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Author(s)	Moriarty, Orla; Roche, Michelle; McGuire, Brian E.; Finn, David P.
Publication Date	2011-11
Publication Information	Moriarty O, Roche, M, McGuire BE, Finn DP (2011). Validation of an air-puff passive-avoidance paradigm for assessment of aversive learning and memory in rat models of chronic pain. <i>Journal of Neuroscience Methods</i> , 204(1):1-8.
Publisher	Elsevier
Link to publisher's version	http://dx.doi.org/10.1016/j.jneumeth.2011.10.024
Item record	http://hdl.handle.net/10379/2528

Downloaded 2022-05-19T01:26:37Z

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Title **Validation of an air-puff passive-avoidance paradigm for assessment of aversive learning and memory in rat models of chronic pain**

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Abstract

Chronic pain is associated with cognitive deficits. Considerable overlap in brain regions involved in pain and aversion suggests that aversive learning and memory may be affected during chronic pain. Passive-avoidance paradigms traditionally use foot-shock to induce context-conditioned avoidance and may be unsuitable for use in animal models of chronic pain, which are commonly associated with hypersensitivity of the hind-paws. The aim of the present study was to develop and validate a novel passive-avoidance paradigm in rats, employing air-puff as the aversive stimulus, and to use this paradigm to assess aversive learning and memory in rat models of chronic inflammatory and neuropathic pain. Air-puff exposure produced a significant passive-avoidance and this response was attenuated following administration of scopolamine. Nerve-ligated rats and rats injected with complete Freund's adjuvant developed allodynia and hyperalgesia. Air-puff produced a significant passive-avoidance response in both chronic pain models. However, there was no difference in the response between either model and its respective control group. Thus, air-puff can be used as an alternative to foot-shock to induce a passive-avoidance response. The data generated using this model suggest that aversive learning and memory remain intact in the rat spinal nerve ligation and complete Freund's adjuvant models of chronic neuropathic and inflammatory pain, respectively.

Keywords: Passive avoidance; conditioned aversion; learning; memory; chronic pain

Abbreviations: SNL, spinal nerve ligation; CFA, complete Freund's adjuvant

1. Introduction

Chronic pain is characterised by somatosensory sensitisation including allodynia and hyperalgesia, and may be associated with effects on cognitive functioning (Hart et al., 2000; Moriarty et al., 2011). It is hypothesised that neural systems involved in cognition and pain processing are linked, and may modulate one another (Moriarty et al., 2011). Chronic pain has been likened to the continuous presence of an unconditioned aversive stimulus, which can become associated with random conditioned events and inhibit extinction of associative memory on re-exposure to the conditioned events (Apkarian, 2008). Thus, impaired associative learning for other aversive stimuli may be expected.

Clinical studies have shown that chronic pain is associated with impairments in cognitive parameters such as learning, memory and decision-making tasks (Apkarian, 2008; Apkarian et al., 2004; Dick et al., 2002; Dick and Rashiq, 2007; Dufton, 1989; Eccleston, 1994; Hart et al., 2000; Kewman et al., 1991; McCracken and Iverson, 2001; Oosterman et al., 2010; Povedano et al., 2007), but few studies have investigated the effect of pain on aversive learning or recall of aversive memory. However, reporting of previous pain is known to be distorted in chronic pain patients (Bryant, 1993; Eich et al., 1985). Other studies have shown that both clinical and experimental pain enhance recall of aversive words in a memory task (Pearce et al., 1990; Seltzer and Yarczower, 1991), and increased pain levels are associated with increased re-experiencing symptoms in post-traumatic stress disorder (Beckham et al., 1997), suggesting that pain may actually enhance aversive memory. In animal models of pain, cognitive impairments have been demonstrated in novel-object exploration, operant learning tasks and the Morris Water Maze tests of spatial learning and memory (Boyette-Davis et al., 2008; Hu et al., 2010; Leite-Almeida et al., 2009; Millecamps et al., 2004; Pais-

Vieira et al., 2009a). As with clinical research, few studies specifically investigated aversive learning and memory in animal models of chronic pain. It has been shown that rats acquire an active avoidance response in place-escape/avoidance paradigms following context-specific, aversive mechanical stimulation of the injured hind-paw (with noxious von Frey filaments) in models of chronic pain (LaBuda and Fuchs, 2000; Pedersen and Blackburn-Munro, 2006). However, such paradigms do not typically include a retention trial to measure recall of the learned response. Spinal nerve ligation, a model of chronic neuropathic pain, did not affect aversive learning and memory in a foot-shock-induced passive-avoidance paradigm in mice (Suzuki et al., 2007). Passive avoidance, a fear-motivated classical conditioning paradigm, is used to measure aversive learning and memory in rodents. Rodents learn to associate a conditioned stimulus or context with an aversive or noxious stimulus, resulting in subsequent avoidance of the conditioned stimulus. Simple paradigms involve a single acquisition trial, during which an instinctive response (moving from a light to a dark compartment of an arena) is punished by administering an aversive stimulus in the dark compartment. In the retention trial (carried out after a specified interval) the animal is returned to the arena and avoidance of the punished (dark) context is observed. Traditionally-used, noxious foot-shock-induced passive avoidance is complicated in models of chronic pain as such models are commonly associated with hypersensitivity of the hind-paw(s). Because foot-shock stimulates peripheral nociceptors in the paws, the stimulus is likely to be perceived differently in models of chronic pain, which may confound interpretation of cognitive performance.

A brief, non-noxious exposure to compressed air (“air-puff”) applied to the head of a rodent induces fear-related behaviour such as a startle response (Engelmann et al., 1996), 22kHz ultrasonic vocalisation (Sanchez, 2003) and avoidance behaviour (Cimadevilla et al., 2001; Karlsson et al., 2009; Koistinaho et al., 2001). Therefore, we hypothesised that the use of an

air-puff stimulus may be a suitable non-noxious alternative to foot-shock in passive-avoidance paradigms designed to assess aversive memory in rodent models of chronic pain.

The aims of the present study were: to establish a rat passive-avoidance paradigm using an aversive air-puff stimulus as an alternative to noxious foot-shock; to validate the paradigm using scopolamine (which inhibits memory acquisition); and to use this validated paradigm to assess aversive learning and memory in two rat models of chronic pain, the L5-L6 spinal nerve ligation (SNL) model of neuropathic pain (Kim and Chung, 1992), and the complete Freund's adjuvant (CFA) model of chronic inflammatory pain (Stein et al., 1988).

2. Materials and Methods

2.1 Animals

Male Sprague-Dawley rats (CD sub-strain, Charles River, UK) weighing 175-250g (6-9 weeks old) on arrival were used for these experiments. Rats were singly-housed in plastic-bottomed cages 40 cm (length) x 25 cm (width) x 20 cm (height) containing wood-shavings for bedding. Rats were maintained under standard laboratory conditions of temperature ($20 \pm 2^\circ\text{C}$), humidity (40-60%) and lighting (12:12 hour light/dark cycle, lights on at 08:00 h). Food and water were available *ad libitum*. Baseline testing began a minimum of 3 days following arrival of rats to the unit and all experiments were carried out during the light phase. All experiments were carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee of the National University of Ireland, Galway, under licence from the Irish Department for Health and Children and in compliance with European Communities Council Directive 86/609 and International Association for the Study of Pain (IASP) guidelines.

2.2 Experiment 1: Validation of an air-puff passive-avoidance paradigm

2.2.1 Passive-Avoidance Apparatus

Passive avoidance was carried out in a specially constructed light/dark arena. The light compartment was made of white melamine-coated chipboard with dimensions of 30cm (length) x 30cm (width) x 40cm (height) and was lit from above using a standard 60W bulb, such that the compartment was maintained at a constant light intensity of 100 ± 10 lux. The dark compartment was made of dark grey Perspex and its dimensions were identical to those of the light compartment. The dark compartment was also fitted with a lid made of black wood to minimise the entry of light to the compartment. The two compartments were separated by a manually-controlled guillotine door. Video cameras were fitted above the light compartment and in the lid of the dark compartment to monitor rat behaviour. For delivery of air-puff directed at the rat's face, an air-duster canister (Electrolube, GDP, UK) was mounted to the outside of the dark compartment, such that the nozzle was 3 cm from the floor of the arena (approximately nose-height of the rat) and could protrude up to 3.5 cm into the dark compartment through a 5 mm diameter hole in the compartment wall opposite the guillotine door. The vapour pressure of the compressed air in the air-duster canister used to deliver the air-puff was 62.8 kPa at 20°C.

2.2.2 Passive-avoidance paradigm

Rats first underwent an habituation period of 5 minutes, during which they were placed in the light compartment of the arena, facing away from the guillotine door and allowed to freely

explore both the light and dark compartments of the arena. After this period, the rat was removed from the arena and returned to its home-cage. The following day, rats were subjected to an acquisition trial. The rat was again placed in the light compartment of the arena, facing away from the guillotine door and the latency to enter the dark compartment was recorded up to a maximum of 300 seconds. Once the rat had entered the dark compartment, the guillotine door was lowered by the experimenter. Once the rat was facing the wall opposite the guillotine door, a single, brief puff of air (duration of ~1 second) was administered to the face. The rat was confined to the dark compartment for a further 90 seconds post-air-puff, after which it was removed from the arena and returned to its home-cage. If the rat did not enter the dark chamber within the 300-second period it was removed from the arena, returned to its home-cage and excluded from further testing. Control rats went through an identical procedure to that described above, but were not exposed to air-puff.

The retention trial was carried out 24 hours following the acquisition trial. The rat was again placed in the light compartment of the arena, facing away from the guillotine door and the step-through latency to enter the dark compartment was recorded. If the animal did not enter the dark compartment within 300 seconds they were removed from the arena and returned to their home cage, and latency was recorded as 300 seconds. No air-puff was administered during the retention trial. In all trials, the arena was cleaned with mild detergent between rats to remove olfactory cues.

2.2.3 Scopolamine validation

Passive-avoidance habituation was carried out as described above. Rats were divided into air-puff ($n=24$) and no-air-puff ($n=8$) groups. No-air-puff rats received a single intraperitoneal (i.p.) injection of physiological saline (0.89% NaCl) 30 minutes before the acquisition trial.

Air-puff rats received either saline ($n=8$) or scopolamine hydrobromide (Sigma, Ireland) at a dose of 1mg/kg ($n=8$) or 3mg/kg ($n=8$), also administered i.p. 30 minutes prior to the acquisition trial. Scopolamine or saline was administered in an injection volume of 1mL/kg.

2.3 Experiment 2: Assessment of aversive learning in rat models of chronic neuropathic and inflammatory pain

2.3.1 Spinal Nerve Ligation Surgery

Spinal nerve ligation (SNL) was used to model chronic neuropathic pain and was carried out as described previously (Kim and Chung, 1992). Rats were randomly assigned to SNL ($n=11$) or sham control surgery ($n=12$). Briefly, the rats were anaesthetized with isoflurane (Isoflo®, 2.5% in 0.6 L/min oxygen), the fur lateral to the midline on the left-hand side at the lower lumbar and sacral regions was clipped closely, and an incision was made through the skin between the spinal column and the left iliac crest. Paraspinal muscles were removed using a toothed forceps to visualise the L6 transverse process. This bone was then removed using a small rongeur to expose the L4 and L5 spinal nerves. The L5 nerve was tightly ligated using 6-0 (0.07-mm diameter) silk suture. The L6 nerve, which is located underneath the sacrum, was also ligated. In the sham-operated rats, the L5 and L6 nerves were exposed but were not ligated. The total surgery duration was approximately 1.5 hours. Rats were allowed to recover from anaesthesia in recovery cages maintained at a constant temperature on a heating pad and then re-housed singly with fresh bedding in their home-cages.

2.3.2 Complete Freund's adjuvant (CFA) injection

Immunogenic CFA (dissicated *Mycobacterium tuberculosis* in an 85% mineral oil, 15% mannide monooleate suspension, Sigma, Ireland) was used to induce a chronic inflammatory pain state (Stein et al., 1988). Rats received a single 100 µL intraplantar injection of CFA (1mg/mL) into the left hind-paw ($n=9$), under brief isoflurane anaesthesia (5% in 0.8 L/min Oxygen). Control rats underwent intraplantar needle insertion to the left hind paw ($n=9$), also under isoflurane anaesthesia. The duration of anaesthesia was approximately 2 minutes. After a brief recovery period, rats were re-housed singly in their home-cages.

2.3.3 Von Frey testing for mechanical allodynia

The arena used for von Frey testing consisted of a six-compartment Perspex arena. Each compartment was 11 cm (length) x 20cm (width) x 15 cm (height), and was separated from the adjoining compartment by a Perspex partition. The arena was placed on a wire mesh flooring so that the tester could easily access the hind paws from beneath. Rats were habituated to the arena for 30 minutes the day before testing began, and on test days were habituated to the arena for 20 minutes prior to testing.

von Frey filaments (Touch-Test ®Sensory Evaluators, North Coast Medical, Inc.) of different weights (0.16g – 180g), were applied perpendicular to the plantar surface of the hind-paw, with sufficient force to cause slight buckling of the filament, for approximately 6 seconds or until a positive result was observed. A positive result was recorded if flinching, licking or withdrawal of the paw occurred on application of the filament or immediately after removal of the filament. Filaments were applied to both left and right hind-paws five times

(alternating between paws) in order of increasing weight until a 100% positive response (5 positive responses to 5 applications) was observed. The filament weight eliciting 50% response was calculated by plotting the % response versus filament weight for each rat. von Frey testing was carried out on days 1, 3, 5, 7, 9, 11 and 13 post-injury (SNL surgery or CFA injection) by an experimenter blind to the treatment procedure. The arena was cleaned with mild detergent between testing each group of rats.

2.3.4 Hargreaves' test for thermal hyperalgesia

The Hargreaves' test apparatus (IITC Life Science Inc., Woodland Hills, CA) consisted of a six-chamber Perspex arena placed on top of a heated glass plate ($30 \pm 1^\circ\text{C}$). Rats were habituated to the pain test apparatus for 30 minutes on the day before baseline testing began, and on test days were habituated to the arena for 20 minutes prior to testing. A moveable radiant heat source was positioned underneath the glass and could be focused on the rat's hind-paw. The heat source was set to an active intensity of 30% and focused from below on the plantar surface of the rat's hind-paw. The thermal stimulus was applied until a positive response (criteria similar to those used for von Frey testing) was recorded or until a cut-off time of 20 seconds was reached. This cut-off time was selected to prevent tissue damage. Right and left hind-paws were tested 4 times, alternating between paws, and the average withdrawal latency for each paw was calculated. Hargreaves' testing was carried out on days 8 and 16 post-injury by an experimenter blind to the treatment procedure. The arena was cleaned with mild detergent between testing each group of animals.

2.3.5 Acetone drop test for cold allodynia

The arena used for this test was identical to that used for von Frey testing. A 1 mL syringe with a short length of polyethylene Portex® tubing (1mm internal diameter) attached was used to apply 0.2 mL of acetone (Sigma, Ireland) to the plantar surface of the hind-paw, without stimulating the paw mechanically. Each hind-paw was tested 3 times, alternating between paws, the number of positive responses within 60 seconds of acetone application for each trial was recorded, and the total across trials calculated. Testing for cold allodynia was carried out on days 4 and 12 post-injury by an experimenter blind to the treatment procedure. The arena was cleaned with mild detergent between testing of each group of animals.

2.3.6 Passive-avoidance testing in pain models

Following surgery, nerve-ligated animals and sham SNL controls were subdivided into air-puff and no air-puff group (SNL air-puff: n=6, SNL no-air-puff: n=5, sham air-puff: n=6 and sham no-air-puff: n=6). All CFA-treated rats and controls were exposed to air-puff (CFA air-puff n=9 and control air-puff n=9). Passive-avoidance testing was carried out as described above on days 18 (habituation), 19 (acquisition) and 20 (retention) post-SNL surgery or post-CFA injection.

2.4 Data analysis

Data were analysed using PASW software for Windows and results were depicted graphically with the aid of GraphPad Prism software. All data were tested for normality using Shapiro-Wilk test and for equality of variance using Levene's test. Parametric data are expressed as means \pm SEM and non-parametric data as medians and interquartile ranges. Parametric data were analysed using student's unpaired two-tailed t-tests or two-way repeated measures

analysis of variance (ANOVA). Student-Newman-Keuls (SNK) tests were used to make post-hoc comparisons, as appropriate. Where possible, non-parametric data were square-root or log transformed and analysed similarly to parametric data. If non-parametric data could not be transformed, they were analysed using Friedman's two-way ANOVA by ranks, followed by Mann-Whitney U or Wilcoxon signed-ranks tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Validation of air-puff passive-avoidance paradigm

Step-through latency during the acquisition trial did not differ between the experimental groups (Fig. 1). Saline-treated rats exposed to air-puff exhibited a significant passive-avoidance response, measured as a significant increase in the step-through latency in the retention trial compared with the acquisition trial (Fig. 1). There was no increase in step-through latency in saline-treated rats not exposed to air-puff, indicating that the avoidance response was a specific consequence of prior exposure to air-puff. Although scopolamine-treated rats also exhibited an increased step-through latency during the retention trial relative to the acquisition trial, the magnitude of the increase was less than that observed in saline-treated rats. Thus, scopolamine treatment resulted in a dose-related, partial attenuation of the passive-avoidance response, with a significant effect at the 3 mg/kg dose (Fig. 1).

3.2 Mechanical and thermal hypersensitivity in the SNL and/or CFA models of chronic pain

SNL and CFA rats developed mechanical and cold allodynia and thermal hyperalgesia as evidenced by the ipsilateral hind-paw responses to mechanical and thermal stimuli (Fig. 2, 3,

and 4). SNL (Fig. 2a) and CFA (Fig 2b) rats displayed a significant decrease in the 50% response threshold compared with sham or control rats, respectively, at all post-injury time points. In the Hargreaves' test, SNL (Fig. 3a) and CFA (Fig. 3b) rats showed a decrease in withdrawal latency in the ipsilateral hind-paw in response to a noxious thermal stimulus on days 8 and 16 post-injury compared with sham or control groups, indicating the expression of thermal hyperalgesia. Cold allodynia, expressed as an increase in the number of withdrawals following application of acetone to the ipsilateral hind-paw, was also evident in SNL (Fig. 4a) and CFA (Fig. 4b) rats. The number of responses increased in the SNL and CFA groups compared with the sham and control groups on days 4 and 12 post-injury.

3.3 Passive-avoidance in SNL and CFA models of chronic pain

Exposure to air-puff induced a passive-avoidance response in SNL, CFA, sham and control groups, as shown by significant increases in the step-through latency in the retention trial compared with the acquisition trial (Fig. 5a and 5b). The groups not exposed to air-puff did not demonstrate passive avoidance (data not shown). No between-group differences were observed in the step-through latency in the retention trial, or in the relative change from acquisition for either pain model and its respective control (Fig. 5a and 5b). Thus, aversive learning and memory, assessed with this air-puff paradigm, were intact and unaltered in the SNL model of neuropathic pain and in the CFA model of inflammatory pain.

4. Discussion

These results demonstrate that a single brief air-puff exposure is capable of producing a robust passive-avoidance response in rats in a simple context-conditioning paradigm and that this response is partially attenuated by pre-acquisition administration of the muscarinic receptor antagonist scopolamine, at a dose of 3 mg/kg. The present findings also show that this validated air-puff paradigm successfully produces a passive-avoidance response in two different models of chronic pain: SNL, which models chronic neuropathic pain, and intraplantar injection of CFA, which models chronic inflammatory pain. Allodynia and hyperalgesia, confirmed behaviourally, indicated that SNL surgery and intraplantar CFA injection successfully resulted in neuropathic and inflammatory pain-like phenotypes, respectively. However, passive-avoidance responding was not altered in either pain model with respect to controls, suggesting that aversive learning and memory remained intact in both models.

There is a substantial body of evidence suggesting that chronic pain is associated with cognitive impairment in human patients (Eccleston and Crombez, 1999; Hart et al., 2000; Moriarty et al., 2011; Povedano et al., 2007). As such, valid disease models of chronic pain should mirror this clinical phenomenon. A small number of studies have attempted to model pain-related cognitive impairment pre-clinically. Deficits in attention have been demonstrated in models of tonic persistent (formalin injection) and chronic (2,4,6-trinitrobenzene-induced colitis and CFA) inflammatory pain in rats (Boyette-Davis et al., 2008; Millecamps et al., 2004). Inflammatory pain, induced by intra-articular kaolin and carrageenan into the knee joint (Ji et al., 2010) or CFA injection into the hind paw tibiotarsal joint (Pais-Vieira et al., 2009b) was also shown to affect the performance of rats in a gambling task which assessed emotional decision making. In these studies increased pain responding was associated with a preference for a “high-risk” lever associated with larger but more infrequent rewards than the

To our knowledge, only one published study has investigated passive avoidance aversive learning and memory in an animal model of chronic pain. Suzuki et al. (2007) tested performance in a foot-shock-induced passive-avoidance paradigm in nerve-injured mice. The paradigm used a step-through light/dark arena and the aversive stimulus consisted of a 0.2 mA electric shock. This study revealed that nerve injury was not associated with a deficit in aversive learning and memory under those conditions. However, as discussed in section 1, in circumstances where there is pre-existing hypersensitivity of the paws to noxious stimulation (as occurs in many animal models of chronic neuropathic and inflammatory pain), interpretation of a foot-shock-induced passive-avoidance response in the context of learning and memory is complicated and potentially confounded, as any change (or lack of change) in passive-avoidance responding could be a consequence of paw hypersensitivity to foot-shock rather than being due to alterations in cognitive processing. The present study demonstrates that air-puff directed at the head/face can be used successfully as an alternative to foot-shock to produce a passive-avoidance response and avoid this potential confound, allowing for a more objective and ethically acceptable analysis of passive-avoidance responding in rodent

models of chronic pain. In our validation experiment, retention trial step-through latency in the air-puff saline group was 264.7s (119.5 – 300s) compared with 9.34s (8.01 – 11.01s) in the acquisition trial (data expressed as median \pm interquartile ranges). Notably, the magnitude of this avoidance response was similar to that observed in similar one-trial paradigms which used foot-shock to induce passive avoidance (Graham and Buccafusco, 2001; Holloway and Wansley, 1973). As with foot-shock (Elrod and Buccafusco, 1988), air-puff-induced passive avoidance was sensitive to the pre-acquisition administration of the muscarinic receptor antagonist scopolamine, indicating a learning-mediated phenomenon. Our results suggest that aversive learning and memory assessed in our air-puff passive-avoidance paradigm were not affected in either the inflammatory (CFA) or neuropathic (SNL) pain models under the conditions and at the time-point described. This result supports the findings of Suzuki et al. (2007) and other related research which has shown that learned helplessness in the forced-swim test and active avoidance in the place-escape/avoidance paradigm can be acquired in rat models of chronic pain (Goncalves et al., 2008; Hu et al., 2009; Leite-Almeida et al., 2009; Suzuki et al., 2007). These findings relating to aversive learning and memory are somewhat surprising given the evidence, discussed above, that chronic pain is associated with impairment in other cognitive domains, both clinically and preclinically. It is important to note that cognitive testing was performed at only one time-point relative to spinal nerve ligation surgery/CFA injection. The mechanism underlying cognitive impairment in chronic pain has yet to be elucidated, but it has been hypothesised that pain-related alterations in brain structure and plasticity may in turn affect cognition (Moriarty et al., 2011). A study by Seminowicz et al. (2009) found anatomical alterations in brain regions associated with cognition following peripheral nerve injury in rats, but these alterations were not observed until several months post-surgery. Therefore, although we observed robust, consistent expression of chronic pain-like behaviours at the post-surgery time-point selected for passive-

avoidance testing, it is possible that cognitive impairment, as assessed using this paradigm, may not have developed by this time-point. However, the time-point selected in the present study (days 18-20 post-SNL or post-CFA) was based on previous studies that have demonstrated pain-related alterations in both emotional (Suzuki et al., 2007) and cognitive (Pais-Vieira et al., 2009a) behaviours within a similar timeframe relative to injury. A recent study by Leite-Almeida et al. (2009) has indicated that age is another important factor in expression of pain-related cognitive impairment, with mid-aged (9 month-old) rats, but not young (3 month-old) or old (22 month-old) rats, showing an increased susceptibility to impaired performance in a Morris Water maze reversal task. This impairment may be due to a variety of age-related alterations in neural plasticity, neurotransmitter and neuromodulator levels, structural changes in the brain, and neuroendocrine alterations. Although aversive learning and memory were not investigated by Leite-Almeida and colleagues, it is possible that pain-related deficits in these may also be detectable in mid-aged rats, despite failure to detect any deficits in the young adult rats (6-9 weeks old on arrival) used in the present study. However, it is also possible, given the results of the present study and the findings of Suzuki et al. (2007), that aversive memory is simply unaffected in rodent models of chronic pain, or that passive-avoidance responding is not a reliable predictor of pain-related cognitive-impairment. The learning and memory mechanisms affected by chronic pain may be distinct from those affected by scopolamine, and thus more specific behavioural paradigms may be required to detect cognitive deficits associated with chronic pain.

The use of air-puff to induce avoidance responding and to evaluate cognition has been described previously (Cimadevilla et al., 2001; Karlsson et al., 2009; Koistinaho et al., 2001). However, the apparatus and test protocols differ from those described in the present study. Karlsson et al. (2009) tested cognition in a multivariate concentric square-field paradigm,

which was also used to investigate locomotor activity, exploration, risk assessment, risk-taking and security-seeking. Air-puff was administered when rats entered a defined zone within the test apparatus. The memory retention trial was carried out 2 weeks later and the percentage of rats returning to the air-puff-associated zone was recorded. The paradigm used by Cimadevilla et al. (2001) and Koistinaho et al. (2001) tested both active and passive avoidance in mice. The apparatus consisted of a circular arena, where entry into a segment of the arena was punished by an air-puff stimulus. In the passive-avoidance trial, mice spent less time in the punished segment of the arena, indicating memory of the aversive air-puff stimulus. In the active-avoidance test, the arena was rotated such that animals were required to avoid the air-puff-associated segment not only by not entering it, but also by not allowing themselves to be transported into it. This part of the test also involved a spatial component as subjects had to learn the location of the punished segment according to extra-maze cues. Passive and active avoidance were tested over a period of 4 days. The air-puff passive-avoidance paradigm described in the present study is relatively simple, and can be carried out over a shorter period of time, in comparison with those described above. Thus it lends itself to high-throughput cognitive screening of passive-avoidance behaviour. In addition, our paradigm is more similar to commonly used foot-shock paradigms, with the principal modification being the replacement of foot-shock with air-puff, allowing for a more direct comparison of results across paradigms.

A noteworthy feature of the passive-avoidance paradigm presented here is its ability to assess aversive learning and memory without directly activating peripheral nociceptive pathways and this may be of particular utility in certain circumstances. Clinical and preclinical studies have demonstrated that altered sensitivity to noxious stimuli, or increased incidence and severity of chronic pain, is associated with a large number of disorders of the central nervous

system, including depression (Boettger et al., 2010; Dworkin et al., 1995; Klauenberg et al., 2008; Shi et al., 2010a; Shi et al., 2010b; Terhaar et al., 2010), anxiety disorders (Asmundson and Katz, 2009; Finn et al., 2006; Geuze et al., 2007; Ploghaus et al., 2001; Rivat et al., 2010), multiple sclerosis (Kenner et al., 2007; Olechowski et al., 2009; Solaro et al., 2003) and others. Thus, a passive-avoidance paradigm using air-puff in place of noxious foot-shock may also be preferable for assessment of aversive learning and memory in rodent models of these disorders. In addition, many pharmacological agents, including of course analgesic drugs, may alter threshold to noxious stimuli. Therefore, passive-avoidance testing using an air-puff paradigm may yield a more accurate indication of the effect of such drugs on aversive learning and memory than testing using a foot-shock paradigm.

In conclusion, air-puff can be used to produce a reliable, robust passive-avoidance response in a context-conditioned aversive learning paradigm. Aversive learning and memory, as tested using the air-puff passive-avoidance paradigm, appear to be intact in models of chronic inflammatory and neuropathic pain, as step-through latency was not altered in SNL or CFA models compared with their respective controls. The paradigm developed here may be useful in future studies aimed at assessing aversive learning and memory in rodent models of disease where pain processing or sensitivity to noxious stimuli is altered.

Acknowledgements

The authors are grateful for technical assistance from Mr. Ambrose O' Halloran, Mr. Daniel Kerr and Mr. Mark Webber. This work was supported financially through the National Biophotonics and Imaging Platform, Ireland, and funded by the Irish Higher Education Authority's Programme for Research in Third Level Institutions Cycle 4 (National Development Plan 2007-2013).

Figure Legends

Figure 1: Demonstration of air-puff-induced passive-avoidance and pharmacological validation using scopolamine. Friedman's test revealed a significant overall effect ($P < 0.001$, $\chi^2 = 32.225$). Wilcoxon tests showed that air-puff produced a significant passive-avoidance response in the air-puff saline and in the air-puff scopolamine 3mg/kg group ($*P < 0.05$ retention vs. acquisition), and a trend for a passive-avoidance response in the air-puff scopolamine 1mg/kg group ($P = 0.09$). There was no passive-avoidance response in the no-air-puff saline group and Mann-Whitney U tests revealed that the latency in the retention trial was significantly greater in the air-puff saline group than in the no-air-puff saline group ($+P = 0.001$). The retention trial step-through latency in the scopolamine 3mg/kg group was significantly shorter than that of the air-puff saline group ($\#P < 0.01$, Mann-Whitney U test). $n = 8/\text{group}$.

Figure 2: (a) SNL-operated rats expressed mechanical allodynia: Friedman's test revealed a significant overall effect ($P < 0.001$, $\chi^2 = 48.379$). (b) CFA-treated rats expressed mechanical allodynia: Friedman's test revealed a significant overall effect ($P < 0.001$, $\chi^2 = 97.808$). Mann-Whitney U tests revealed that thresholds were significantly lower in the injured groups than in the respective control groups at all post-SNL/CFA time points ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). $n = 9-12/\text{group}$.

Figure 3: (a) SNL-operated rats expressed thermal hyperalgesia: Two-way repeated measures ANOVA revealed a significant main effect of surgery group ($P < 0.001$, $F_{(1,21)} = 19.960$). (b) CFA-treated rats expressed thermal hyperalgesia: Two-way repeated measures ANOVA revealed a significant main effect injection group ($P < 0.001$, $F_{(1,16)} = 58.241$). SNK post-hoc tests revealed that injured groups had a shorter response latency than their respective control groups at all post-SNL/CFA time points ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). $n = 9-12/\text{group}$.

Figure 4: (a) SNL-operated rats expressed cold allodynia: Two-way repeated measures ANOVA of square-root-transformed data revealed significant main effect of surgery group ($P=0.001$, $F_{(1,21)}=13.616$). (b) CFA-treated rats expressed cold allodynia: Two-Way Repeated Measures ANOVA revealed significant main effect of injection group ($P<0.01$, $F_{(1,16)}=12.592$). SNK post-hocs revealed a significantly greater number of responses in the injured groups at all post-SNL/CFA time-points compared with their respective control groups (** $P<0.01$). $n=9-12$ /group.

Figure 5: (a) SNL and sham rats exhibited a passive-avoidance response following air-puff but aversive memory was not affected by SNL surgery: Friedman's test showed a significant overall main effect ($P<0.01$, $\chi^2=12.536$). Wilcoxon tests showed that air-puff produced a significant passive-avoidance response in both sham and SNL groups ($*P<0.05$), but there was no difference in responding between sham and SNL groups. (b) CFA and control rats exhibited a passive-avoidance response following air-puff but aversive memory was not affected by CFA treatment: Two-way repeated measures ANOVA of log-transformed data revealed a significant main effect of time ($P<0.001$, $F_{(1,16)}=33.927$). SNK post-hoc tests showed that air-puff produced a significant passive-avoidance effect in the CFA and control groups (** $P<0.01$, *** $P<0.001$), but there was no difference in responding between the CFA and control groups. $n=6-9$ /group.

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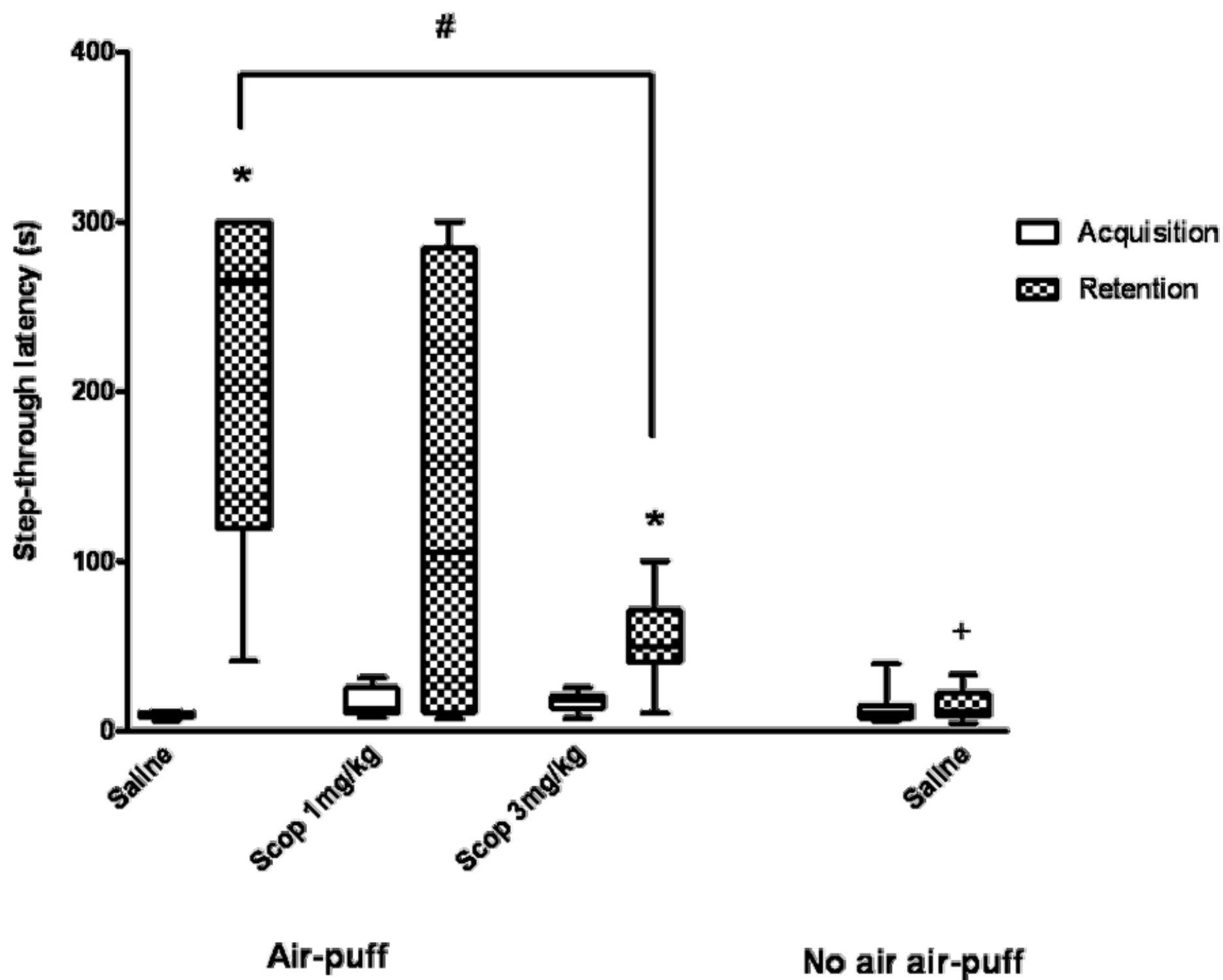
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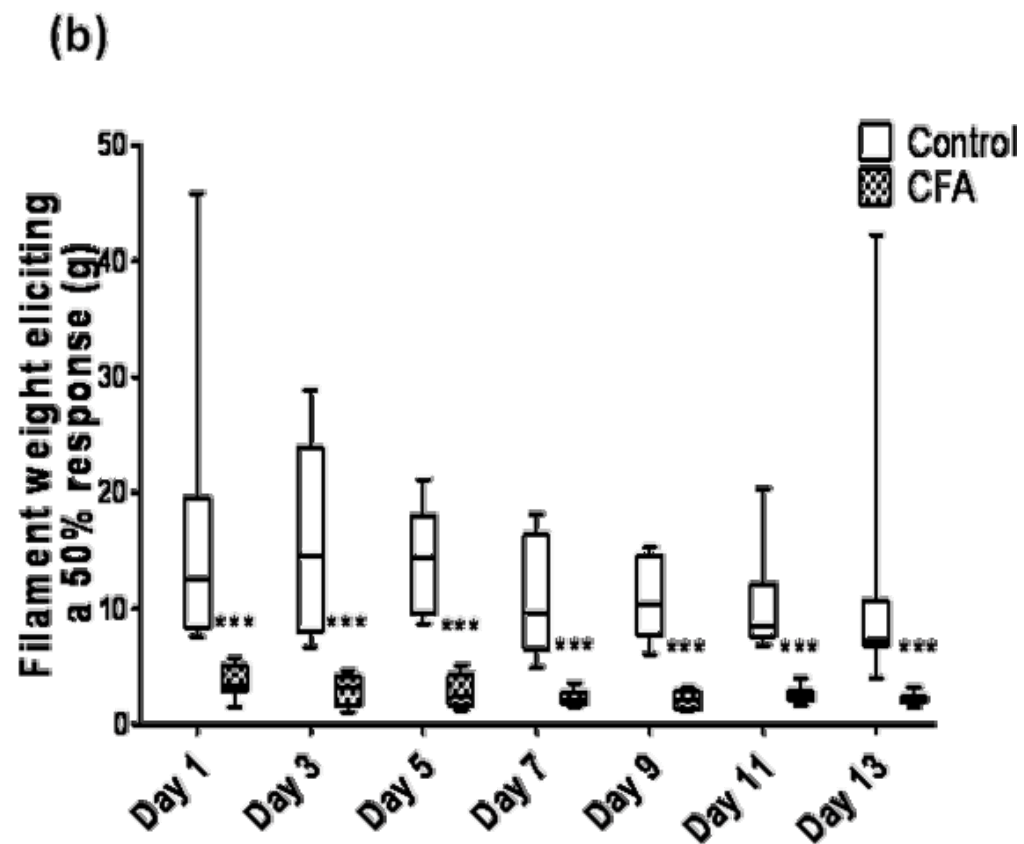
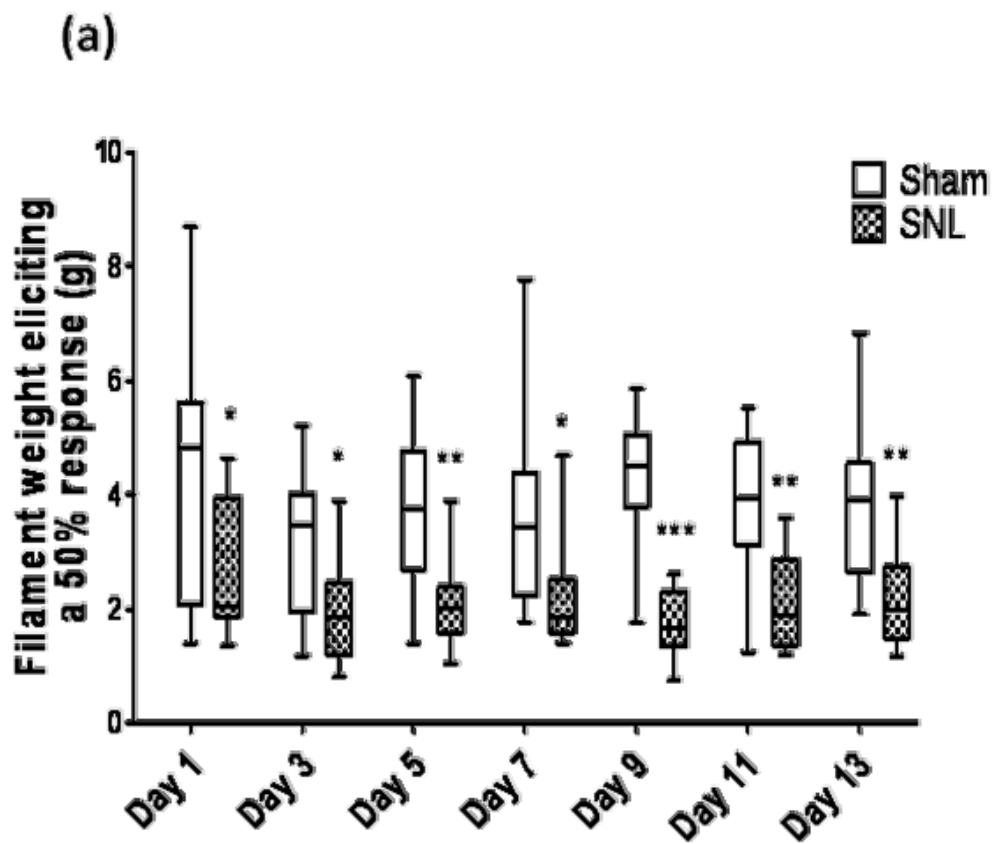
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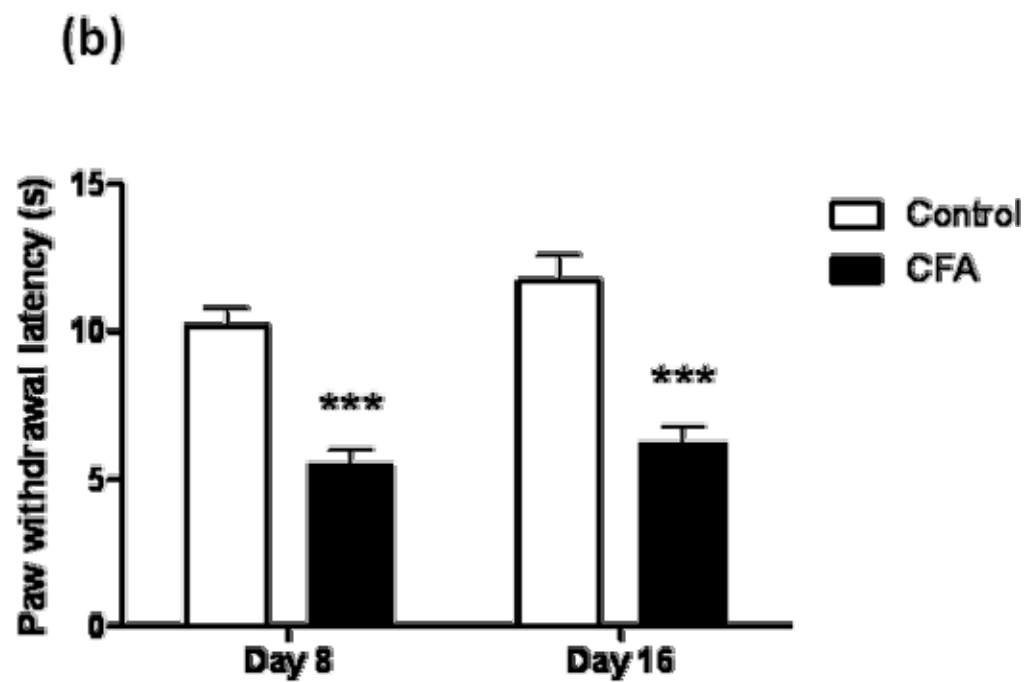
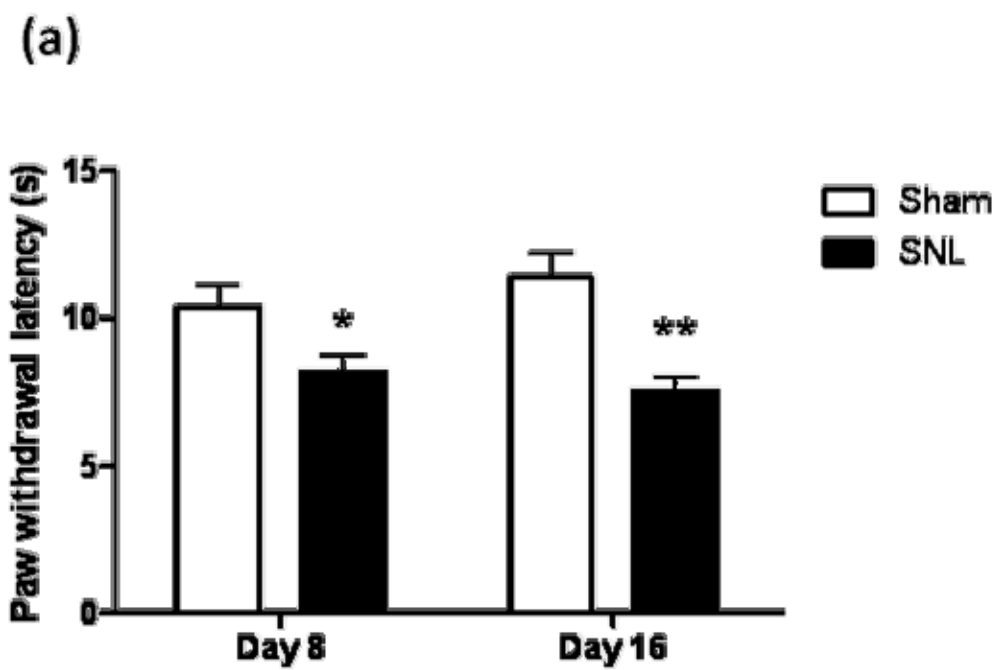
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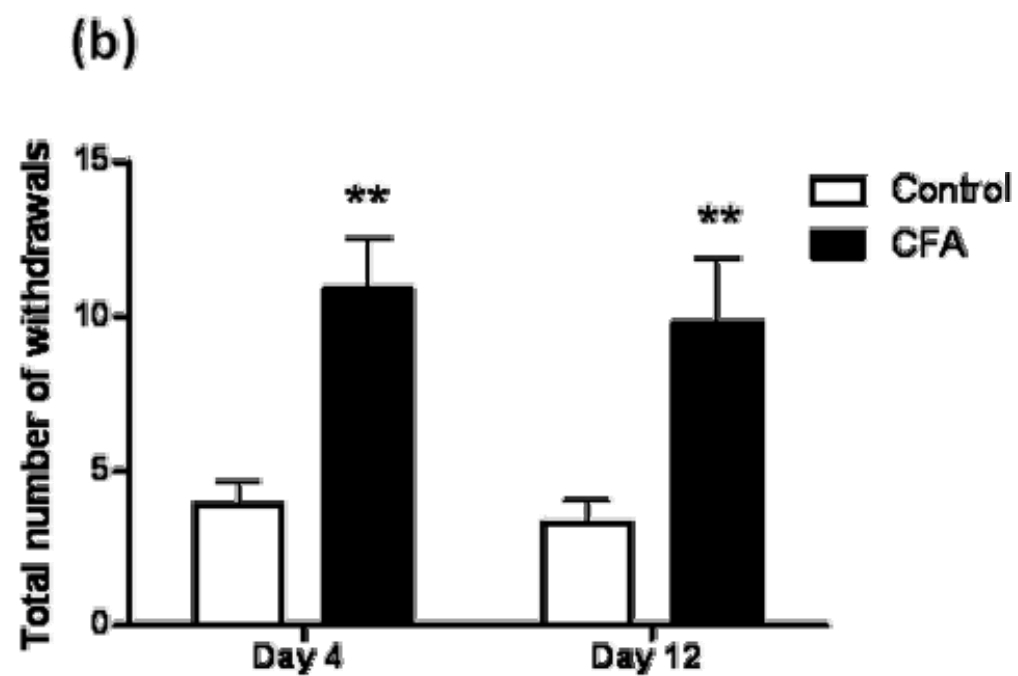
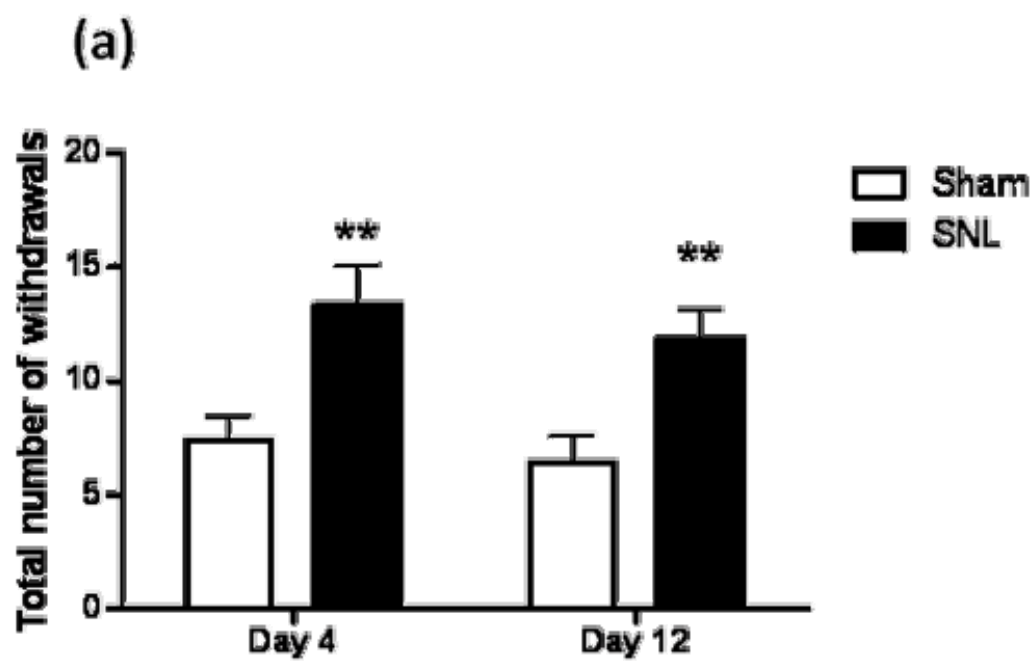
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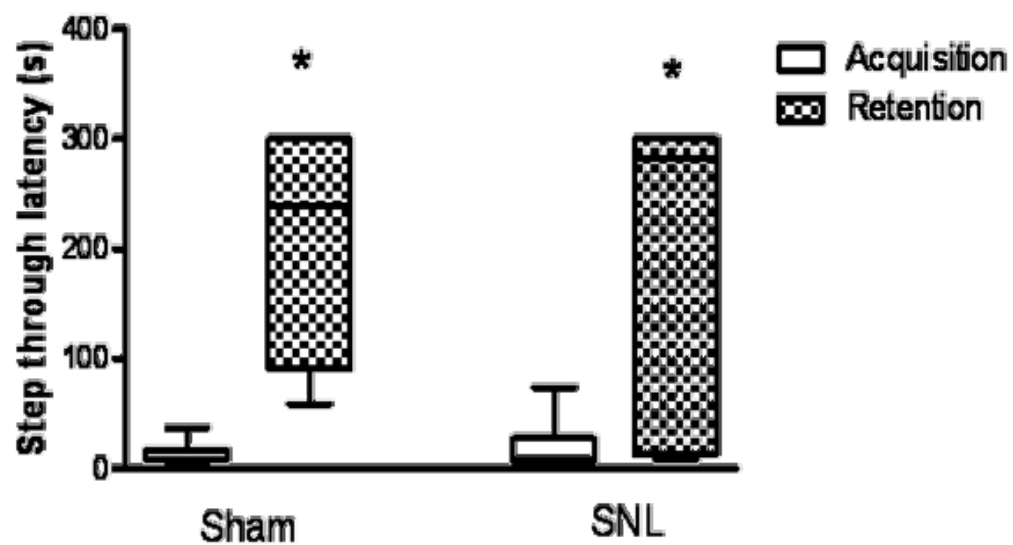








(a)



(b)

