Evaluation of Animal Behavior: Problems and Pitfalls

focus on pain research

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Animal behavior
vs.
Behavioral laboratory work

Behavior (ETHOLOGY) is the science that studies animal behavior: communication, social structure, aggression, activity patterns, mating, etc ...

Ethology is based on the observation, interpretation and quantification of **spontaneous** behavior, commonly in **natural** conditions, without **human intervention**.

Most of the laboratory work in neuroscience is not ethology

Animal behavior
vs.
Behavioral laboratory work

Behavior in laboratory work is usually something very different: we use **not spontaneous** behavioral responses as measurable indicators for parameters that are noncommunicable otherwise (ex: memory, anxiety, vision, pain ...).

We cannot forget what - ultimately - what is evaluated experimentally, are always MOTOR RESPONSES

We do not observe the mental states of ***memory, fear, pain…***

We only observe the motor responses that we use to quantify ***memory, fear, pain***.

Animal behavior
vs.
Behavioral laboratory work

The goal of this class is to alert to the problems that ALWAYS exist when we use **motor responses** to evaluate **something more complex**.

We have to ask ourselves:

Is the motor response of this test **adequate** to evaluate what I want to study (ex: memory, fear, pain)?

Is the motor response **affected** only by what we want to evaluate (ex: memory, fear, pain)?

Take-Home Messages

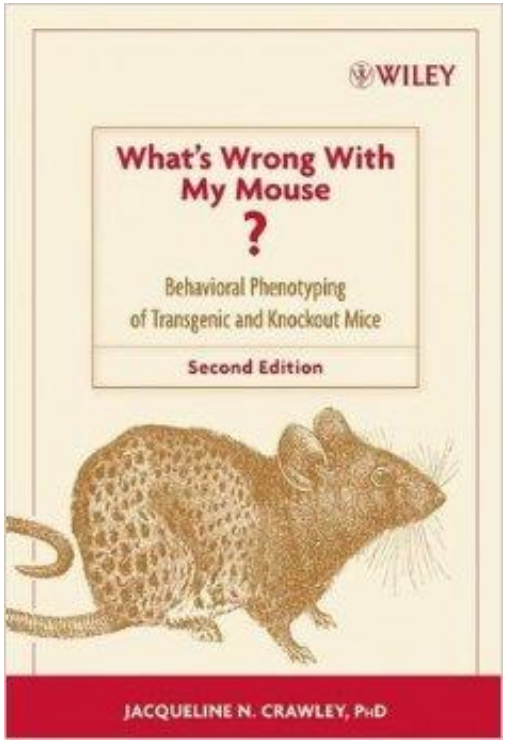
Repeat, Repeat, Repeat

Observe, Observe, Observe

Question, Question, Question

Recommended reading for those starting studies based in animal models

Addresses laboratory protocols, principles of ethology and even veterinary care for animal welfare



Problems and Pitfalls in Behavioral Experiments

cautions in the planning, execution and interpretation of behavioral experiments

Caution # 1

Observe the behavior of EACH animal:
locomotion, attention, excitement ...

Are the GROUP changes
also observed at the INDIVIDUAL level?

Be on the lookout for
FALSE-POSITIVE or FALSE-NEGATIVE
effects of the experimental protocol...

The problem of false positives

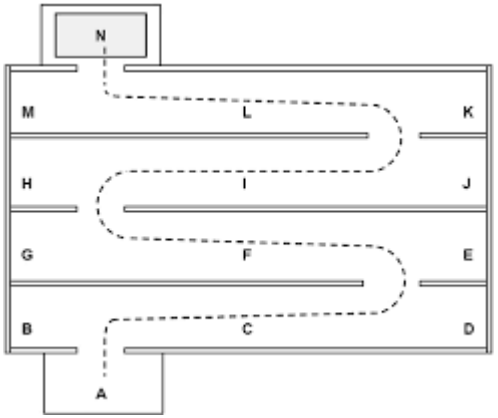
The most common type of false positive is an incorrect **attribution** of an observed behavioral change.

"if all you have is an hammer, everything looks like a nail"

For example, interpreting change in task performance as a change in motivation or in stress, when in fact it is only a change in motor behaviour…

The problem of false positives

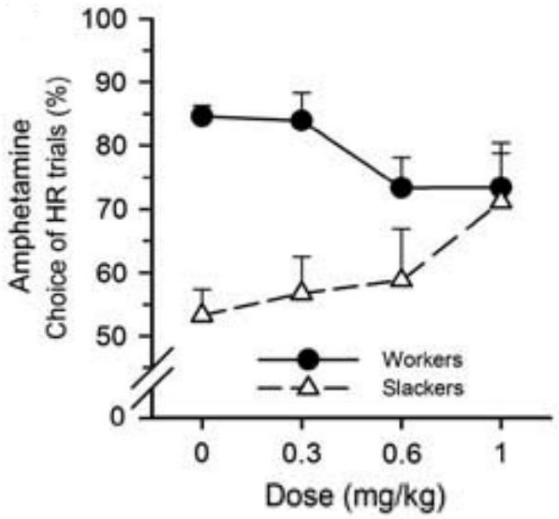
A good example of incorrect interpretation of results is Karl Lashley's conclusion that navigation memories are distributed in the cortex; it is now known that in over-trained animals the cortical memories are transfered to non-cortical areas.



Another exemple is that the hippocampus is crucial for fear conditioning, but it is irrelevant to other types of conditioning such as the eye-blink that is dependent on the cerebellum.

False negatives and group variability

Is each animal strain homogenous, or there may be variability within a group of similar animals?



Variability within a supposedly homogeneous group can hide real differences between groups of individuals

Cocker et al (2012) Neuropsychopharmacology

Caution # 2

Uncontrolled communication
between animals,
may change their behavioral responses

Effect of Direct Communication

Do animals exchange learning information?

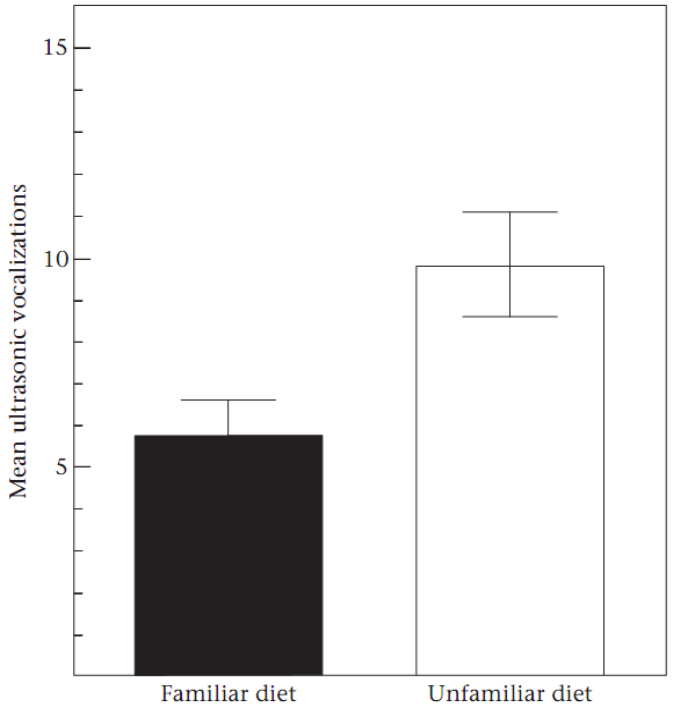


Figure 1. Mean number (±SE) of short (20–80 ms) 50-kHz ultrasonic vocalizations produced by pairs of reunited Norway rats when one member of a pair had eaten an unfamiliar food or both pair members had eaten the same familiar food while pair members were separated.

Pairs of two animals in the same cage were kept separate and individually exposed to two types of food: the usual (normal diet) or a novel diet based on corn starch or milk protein.

During the three minutes immediately following their meeting in the usual cage, the type of **ultrasonic vocalizations** made by the two animals **coded** the type of diet they had been exposed to.

Galef & Jeimy (2004) Animal Behav

Effect of Direct Communication

Do animals exchange learning information?

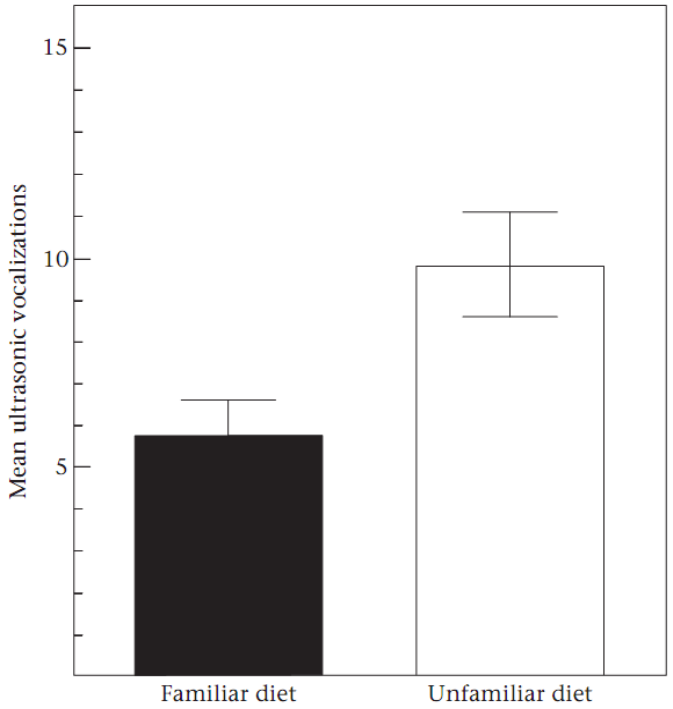


Figure 1. Mean number (±SE) of short (20–80 ms) 50-kHz ultrasonic vocalizations produced by pairs of reunited Norway rats when one member of a pair had eaten an unfamiliar food or both pair members had eaten the same familiar food while pair members were separated.

Adult mice/rats produce two types of vocalizations:

- **Long ultrasonic vocalizations: low frequency (0.3-3 sec, 22 kHz)**

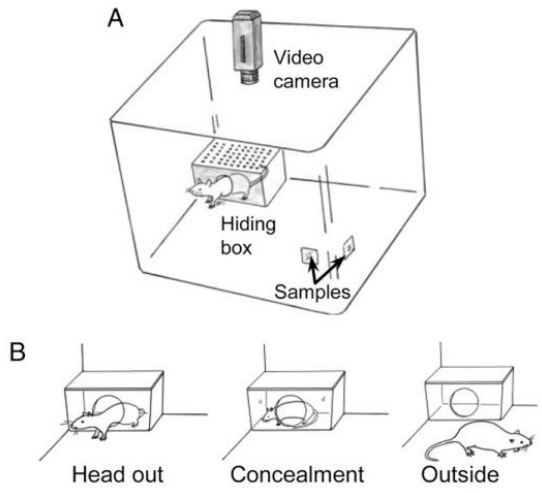
- **Short ultrasonic vocalizations: high frequency (20-80 ms, 50 kHz)**

Slow vocalizations are commonly associated with negative experiences, while short vocalizations typically signal positive experiences and social interaction.

Galef & Jeimy (2004) Animal Behav

Effect of Indirect Communication

Is there indirect transmission of anxiety among animals?



The exposure to samples of volatiles taken from the air during noxious stimulation of an animal, affects the exploratory behavior of a second animal.

Inagaki et al (2014) PNAS

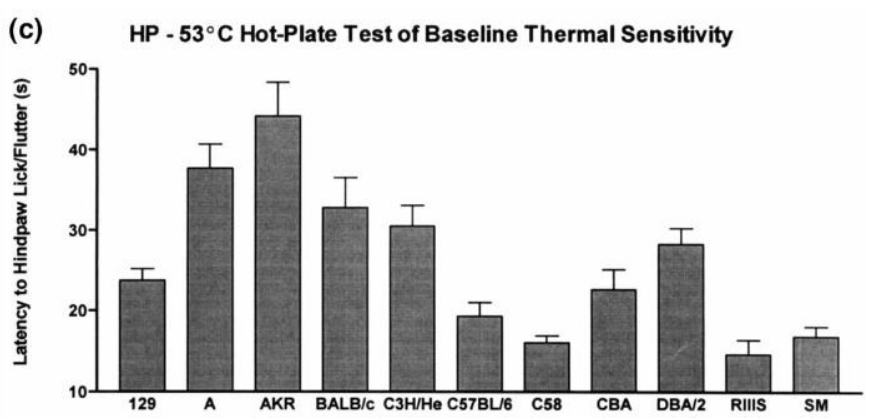
Caution # 3

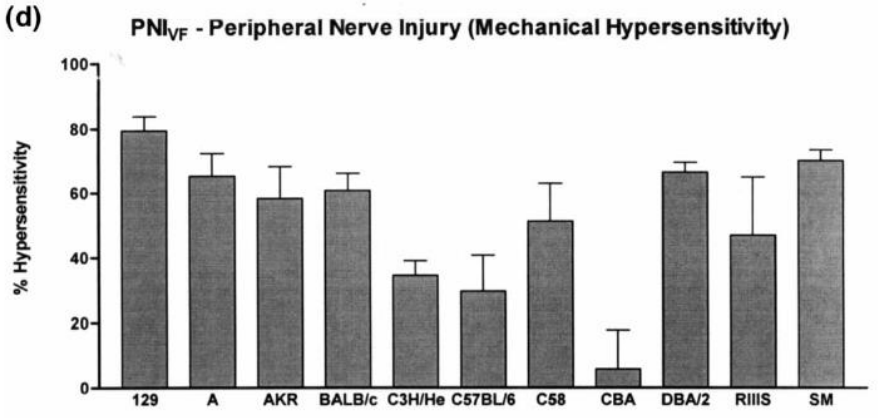
Test the reproducibility of the results

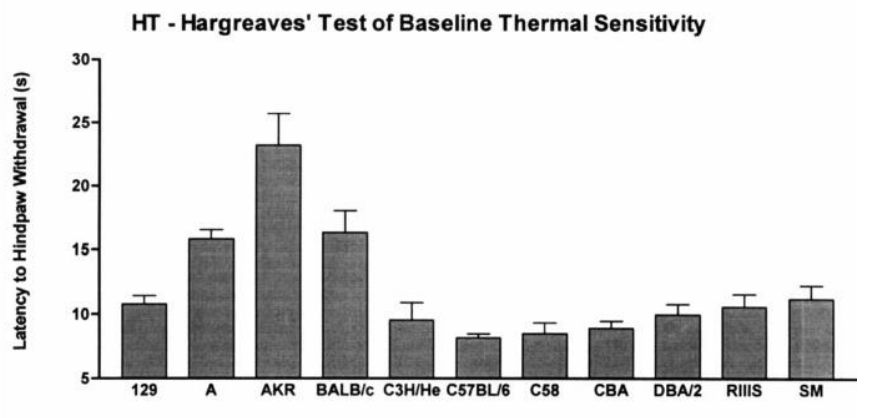
repeat tests under various conditions,
with various groups of animals,
with various experimenters.

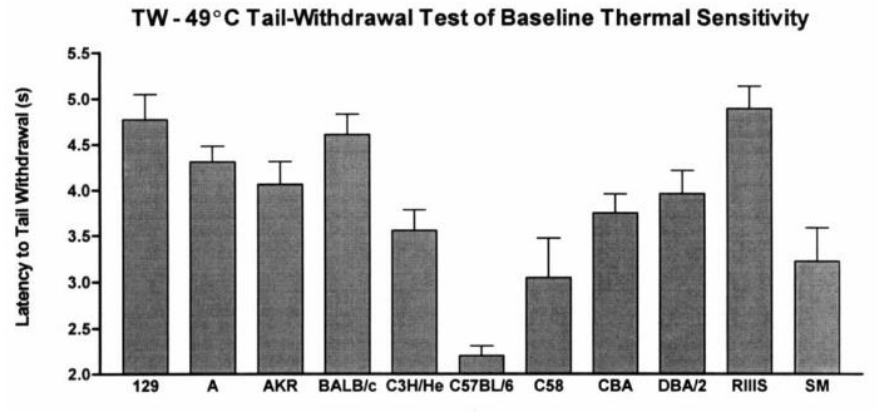
Effects of Genotype

Analysis of 11 mouse strains in pain response tests









Mogil et al (1999) Pain

Effects of Genotype

How different genotypes may explain pain testing variability?

Characteristics of peripheral nerve transmission?

Characteristics of central nervous transmission?

Differences in anxiety?

Cognitive differences: learning, memory, attention?

Differences in the organization of the social hierarchy?

Effect of Animal Behavioral State

"Behavioral State" is the overall level of awareness

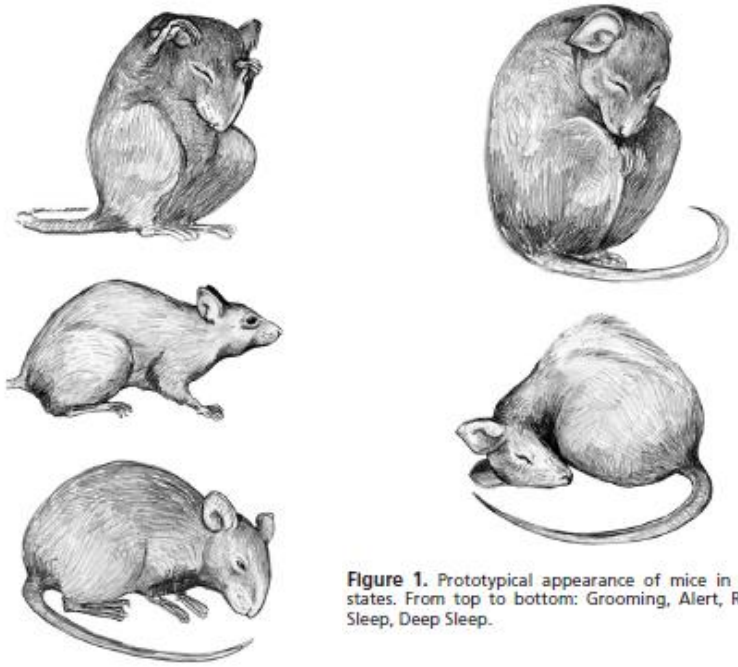


Figure 1. Prototypical appearance of mice in 5 behavioral states. From top to bottom: Grooming, Alert, Resting, Light Sleep, Deep Sleep.

Table 1. Percentage Agreement and Confidence of Behavioral State Coding Among Observers

Behavioral State	*% Agreement**	*Confidence†*
Deep sleep	94.3	4.7 ± 0.1
Light sleep	88.0	4.1 ± 0.1
Resting	72.5	4.0 ± 0.2
Alert	81.5	4.3 ± 0.2
Grooming	93.3	4.9 ± 0.04
Locomotion/exploring	95.6	4.7 ± 0.1

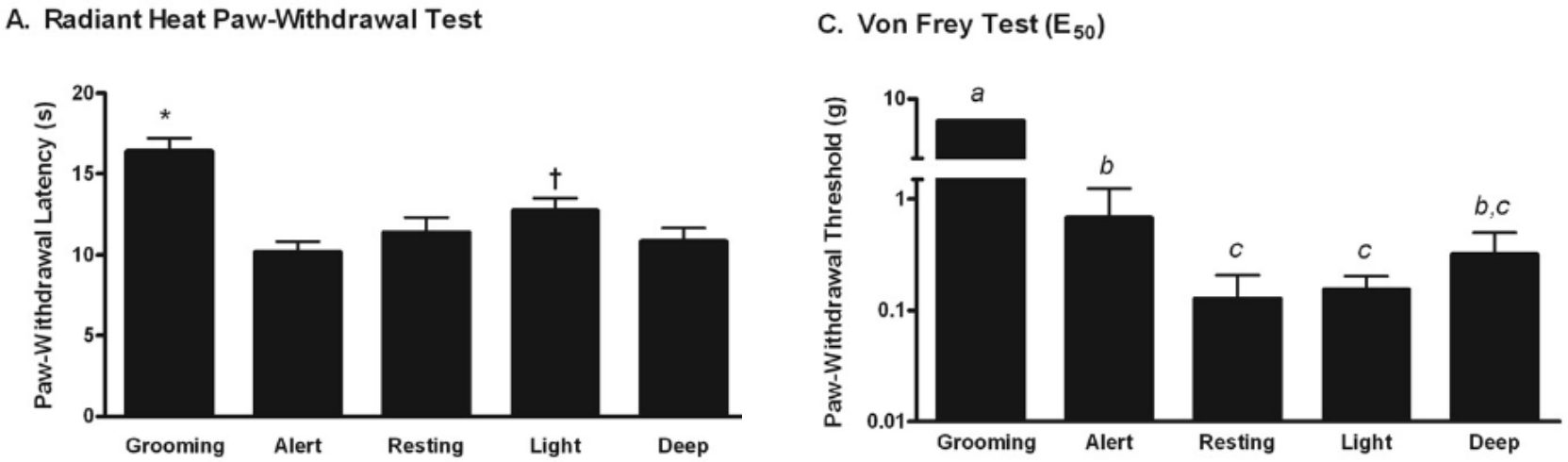
*For each measurement (n = 5) on each mouse (n = 12), the percentage agreement among the 5 observers was calculated; 100% = all in agreement; 80% = 4 of 5 in agreement; 60% = 3 of 5 in agreement. In only 1 case, was there less than 3 of 5 in agreement.

†Mean (±SEM) confidence of the coded behavioral state, on a scale from 1 (no confidence) to 5 (full confidence).

Callahan, Gil, Levesque & Mogil (2008) Journal of Pain

Effect of Animal Behavioral State

Does it affect pain scores?



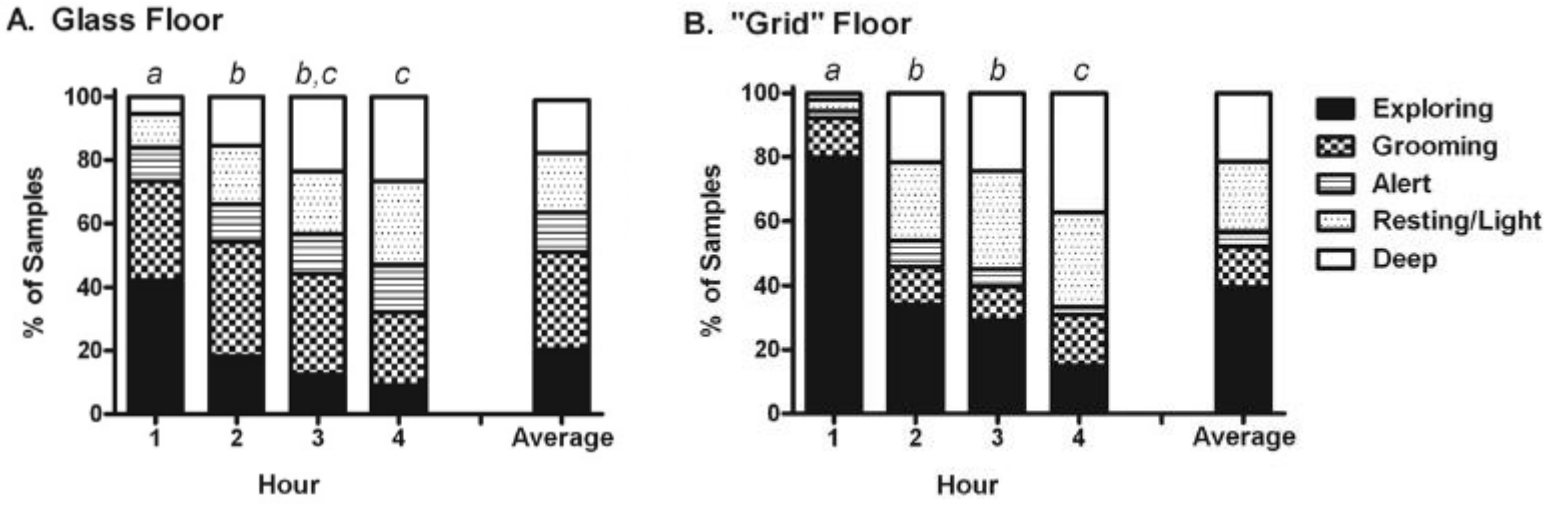
Grooming has a strong analgesic effect ("painkiller")

Is *grooming* analgesic because of a neurobiological mechanism (calming effect that releases endorphins, tactile stimulation of sensory fibers, etc), or simply because it alters the latency of motor response?

Callahan, Gil, Levesque & Mogil (2008) Journal of Pain

Effect of Animal Behavioral State

Laboratory conditions affect behavioral state?



Testing conditions alter the behavioral state of the animal (grooming is reduced while the animal is in a cage with metal grid floors).

Callahan, Gil, Levesque & Mogil (2008) Journal of Pain

Experimental variability

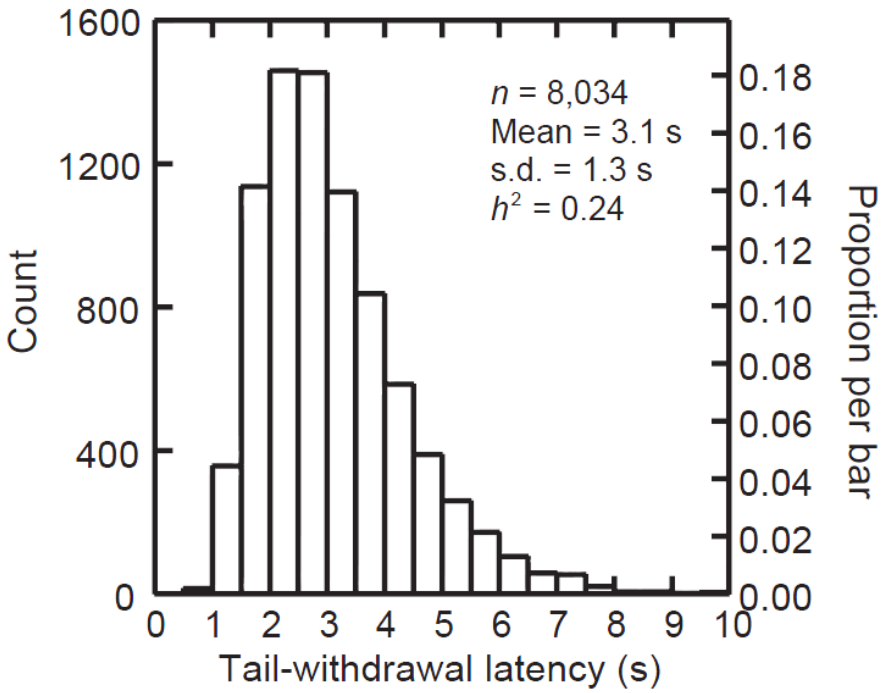


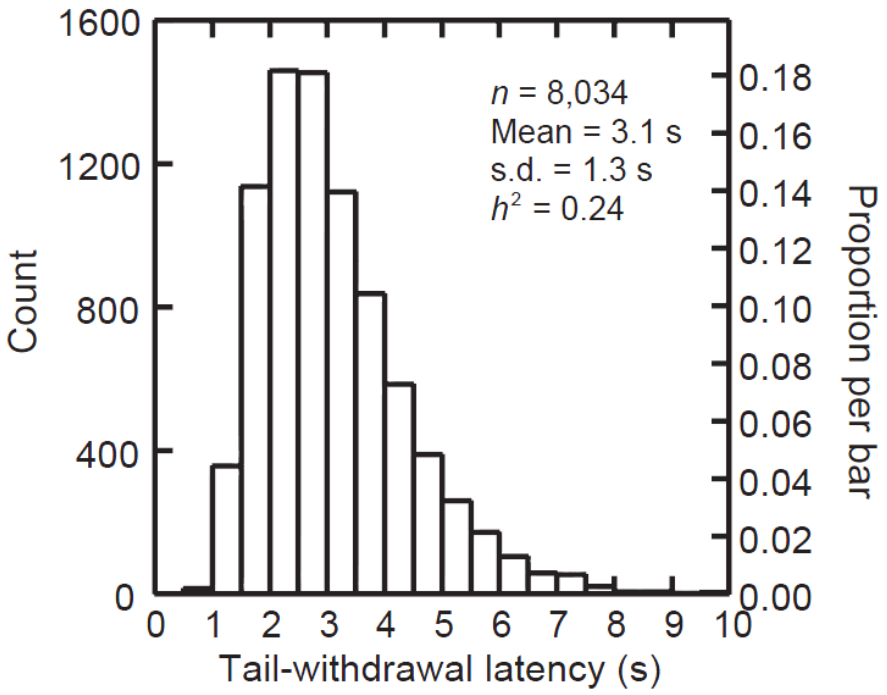
Fig. 1. Frequency histogram of responses on the 49°C tail-withdrawal assay of 8,034 mice tested from 1993 to 2001. Mice were individually removed from their home cage and introduced to a cloth/cardboard 'pocket', which they freely entered. Thus lightly restrained, the distal half of its tail was immersed in water thermostatically controlled at 49 ± 0.2°C. Latency to a vigorous, reflexive tail withdrawal was measured to the nearest 0.1 s with a handheld stopwatch.

Jeffrey Mogil decided to compile data from 8034 thermal nociceptive stimulation tests conducted in the laboratory over 8 years.

He observed a large variability of results (more than 25% of the animals had latency times that were very distant from the average)

Chesler (...) & Mogil (2002) Nature Neuroscience

Experimental variability



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He tested if that experimental variability was correlated with uncontrolled factors of the experimental protocol:

- Hour of testing time
- Day of the year
- Investigator
- Humidity in the room
- Number animals / box
- Order test
- Sex of animals
- Genotype of animals

Chesler (...) & Mogil (2002) Nature Neuroscience

Experimental variability

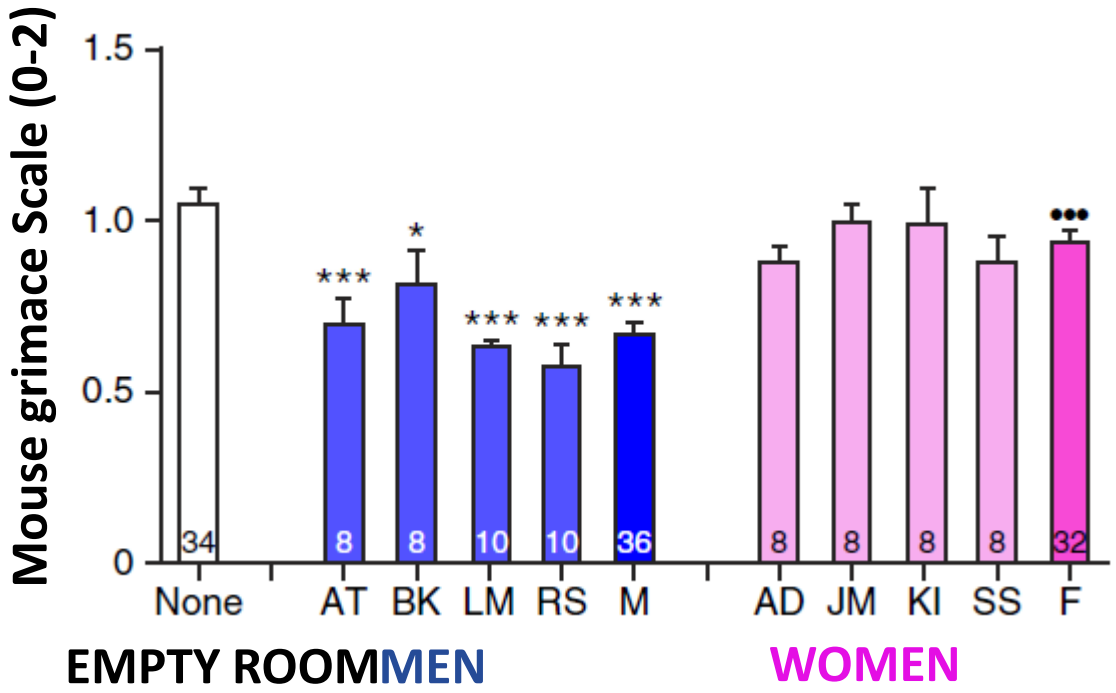
Table 1. Factor importance rankings computed by CART.

Factor[a]	Number of factor levels	Score[b]
Experimenter	11	100.0
Genotype	40	78.0
Season	4	35.8
Cage density	7	20.4
Time of day	3[c]	17.4
Sex	2	14.6
Humidity	4[d]	12.0
Order of testing	7	8.7

The effect **"researcher"** is a larger cause of experimental variability than the genotype or the sex of the animals (even among researchers from the same laboratory trained by the same persons).

Chesler (...) & Mogil (2002) Nature Neuroscience

Researcher Gender

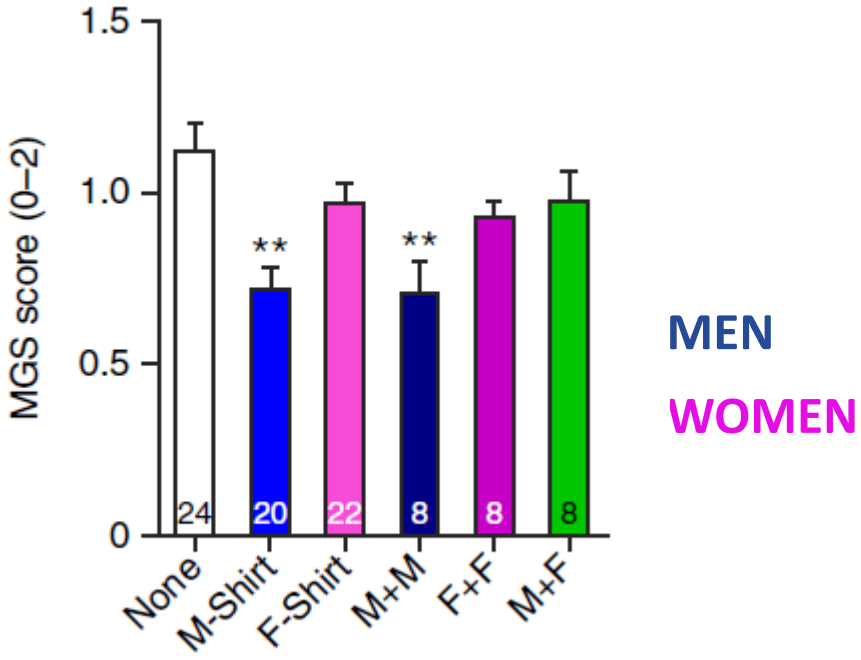


The presence of MALE researchers within half meter from the animal cage, alters pain response after injection of Zymozan

("analgesic" effect is rated by facial expression - Mouse Grimace Scale) in male and female mice.

Sorge..., Mogil (2014) Nature Methods

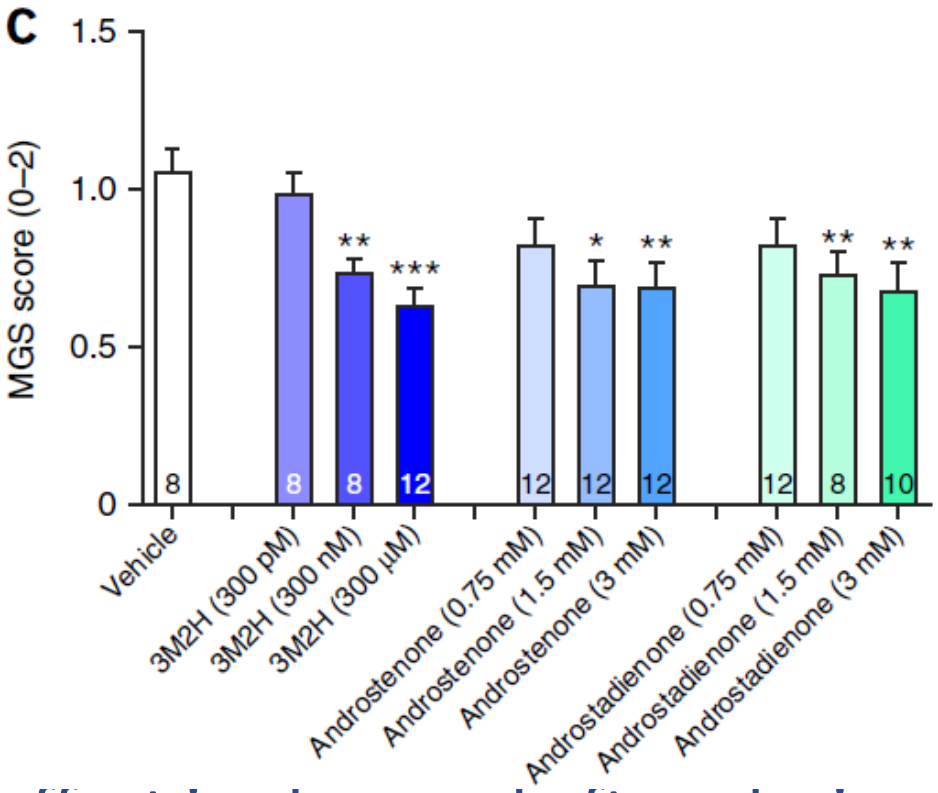
Researcher Gender



The introduction of a previously used t-shirt by a male researcher, placed half a meter from the testing cage, reduces pain response ("analgesic" effect).

Sorge..., Mogil (2014) Nature Methods

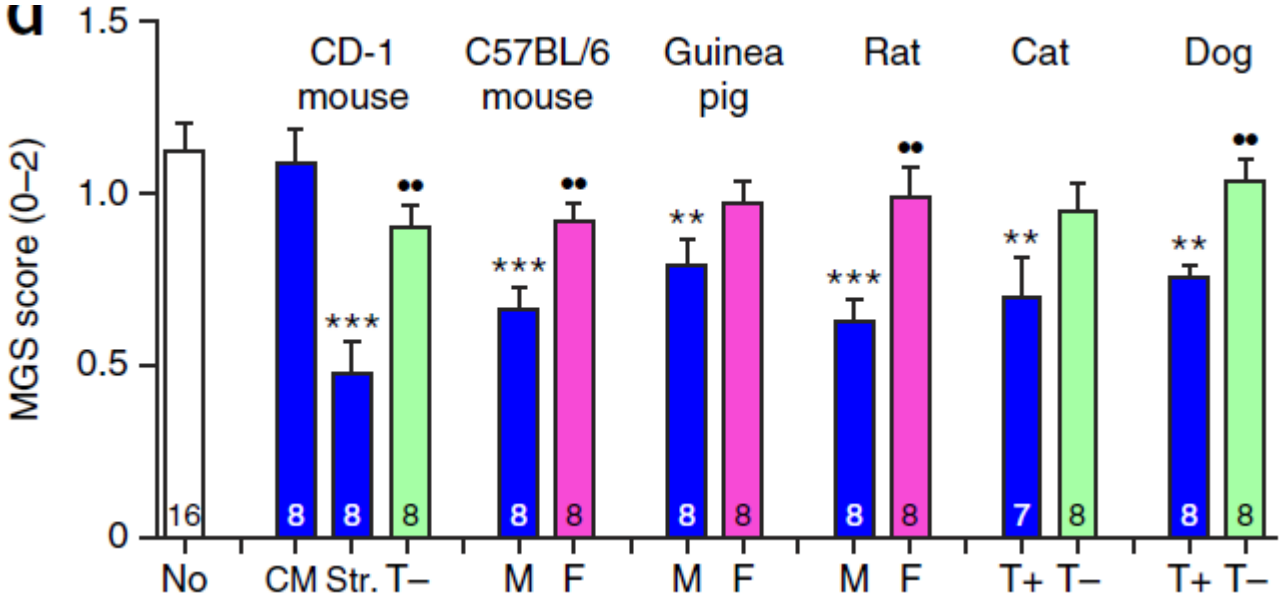
Researcher Gender



The same effect is observed after placing sponges impregnated with male volatile compounds.

Sorge..., Mogil (2014) Nature Methods

Researcher Gender

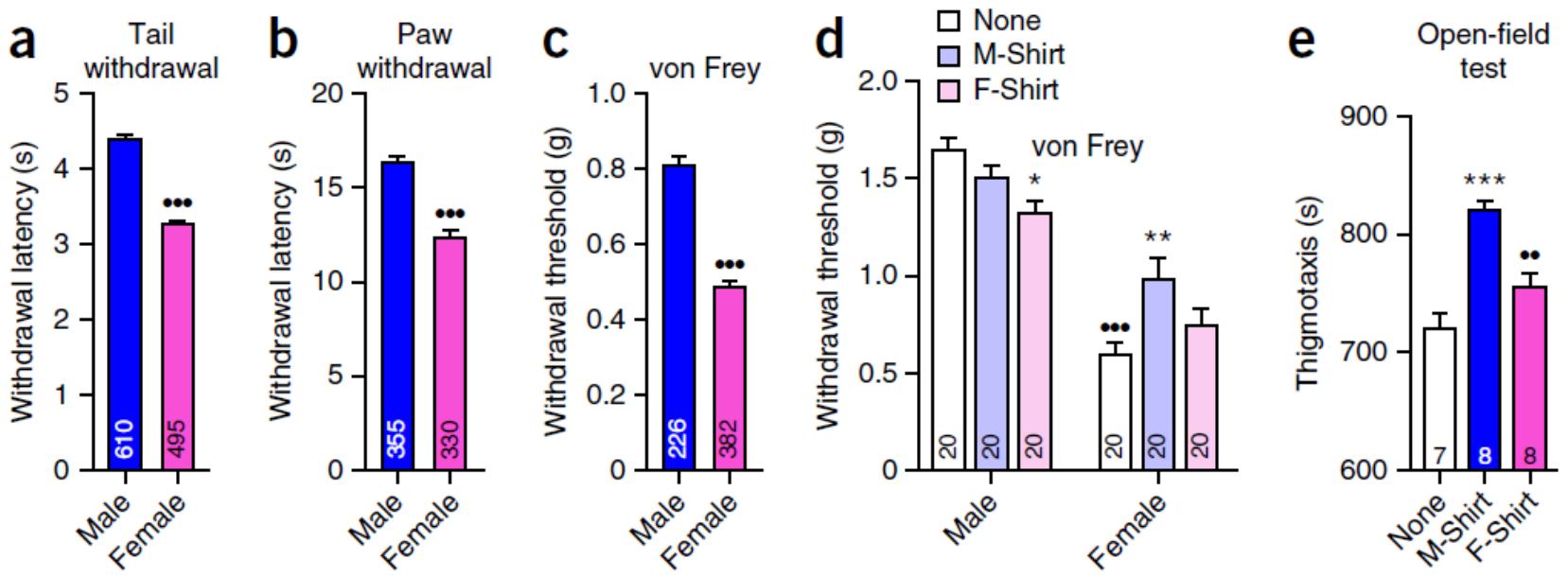


... or after placing "*bedding*" exposed to other males, either mice or other species.

"*Bedding*" exposed to castrated animals (T) or "cagemates"(CM), did not affect the pain response.

Sorge..., Mogil (2014) Nature Methods

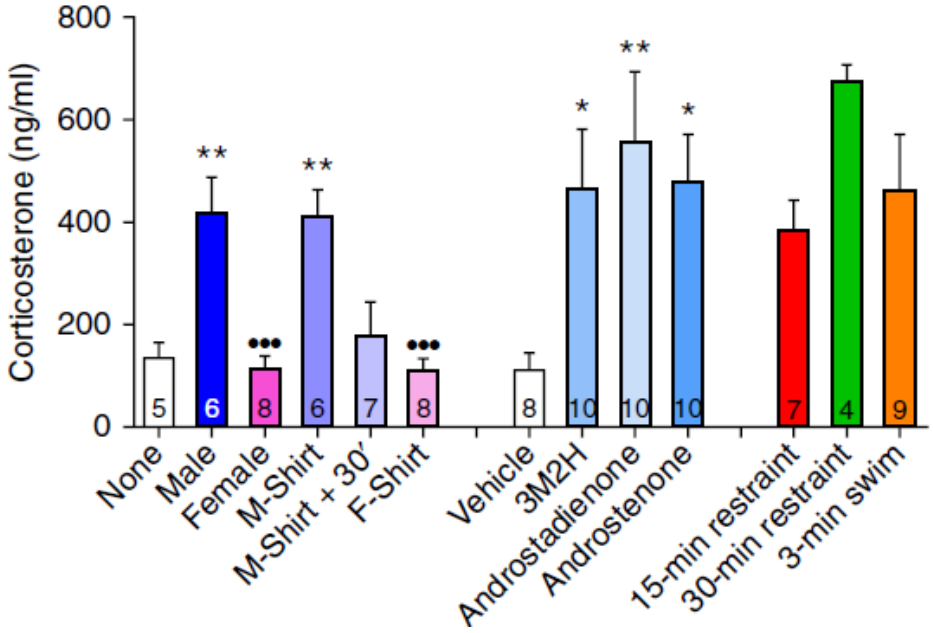
Researcher Gender

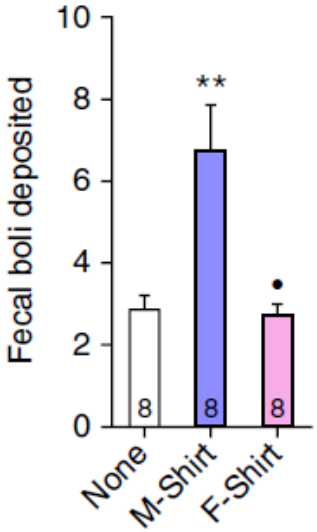


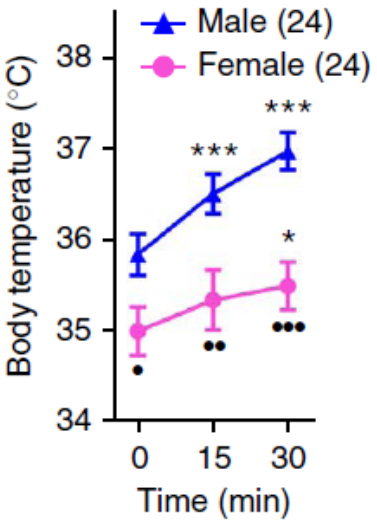
This "analgesic" effect is observed in several tests of nociception.

Sorge..., Mogil (2014) Nature Methods

Effect on stress indicators







This "analgesic" effect is accompanied by an increase in stress indicators.

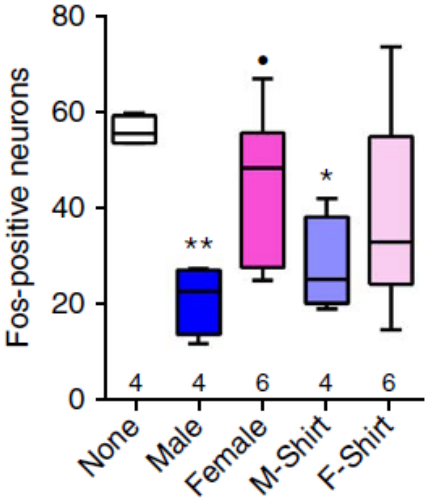
Sorge..., Mogil (2014) Nature Methods

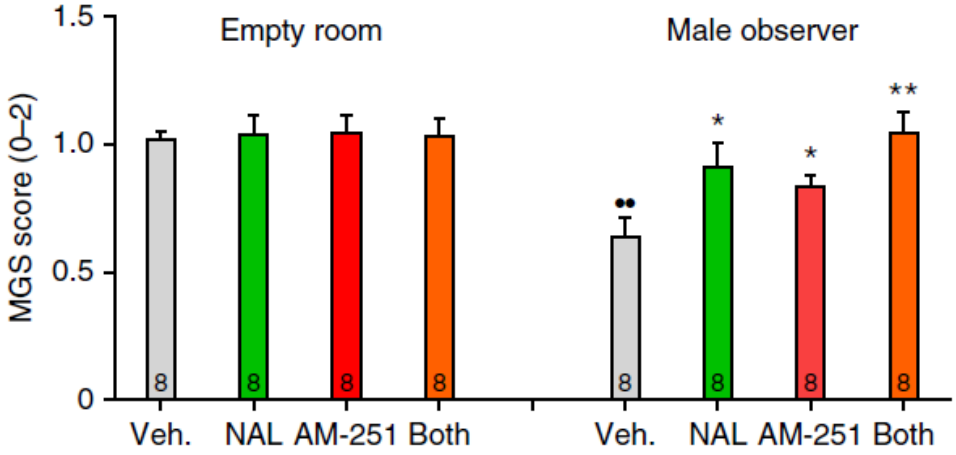
What is the effect of stress in pain testing?

We have seen that stress immobilizes the animals ...
Is the analgesic effect of stress
just an experimental artifact:
decreased motor response / divided attention?

or is there a modulation of the nociceptive system during periods of stress?

Stress Induced Analgesia





The "analgesic" effect also affects the expression of c-fos spinal cord and is reversed by administration of blockers of opioid receptors (naloxone) and CB1 receptors (AM-251).

Sorge..., Mogil (2014) Nature Methods

On the other hand there is also *Stress Induced Hyperalgesia*

Table 1
Summary of commonly used animal models of stress-induced hyperalgesia.

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Forced swim-stress				
Male Sprague-Dawley rats	Day 1: 10 min	Formalin test and hot plate test	Inflammatory and thermal hyperalgesia	Quintero et al. (2011, 2003, 2000), Suarez-Roca et al. (2008), and Suarez-Roca et al. (2006a,b)
	Day 2–3: 20 min	Carrageenan intra-muscular injection followed by grip strength	Mechanical hyperalgesia as determined by reduced grip strength	Suarez-Roca et al. (2006a)
Male Swiss albino mice	Two swim stress sessions 6 min duration, 8 h apart	Hot plate	Thermal hyperalgesia	Suaudeau and Costentin (2000)
Male Wistar rats	5 min sessions daily for 5 days	Tail flick test	Thermal hyperalgesia	Fereidoni et al. (2007)
Adult male and female Swiss Webster mice	5, 15 or 30 min or 15 min for 2 days	Tail flick test and grip strength	Thermal and mechanical hyperalgesia	Abdelhamid et al. (2013a,b)
Male albino mice	6 min sessions daily for 15 days	Tail immersion test	Thermal hyperalgesia	Dhir and Kulkarni (2008)
Repeated cold stress/SART				
Male 4-week old ddY mice	Over 7 days: Alternating 24 °C/4 °C every hour for 7 h; 4 °C for final 17 h	Randall–Selitto apparatus	Mechanical hyperalgesia (days 5–7)	Ohara et al. (1991)
Male Sprague-Dawley rats	Over 5 days: Alternating 24 °C/4 °C or −3 °C every 30 min for 7½ h; 4 °C/−3 °C for final 16½ h	Randall–Selitto test and the von Frey hair test	Mechanical hyperalgesia (greater in −3 °C group than 4 °C group)	Nasu et al. (2010)
Male Wistar Rats	Over 5 days: Alternating 24 °C/−3 °C every hour for 4 h; −3 °C for remaining 20 h	Randall–Selitto test	Mechanical hyperalgesia	Fujisawa et al. (2008)
Male Wistar rats	Over 5 days: Alternating 24 °C/−3 °C every 2 h for 6 h; −3 °C for remaining 18 h	Footshock on one of two floors	Decreased escape latency	Kawanishi et al. (1997)
Restraint stress				
Male and female (mixed estrous phases) Wistar rats	Daily 1 h restraint for 40 days	Tail flick test	Thermal hyperalgesia in males, no effect in females	Gamaro et al. (1998)
Male Wistar rats	Acute: 15 min, 30 min or 1 h restraint Subchronic: 1 h restraint for 3 days Chronic: 1 h daily, 5 days per week for 40 days	Formalin injection into the temporomandibular joint (TMJ)	Increased inflammatory pain in chronic restraint stress rats	Gameiro et al. (2006)
Male Sprague-Dawley rats	Daily 1 h restraint for 4 days over for 5 weeks	von Frey, Randall–Selitto, tail immersion test, acetone-induced cold allodynia, formalin test	Inflammatory, thermal and mechanical hyperalgesia	Bardin et al. (2009)
Adult male Wistar rats	1 h daily restraints for 5 days per week over 8 weeks	Tail flick test[a] Formalin injection into TMJ[b]	Thermal and inflammatory hyperalgesia	da Silva Torres et al. (2003)[a] and Gameiro et al. (2005)[b]
Male Sprague-Dawley rats	6 h restraint once or over 1, 2 or 3 weeks	Tail flick test	Thermal hyperalgesia following 2 and 3 week restraint	Imbe et al. (2004)
Male Sprague[a] Dawley rats Male and female Wistar rats[b]	Acute restraint for 2 h in restraint cage	Colorectal distension	Visceral hyperalgesia	Ohashi-Doi et al. (2010)[a] and Eutamene et al. (2010)[b]
Male Sprague Dawley rats	2 h restraint stress 4 days	Colorectal distension	Visceral hyperalgesia	Shen et al. (2010)
Male Wistar rats	1 h restraint 5 days a week for 11 weeks	von Frey Test and hot plate	Mechanical allodynia and thermal hyperalgesia	Spezia Adachi et al. (2012)
Immobilisation stress				
Adult male Sprague Dawley rats	90 min daily for 7 days	Tail-Flick test	Thermal hyperalgesia	Costa et al. (2005)
Male ICR mice	1 h daily for 5 days	Formalin test	Inflammatory hyperalgesia	Seo et al. (2006)
Social defeat				
Male Sprague-Dawley rats (Long Evans rats as intruder)	Four daily intruder sessions divided into two periods (see above)	von Frey, Randall–Selitto test and formalin test	Mechanical and inflammatory hyperalgesia	Rivat et al. (2010)
Male Long Evans rats	Resident rats were vasectomised prior to testing. Five daily intruder sessions divided into two periods (see above)	Formalin test[a], thermal preference and thermal escape test[b]	Inflammatory and thermal hyperalgesia	Andre et al. (2005)[a] and Marcinkiewcz et al. (2006)[b]
Water avoidance				
Male Wistar rats	1 h per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	Bradesi et al. (2006, 2007, 2009, 2005), Larauche et al. (2008) and Wang et al. (2013)
Male Sprague-Dawley rats	1 h per day for 10 consecutive days	von Frey test[a], colorectal distension[b]	Mechanical and visceral hyperalgesia	Chen et al. (2011)[a] and Green et al. (2011a,b)[b]
Adult male C57Bl/6 mice	1 h per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	Hong et al. (2009) and Larauche et al. (2010)

Table 1 (Continued)

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Male Sprague-Dawley rats	Over three days: tones played over four frequencies over 30 min in time period	Randall Selitto test[a], colorectal distension[b]	Mechanical and visceral hyperalgesia	Khasar et al. (2009, 2005)[a] and Green et al. (2011a,b)[b]
Chronic mild stress				
Male Wistar rats	Unpredictable Chronic stress for 6 weeks;	von Frey and hot plate in normal and complete Freund's adjuvant chronic pain rat model and formalin test	Increased mechanical and thermal thresholds and inflammatory hyperalgesia	Shi et al. (2010a)
Male Wistar rats	Unpredictable chronic stress for 6 weeks	Hot plate and von Frey tests in naive and SNL rats	Increased thermal and inflammatory pain thresholds for both normal and SNL rats	Shi et al. (2010b)
Rotation stress				
Male CBA/J mice	Rotational movement in spinning cages at 45 rpm for 10 min every hour daily for 2 weeks	Formalin test	Inflammatory hyperalgesia	Boccalon et al. (2006)
Maternal separation/Deprivation/Early life stress				
Wistar male rats[a] Sprague Dawley[b] male rats	Pups separated from mother for 180 min from days 2 to 14	Colorectal distension	Visceral hyperalgesia	Chung et al. (2007a,b)[a] and Zhang et al. (2009a,b, 2008)[b]
long-Evans rats	Pups separated from mother for 180 min from days 2 to 14	Colorectal distension	Visceral hyperalgesia	van den Wijngaard et al. (2012), Wouters et al. (2012) and Welting et al. (2005)
Wistar male and female rats	24 h MD on PND 9	Hot Plate, von Frey, acetone test and prior to and after spinal nerve ligation	Thermal hypoalgesia, mechanical allodynia in females	Burke et al. (2013)
Sprague-Dawley rats	Mother and pups are placed in cages fitted with a stainless steel mesh bottom on post-natal day 2–9	Digital force transducer	Mechanical hyperalgesia	Alvarez et al. (2013) and Green et al. (2011a,b)
Noise stress				
Male Sprague-Dawley rats	105 dB tone of mixed frequencies, ranging from 11 to 19 kHz over 30 min over 3–4 days	Paw-withdrawal threshold	Enhanced inflammatory pain	Khasar et al. (2009, 2005)
Vibration stress				
Male and female Wistar rats	4 Hz applied to restraint tube for 5 min	Tail flick test	Hyperalgesia developed at 2–10 min after stress in male rats. Thermal hyperalgesia and female responding was oestrus dependent	Devall et al. (2009) and Devall and Lovick (2010)
Whisker pad stimulation				
Male Sprague-Dawley rats	Light tactile whisker pad stimulation: 10 applications/session, 4 sessions/h in 1 day, sessions on days 1–5 and 8–12	von Frey test	Mechanical hyperalgesia	Reynolds et al. (2011)
Air stress				
Male Sprague-Dawley rats	Continuous stream of air at room temperature was directed at the face for 30 min	von Frey test	Mechanical hyperalgesia	Wagner et al. (2013)
PTSD model				
Male Sprague-Dawley rats	2 h restraint, 20 min swim followed by 15 min rest, inhalation of an ether until unconscious	von Frey test and paw withdrawal to heat stimulus	Mechanical allodyina and thermal hyperalgesia	Zhang et al. (2012)
Male Sprague-Dawley rats	2 h restraint, 20 min swim followed by 15 min rest, inhalation of an ether until unconscious then footshock when conscious	Colorectal distension	Visceral hyperalgesia	He et al. (2013)

Jennings, Okine, Roche & Finn (2014) Progress in Neurobiology

Afterall, is stress hypoalgesic or hyperalgesic?

What is currently accepted is that situations of **acute and unpredictable stress** have an analgesic effect on nociceptive tests.

On the other hand, situations of **chronic and predictable stress** have an hyperalgesic effect in nociceptive tests.

This experimenter-related effect of stress also alters other behavioral tests?

Most certainly yes...

The effect of stress on cognitive and attentional performance is well documented, with sometimes opposite effects depending on the predictable or unpredictable nature of the stressor

Is there indirect transmission of anxiety in human experiments?

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Smelling Chemosensory Signals of Males in Anxious Versus Nonanxious Condition Increases State Anxiety of Female Subjects

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Is there indirect transmission of anxiety in human experiments?

Figure 1 Subject during anxiety odor donation session.

Figure 2 State anxiety scores during neutral and anxiety condition after 5 and 20 min of odor exposure (n = 20). State anxiety differed significantly between neutral and anxiety condition after 20 min but not after 5 min of odor exposure (*P < 0.05).

Albrecht et al (2011) Chem Senses

Take-Home Messages

Repeat, Repeat, Repeat

Observe, Observe, Observe

Question, Question, Question

Evaluation of Animal Behavior: Problems and Pitfalls

focus on pain research

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