2007). At the beginning of the session, the animals are actively exploring both sides of the chamber but can easily escape to the other side. If the animals learn the association between stimulation of the ipsilateral (presumably a painful stimulus) paw and one side of the chamber, they tend to avoid that side by staying increased time on the other side, demonstrating an avoidance behavior.

As compared to the conditioned place preference or conditioned place aversion paradigms described above, the place escape/avoidance paradigm is much more time-efficient (the whole session only lasts 30 min) and therefore studying both acute and chronic pain are possible (Table 1). This procedure is labor-intensive to the experimenters because usually one animal can only be tested at one time and the experimenter has to apply the von Frey filament stimulation 120 times during a 30 min session and the stimulations have to be separated as evenly as possible (15 sec intevals). Currently there is no automated apparatus that is commercially available for this test. Unlike the conditioned place preference and conditioned place aversion, there are no training sessions for this procedure and the training session is also the test session. The final behavioral readout is the temporal changes of the time the animal spends in a specific environment during the whole session.

It has been shown that in rats receiving spinal nerve ligation surgery, chronic constriction injury or complete Freund’s adjuvant treatment, they demonstrate marked place avoidance behavior (LaBuda and Fuchs, 2000; Pedersen and Blackburn-Munro, 2006). Clinically used analgesics such as morphine, gabapentin and duloxetine dose-dependently prevent the place avoidance behavior in this procedure at the doses that do not produce sedation (Pedersen and Blackburn-Munro, 2006). It is suggested that this procedure may be able to differentiate the drug classes that demonstrate distinct effectiveness on the affective and sensory components of pain. Anterior cingulate cortex lesion selectively attenuates the place avoidance behavior in the place escape/avoidance paradigm procedure but does not alter the mechanical hyperalgesia (LaGraize et al., 2004), in congruent with the previous studies using conditioned place aversion procedure (Johansen et al., 2001).

This procedure measures the general aversive nature of a mechanical stimulation-induced pain response in animals with hyperalgesia. Unlike the revised conditioned place aversion described by Hummel et al. (2008), in which animals are restrained in an environment that is not escapable, the place escape/avoidance paradigm offers an alternative option for the animals to escape and subsequently avoid the pain-related environment. This procedure is sensitive to pharmacological and anatomical manipulations, and thus may provide a useful method to understand the neural mechanism of affective pain. This procedure is a good example that both Pavlovian conditioning and operant conditioning are involved in the process of training: the rats learn to associate one side with a noxious stimulus and the other side with a relatively innocuous stimulus through Pavlovian conditioning; once this relationship is learned, the rats can emit an operant response (move to the less aversive side) to avoid a noxious stimulus and this response further increases the probability that the same behavior re-occurs through the process of negative reinforcement (escape from and avoidance of the painful mechanical stimulus).

4.4 Passive avoidance test (step-down test)

Visceral pain represents a significant clinical challenge, and the development of valid behavioral procedure of visceral pain will facilitate the understanding and development of effective treatments of various visceral painful conditions. Colonic distension by a balloon evokes noxious and pain sensation in humans (Ness et al., 1990; Ritchie, 1973). In an early attempt to adapt the same procedure to rats, it was found that colorectal distension at intensities of > 40 mmHg produces avoidance behavior in rats (Ness and Gebhart, 1988; Ness et al., 1991). For these studies, a step-down, passive avoidance paradigm was used to measure the avoidance behavior (Ness et al., 1991). In this paradigm, a latex balloon was inserted intra-anally into the colorectal section of the rats. Rats were then tested in two distinct environments with a platform (15 × 15 cm size, 10 cm height) placed in the middle of each environment. Rats were placed on the two platforms in a pseudorandom order at 1 min intervals. Usually rats would quickly step down from the platform (within sec). However, when the rats were placed in one environment and when they stepped down from the platform, a colorectal distending stimulus was given (20 sec); they would not receive such a noxious stimulus when they were tested in the other environment. It was found that rats continued to step down from the platform quickly in the environment where they never received the colorectal distending stimulus. In contrast, rats progressively increased the time they spent on the platform (up to the cutoff time of 120 sec) where they would receive the noxious stimulus if they stepped down, suggesting that they remained on the platform to avoid the noxious stimulus, thus demonstrating the aversive nature of the noxious stimulus (Ness et al., 1991). Importantly, when rats received intrathecal morphine treatment, this “step down latency” was markedly decreased, suggesting that morphine attenuates the magnitude of aversion induced by colorectal distension (Ness et al., 1991).

Modified passive avoidance test was reported with minor differences (Nijsen et al., 2003; Stam et al., 2004). These studies attempted to improve the procedure by using chronically indwelled balloon catheters and transmitters, which can simultaneously and wirelessly measure the visceromotor and cardiovascular responses, two important parameters of visceral stimulation (Ness and Gebhart, 1990). A silicone balloon catheter was surgically implanted into the duodenum and a telemetry transmitter was implanted into the abdominal cavity of the rats. After the rats were fully recovered from the surgery (12 days later), the passive avoidance test began. This test involved one platform in an environment instead of two distinct environments as previously described (Ness et al., 1991). Rats were submitted to habituation on the first day and tested on the second day with an identical procedure. In each session, rats were submitted to 9 trials with an interval of 10 min. During the habituation session, no duodenum distention (via remotely-controlled balloon catheter) was applied. However, on the test day some rats were subjected to duodenum distention when they stepped down from the platform during each trial, while others never received the distention stimulus and served as a control. It was found that rats who received the duodenum distention stimulus progressively stayed longer on the platform as compared to the control group and thus suggested that duodenum distension was perceived as aversive (Nijsen et al., 2003). More importantly, the visceromotor and cardiovascular responses correlated to the passive avoidance response, which can be prevented by morphine treatment, validating this procedure as a useful behavioral assay for the study of one form of visceral pain (Nijsen et al., 2003).

The passive avoidance test seems to measure the aversive aspect of mechanical stimulation of the viscera (colorectal section or duodenum) through operant responding (refrain from stepping down from the platform to avoid the painful stimulus) (Table 1). However, it should be cautioned that the demonstration of avoidance behavior only reflexes the aversive quality of the mechanical stimulus (distention of the hollow organ), but not the “pain-inducing” effects of the stimulus *per se* (Ness et al., 1991). Although both may converge at

the physiological level, this possibility should be kept in mind to avoid over-interpretation of the data. Nonetheless, by combining the visceromotor measures, the passive avoidance test could be a very helpful assay in studies of visceral pain.

4.5 Operant escape paradigm

Mauderli et al. reported an operant based escape procedure to measure pain (Mauderli et al., 2000). In this procedure, animals are confronted with two environments: one environment with dim lighting but the floor temperature is adjustable, and another environment equipped with bright lighting and floor of room temperature. Rats are always confronted with a choice situation such that when the floor temperature on the dim lighting side is below aversive level, the rats spend the majority of time in that side while they could jump to the bright lighting side to escape from a high and aversive floor temperature (thermal nociception). This procedure requires extensive training because animals have to learn the differences between the two environments and the other option when one environment is considered aversive (Mauderli et al., 2000). Two response variables are usually taken: the latency of the rats to escape from the dim lighting side to the bright lighting side and the cumulative time the animals spend on the escape side (bright lighting side). It was found that both systematically increasing and decreasing the floor temperature on the dim lighting side decrease the latency of rats to escape to the other side and increase the time spent at the other side. Morphine dose-dependently increases the latency and decreases the time the animal spends at the bright lighting side, demonstrating the anti-aversive and/or antinociceptive effects of morphine (Mauderli et al., 2000).

This procedure offers an indirect measurement of pain which may represent an integrated response of both sensory-discriminative and affective-motivational components, and the incorporation of the more widely used behavioral signs of pain (e.g., defensive behaviors) in the test is possible (Mauderli et al., 2000). However, although modified versions of this paradigm have been reported (Baliki et al., 2005; Ding et al., 2005), it requires specialized apparatus that is not commercially available and thus has not been used by many other laboratories (Table 1). In addition, the predictive validity of this procedure for the assessment of established and new analgesics except opioids has not been systematically evaluated and thus is unknown. This procedure can be used in freely moving animals both for acute nociception (thermal stimulus) and for thermal hyperalgesia in animals with chronic pain (Mauderli et al., 2000; Vierck et al., 2002; Vierck et al., 2005) and thus does provide flexibility and applicability for the study of various painful conditions.

4.6 Drug self-administration procedure

It was first demonstrated in 1962 that rats equipped with intravenous catheters can actively engage in an operandum to receive morphine injections, a phenomenon termed drug self-administration (Weeks, 1962), which was the first line of direct preclinical evidence that drugs can serve as positive reinforcers. This phenomenon has been quickly confirmed in various species and across several different pharmacological classes. Interestingly, it was soon realized that almost all the drugs that are abused in human population can maintain self-administration behavior in experimental animals whereas most drugs that have low abuse liability in humans cannot be reliably self-administered by animals. Therefore, this procedure has since been increasingly used as the golden standard to evaluate the reinforcing properties and abuse potentials of known or new drugs both in animals and in humans (Ator and Griffiths, 2003; Griffiths et al., 2003).

If a drug has pain-relieving activity and intake of the drug can alleviate pain, it should be expected that animals with pain may increase the drug intake in a magnitude that is greater than in normal pain-free animals (Colpaert et al., 1982). In support of this hypothesis, it was

found that in rats with inflammatory pain (complete Freund's adjuvant treatment), the animals drank significantly more solution dissolved with suprofen or fentanyl as compared to no-drug solution when given a choice, and this dynamic analgesic-containing solution intake was highly correlated to the magnitude of tissue inflammation (diameter of paws and joints) and frequency of vocalization (Colpaert et al., 1980; Colpaert et al., 1982). In addition, continuous infusion of dexamethasone greatly attenuated the inflammatory magnitude and also substantially decreased the oral fentanyl intake to a level that was not different from that of pain-free animals (Colpaert et al., 2001). These data imply that complete Freund's adjuvant-treated animals experience chronic pain and that they opt to self-administer an analgesic to alleviate pain when the drug is available. However, these studies only demonstrate that rats prefer the analgesic-containing solution during pain when given a choice through Pavlovian learning as the total liquid intake in those studies typically does not change, but do not show the reinforcing effects of analgesics in animals with pain.

It was soon found that animals could be trained to self-inject more analgesics through an operant apparatus when a painful stimulus was applied (Dib, 1985). In this study, rats were prepared with intrathecal catheter and trained to press an operant lever under a fixed ratio 1 schedule to receive an intrathecal injection of morphine. Under control conditions when no painful stimulus was applied, the rats did not reliably press the lever for morphine injection. However, electric stimulation markedly increased the intrathecal morphine injection but has no effect on saline injection. Moreover, the dose of self-administered morphine was adequate to significantly increase the tail flick latency, a widely used behavioral endpoint of acute nociception (Dib, 1985). It was proposed that morphine is more reinforcing under the condition that the rats are experiencing noxious stimulus (electric shock) and that the increased morphine intake is likely directly related to the decrease or suppression of the painful sensation (Dib, 1985). However, an alternative interpretation of these results could be that it is the electric shock as a stress but not shock-induced painful sensation *per se* that drives the morphine injection in those rats, because mild foot shock increases the reinforcing effectiveness of both intravenous heroin and oral fentanyl self-administration as measured by a progressive ratio schedule (Shaham et al., 1993; Shaham and Stewart, 1994). In support of this possibility, immobilization stress also significantly increases oral morphine self-administration (Shaham, 1993), and it is difficult to argue that this increased morphine intake is driven by increased pain sensation. This makes sense because in drug abusers, stressful events can increase continued drug use and lead to relapse in those who stop using drugs (Shaham, 1993).

One way to truly define the driving force attributable to the increased analgesic intake in animals with pain is to let rats with pain to self-administer an analgesic that normally is not reinforcing. Thus, Martin et al. found that in rats following spinal nerve ligation, a commonly used animal model of peripheral neuropathic pain, intrathecal clonidine maintained stable self-administration behavior while clonidine could not be reliably self-administered by control normal rats (Martin et al., 2006). This effect was dose-dependent and can be antagonized by the nonselective α_2 adrenoceptor antagonist, idazoxan. Moreover, the self-administered clonidine produced marked anti-hyperalgesic effects and the self-administration behavior went into extinction when clonidine was replaced by saline, suggesting that the self-administration behavior was controlled by clonidine (Martin et al., 2006). These data suggest that the known α_2 adrenoceptor agonist analgesic, clonidine, is reinforcing in animals with neuropathic pain but not in pain-free animals. A recent study utilized the same principle and found that a selective cannabinoid CB_2 receptor agonist, (R, S)-AM1241, is self-administered by rats that received spared nerve injury but not by sham-operated rats (Gutierrez et al., 2011). In addition, when a higher fixed ratio was used, rats with neuropathic pain worked harder than control rats to receive drug injection, and the self-administered (R, S)-AM1241 markedly attenuated mechanical hypersensitivity (Gutierrez et

al., 2011). These results support the notion that pain conditions alter the reinforcing properties of drugs and that rats are taking analgesics to "self-medicate" when experiencing pain.

Although noxious stimulation increases opioid intake (Dib, 1985; Shaham and Stewart, 1994), the presence of persistent chronic pain (e.g., spinal nerve ligation) decreases opioid self-administration behavior in rats (Martin et al., 2007), which is consistent with studies of opioid-induced conditioned place preference in rats with chronic pain (Niikura et al., 2010). Because chronic pain decreases the efficacy of opioids for activating opioid receptors, it is not surprising that opioid maintained self-administration behavior is attenuated (Martin et al., 2007). As discussed above (see Section 3.1), the observed self-administration behavior of opioids is most likely the integrated behavioral output of two separate and opposing processes: the decreased reinforcing effectiveness of opioids in rats with chronic pain and the reinforcing effects of opioids (as effective analgesics) due to the alleviation of chronic pain. The complicated interaction between the two processes is poorly understood and future studies on this question may reveal fresh information to better understand the neurobiological mechanisms of drug-induced analgesia and reinforcement.

It seems that the self-administration procedure can be a useful tool to reveal the true "self-motivated medication" for chronic spontaneous pain as the operant drug-taking behavior is highly correlated to drug-induced analgesia in animals. However, the self-administration procedure does have its disadvantages in the process of analgesic screening (Table 1). For intravenous self-administration, an intravenous catheter is required to be surgically implanted and carefully maintained daily and the catheter life (patency failure) may become a limiting factor in studies that require long term dosing. This is technically much more challenging than the traditional measurements of reflex responses. Drug solubility may also become an issue for the compounds that do not dissolve in common vehicles. Nevertheless, given the successful application of this procedure in the field of drug abuse in the past half a century and the large body of literature accumulated on this topic, this procedure offers a potentially powerful behavioral demonstration of drug-induced suppression of spontaneous pain and, combined with the traditional reflex-based measurements of evoked pain, it could decrease the false positive hits and increase the success rate of developing novel analgesics in the process of analgesic drug discovery.

4.7 Intracranial self-stimulation procedure

In 1954, Olds and Milner observed that rats could be trained to actively press a lever in an operant chamber to emit an electric stimulation through electrodes implanted into septal nuclei, and thus the positive reinforcing property of brain electrical stimulation was discovered (Olds and Milner, 1954). It was soon found that electric stimulation of several brain regions is reinforcing in both animals and humans (Andrew, 1967; Bishop et al., 1963; Wilkinson and Peele, 1963). This procedure was then adapted to assess the abuse potential of drugs as known drugs of abuse facilitate intracranial self-stimulation (Holtzman, 1976; Kornetsky et al., 1979; Phillips and Fibiger, 1973). Because electrical stimulation of certain brain regions is rewarding, and because pain sensation is aversive by nature, one may posit that exposing animals to a painful stimulus may alter the intracranial self-stimulation performance. Indeed, Pereira Do Carmo et al. found that an i.p. injection of lactic acid suppressed the inctrcranial self-stimulation (lateral hypothalamus) and shifted the intracranial self-stimulation frequency-rate curve rightward (Pereira Do Carmo et al., 2009). Moreover, morphine and ketoprofen alone have little effect on intracranial self-stimulation behavior, but they markedly prevented lactic acid-induced intracranial self-stimulation suppression and also inhibited lactic acid-induced abdominal writhing responses (a commonly used behavioral endpoint of acute visceral pain) in the rats (Negus et al., 2012; Pereira Do Carmo et al., 2009). In contrast, κ opioid receptor agonists, which are known to

produce dysphoric effects in humans (Kumor et al., 1986), exacerbated lactic acid-suppressed intracranial self-stimulation performance, although the writhing responses were blocked (Negus et al., 2010). These data suggest that intracranial self-stimulation paradigm could be a useful complementary assay, when combined with other traditionally used reflex-based assays, to refine the assessment of analgesics in preclinical studies.

Intracranial self-stimulation has also been studied in animals with chronic pain (Ewan and Martin, 2011). Electric stimulation of the ventral tegmental area maintained reliable lever-pressing behavior and clinically used opioid analgesics (methadone, fentanyl, hydromorphone and oxycodone) facilitated this intracranial self-stimulation performance and shifted the intracranial self-stimulation frequency-rate curve leftward (Ewan and Martin, 2011). However, the magnitude of leftward shift was significantly smaller in spinal nerve ligated rats (a model of neuropathic pain) as compared to control rats, suggesting that rats with chronic pain have a decreased response to opioids (Ewan and Martin, 2011). This finding is consistent with the studies of conditioned place preference and drug self-administration (Martin et al., 2007; Niikura et al., 2010). Interestingly, this effect may depend on which brain region is stimulated, as when the electrodes were implanted into the paraventricular nucleus, the facilitating effects of morphine on intracranial self-stimulation were not different in control rats and in spinal nerve ligated rats (Ewan and Martin, 2012).

One advantage of this procedure for the study of pain and evaluation of analgesics is that intracranial self-stimulation generates highly quantitative and reliable data (Table 1). The animals with electrode implantation are relatively easy to maintain and, once trained, they can be used for a long period of time. Thus, important pharmacological studies such as dose-effect curves, time courses, and the development of tolerance or sensitization can be evaluated in a small number of animals. The disadvantage of this procedure is that the apparatus required is comparatively expensive and special training is required to conduct operant studies efficiently.

It is unclear what aspect of pain intracranial self-stimulation measures. The intracranial self-stimulation performance is not different in control rats and in spinal nerve ligated rats (Ewan and Martin, 2011). Rats with neuropathic pain are thought to experience tonic ongoing pain (King et al., 2009), and the fact that the intracranial self-stimulation frequency-rate curves are similar in pain-free rats and in rats with ongoing pain may imply that intracranial self-stimulation does not measure ongoing pain and spontaneous pain. Because a noxious stimulus (i.p. lactic acid injection) decreases intracranial self-stimulation performance (Pereira Do Carmo et al., 2009), the change of intracranial self-stimulation behavior may reflect the aversive aspect of evoked pain. Future studies that examine how a noxious stimulus alters the intracranial self-stimulation performance in rats with chronic pain will help clarify this question.

5. Conclusions

Reflex-based pain measurements have been adapted in the study of pain and evaluation of analgesics for nearly a century. However, these measures have received increasing critiques for their questionable predictive validity (Le Bars et al., 2001; Mogil, 2009). Against this background, many models have been proposed attempting to measure different aspects of pain other than nociceptive stimulus-induced reflexive response. In this wave of modeling revolution, conditioning principles have been increasingly used in several behavioral procedures to measure aspects of pain that are difficult to reach with traditional reflex-based endpoints. These procedures all require complex learning and adaptation, thus information processing from the high level of brain hierarchy is thought to be intimately involved. This is critical because as many agree that pain is a complicated experience that involves both

supraspinal and spinal processing of nociception while most reflex responses may only involve spinal level pain processing and thus may not reflect true pain experience.

The procedures discussed above offer a fresh opportunity to begin to unveil aspects of pain that are critical for the understanding of pain experience in animals and humans from ongoing pain to pain affect. Increasing applications of these paradigms in combination with traditional reflex-based measurements will greatly facilitate the understanding of the neurobiology of pain as a whole and improve the prediction of true analgesics in the process of drug discovery. However, it should be noted that all these procedures do not measure the same process (Table 1), and in future studies it is important to choose the appropriate models to measure the aspect of pain that the procedure is designed to measure. While these procedures are exciting new advancements in the field of pain research, interpretation of data generated from them should always be careful and also take other endpoints into consideration. In short, pain should be treated and studied as wholeness rather than a piece of behavior, and that requires the simultaneous measurement of multiple aspects of pain, including both sensory aspect that is long thought to be measured by reflexive responses and pain affect that can be unveiled by procedures that involve affective learning.

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Table 1

Procedures used in pain studies that involve conditioning

Procedure	What aspect of pain is measured?	Advantages	Disadvantages
CPP	Ongoing pain, movement-evoked affective pain	Well validated, simple, apparatus commercially available	Cumbersome
CPA	Pain aversion of ongoing or evoked pain	Well validated, simple, apparatus commercially available	Cumbersome
PEAP	Pain aversion of evoked pain	Reasonably validated, simple, time-efficient, easy to adapt	Not automated and physically demanding
Passive avoidance test	Pain aversion of evoked visceral pain	Time-efficient, reasonably validated, easy to adapt	Only limited to visceral pain studies
Operant escape test	Pain aversion	Reasonably validated	Cumbersome, animals require extensive training
Drug self-administration	Analgesia induced reinforcement	Well validated, apparatus commercially available	Costly, technically sophisticated
ICSS	Pain aversion of evoked pain (?)	Apparatus commercially available, reliable behavioral data, animals are easy to maintain	Costly, technically sophisticated

Note: CPP, conditioned place preference; CPA, conditioned place aversion; PEAP, place escape/avoidance paradigm; ICSS, intracranial selfstimuiation.

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