Alternatives for Analgesiometric Tests in Animals: The Feasibility to Reduce Discomfort by Anaesthesia

by *Mathieu G. Sommers[1,*], Jan van Egmond[2], Jan G. Veening[3,4], Kris C. Vissers[2] & Merel Ritskes-Hoitinga[1]*

[1]Central Animal Laboratory; [2]Department of Anesthesiology, Pain and Palliative Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [3]Department of Anatomy; [4]Department of Psychopharmacology, UIPS, Utrecht University, The Netherlands

Summary

Animal pain and nociception studies have greatly contributed to our understanding of acute and chronic pain processing and thereby contributed to the reduction of suffering of patients in pain. In classic analgesiometric tests in conscious animals, animal suffering is inevitable as pain behaviour is the primary outcome. Therefore, the feasibility of refining analgesiometric tests by anaesthesia is reviewed. The influence on analgesiometric tests of different anaesthetics is described. Other objective primary outcome measures than pain behaviour, including quantification of neural activation with c-fos and functional MRI (fMRI), are suggested to reduce animal discomfort for pain testing. In conclusion, reflex analgesiometric tests may be refined by choosing the right anaesthetics and alternative outcome measures such as c-fos or fMRI. Complex, higher order pain behaviour testing still requires conscious animals and can currently not be refined by the use of anaesthetics.

Introduction

Although our understanding of pain and effective pain management has increased greatly over the last decade, many patients still suffer from unmanageable chronic pain and many patients still experience post-operative hyperalgesia and suboptimal pain control (Wilder-Smith & Arendt-Nielsen 2006). Animal pain studies have greatly contributed to our understanding of acute and chronic pain processing. They resulted in validated and reproducible analgesiometric animal tests for research and development of new analgesics for patients, but also were accompanied by suffering in a large number of animals in these tests. An optimal balance between scientific progress and animal welfare is of utmost importance.

Correspondence: Mathieu G. Sommers, DVM, PhD
P.O. Box 2040, 3000 CA Rotterdam,
The Netherlands
Tel: +31 (0)10-7038863
Fax: +31 (0)10-7044772
Email: M.Sommers@erasmusmc.nl

The acceptance of animal experiments by the public is strongly related to the degree of animal suffering. This acceptance has been the subject of several polls in European countries over the last decade (*MORI / Medical Research Council 1999;Intomart GfK / The Dutch Society for the Protection of Animals, 2004; MORI / Coalition for Medical Progress, 2002; MORI / Coalition for Medical Progress, 2005*).

A reduction in the degree of animal suffering in analgesiometric tests can be achieved by the use of anaesthesia as a refinement. The attained improvement in animal welfare by the use of anaesthesia would be welcomed by both researchers and the general public as long as the validity of the analgesiometric tests remains. Refinement is one of the three Rs (Replacement, Reduction and Refinement) criteria as first described in " The Principles of Humane Experimental Technique" (*Russell & Burch, 1959*). The pain research community has adapted these principles with the publication of the International Association for the Study of Pain (IASP) guidelines (*Zimmermann, 1983*).

Currently, analgesiometric tests frequently use pain related (reflex) behaviour as the primary outcome and therefore require conscious animals. The debate on optimal choice of behavioural pain tests has been broadened by Vierck et al. (*2008*), who encourage the use of complex behavioural tests for pain assessment. Within the context of the high attrition rate of drugs at the preclinical / clinical interface, the introduction of complex behavioural tests in the preclinical phase may improve the translational validity of preclinical phase tests. At the moment, however, analgesiometric reflex tests are still performed in large numbers of animals by pharmaceutical companies and the search for alternatives therefore is very relevant.

Analgesiometric tests with an optimal animal welfare profile would use unconscious animals and non-invasive techniques. Unconsciousness would prevent animals from experiencing pain. Non-invasive techniques would minimize discomfort in the animals after recovery from the analgesiometric tests. The possibility to reuse the same animals for different tests of different drugs would also greatly reduce the number of required animals.

Analgesiometric tests in unconscious animals with an optimal scientific profile would provide either the same (reflex) behaviour as under conscious conditions or a useful alternative measure for pain related behaviour. Relevant alternative objective measures could be EEG or fMRI (measurement of blood oxygenation changes in the brain by MRI), both non-invasive approaches, or by monitoring c-fos (a protein marker of neuronal activity monitored by immunohistochemical staining). For EEG, a review on refinement options, including the minimal anaesthesia model, has recently been published (*Murrell & Johnson, 2006*). Therefore, this review will focus only on c-fos and fMRI as alternative measures for (reflex) pain related behaviour.

The aim of this review is firstly to present an overview of the influence of anaesthesia on pain related reflex behaviour and on c-fos or functional MRI as alternative measures in analgesiometric tests. Secondly, the implications for animal welfare from the use of anaesthesia in analgesiometric tests will be described.

Analgesiometric tests overview
Analgesiometric tests each have their specific scientific and welfare characteristics (Table 1). Pain testing characteristics include the applied stimulus, the measured response and the applicability for different types of analgesics. Animal welfare characteristics include group size and level of discomfort.

Anaesthesia may influence the outcome of analgesiometric tests. When this influence is present, it is important to find out whether this influence improves or reduces the translational validity of the tests. In general, the translational validity to humans of analgesiometric tests during anaesthesia in animals is indicated by the extent to which analgesic dosages in the animal test correlate with the required dosages for analgesic effects in humans. Anaesthetic influence on pain models depends largely on the chosen outcome measure. Reflex behaviour like a withdrawal reflex will be less susceptible to anaesthetic influence and thus a possible target for refinement than higher order behaviour like aggression. When other response functions than behaviour are chosen as the outcome measure, anaesthetic influence again depends on the particular experimental setup. For example, neural activation as represented by spinal c-fos expression may still be present although pain behaviour has disappeared or *vice versa*. Therefore, different measures for analgesiometric tests will be discussed in relation to anaesthetic influence on testing results.

Anaesthesia effects on pain related behaviour and neural c-fos expression
Several inhalation anaesthetics have been used in published pain test studies, including halothane, isoflurane and sevoflurane. Halothane is a halogen substituted ethane and more or less comparable to chloroform, a halogen substituted methane, while isoflurane and sevoflurane are halogenated ethers. These different molecular structures can result in distinctive anaesthetic/analgetic properties and spe-

Table 1. Analgesiometric test characteristics summary

Model	Stimulus	Type*	Duration	Escape	Discomfort	Response**		Refs
Tail flick	Thermal	TH	Short	Yes	Low	Reflex	W	*(D'Amour & Smith, 1941)*
Tail immersion	Thermal	SM	Short	Yes	Low	Reflex	W	*(Farre et al., 1989)*
Paw flick	Thermal	TH	Short	Yes	Low	Reflex	W	*(Hargreaves et al., 1988)*
Paw pressure	Mechanical	TH	Short	Yes	Low	Reflex	W	
Short Electric Tail Stimulation	Electrical	SM	Short	No	Medium	Complex	TEVD	*(Charpentier 1961; Jourdan et al., 1995)*
Electric Train Tail Stimulation	Electrical	SM	Medium	No	Medium	Complex	WV	*(Carroll & Lim, 1960; Borszcz et al., 1994)*
Electric Train Paw Stimulation (NIWR)	Electrical	SM	Medium	No	Medium	Reflex	W	*(Duysens & Gybels, 1988; Matsumoto et al., 2006)*
Formalin paw test	Chemical	SM	Long	No	High	Reflex	FL	*(Dubuisson & Dennis, 1977; Vissers et al., 2006)*
Writhing test	Chemical	SM	Long	No	High	Reflex	Wr	*(Siegmund et al., 1957)*

* TH: Threshold; **SM**: Supramaximal

** D: Defence; **E**: Escape; **F**: Flinching; **L**: Licking; **T**: Twitching; **V**: Vocalisation; **W**: Withdrawal; **Wr**: Writhing;

cific effects in pain tests.

When both inhalation anaesthetics and injection anaesthetics are equally suitable for analgesiometric testing, the preference should go to inhalation anaesthetics because of better pharmacokinetic control of the administration. Modern inhalation anaesthetics like isoflurane and sevoflurane have the advantage that pharmacokinetics are very stable in time and metabolism is minimal. This stability results in a robust level of anaesthesia. Injection anaesthetics have the disadvantage that plasma concentrations are likely to increase over time. Their pharmacokinetics are also more likely to interact with the pharmacokinetics of the analgesic study drug.

Halothane is a suitable anaesthetic for the study of spinal reflexes e.g. the withdrawal reflex and spinal neural activity as marked e.g. by c-fos expression. Halothane may under certain conditions even improve the sensitivity to detect nociceptive (mechanical) neural responses (c-fos) in rats (*Novikova et al., 2004*). Halothane does not influence tail flick testing of morphine when used at moderate dosages (*Goto et al., 1996*). In the formalin test, halothane alone does not influence spinal c-fos expression and in combination with nitrous oxide it is suitable to reduce discomfort by anaesthesia during phase I and subsequently let the animal recover to study phase II flinching and licking behaviour (*O'Connor & Abram, 1995*).

Isoflurane does not influence the analgesic effect of morphine on tail flick tests (*Goto et al., 1996*), but dose-dependently antagonizes nitrous oxide analgesia.

Sevoflurane at light anaesthesia levels can be used to quantitatively measure the withdrawal reflex in the hindpaw noxious induced withdrawal reflex (NIWR) model, but dose dependently reduces this reflex (*van den Broek et al., 2006*).

Urethane is a suitable anaesthetic for the study of normal physiology. Despite its carcinogenic properties, it is frequently used for terminal physiological measurements. Reflex behaviour can also be reproduced under anaesthesia for the paw flick test (*Yeomans et al., 1996; Yeomans & Proudfit, 1994*) in the rat, but tail flick latencies are fully suppressed in mice (*Banks et al., 1988b*). These observed assay influences may not only be related to species, but also to dose. In general, spinal mechanisms are still intact under light urethane anaesthesia and this provides a good starting point for other spinally mediated reflexes and measures of (nociceptive) neural activity. However, not only clinical signs of inflammatory pain, including swelling and edema, but also spinal c-fos expression are strongly decreased by urethane in the carrageenan hindpaw inflammation model (*Buritova & Besson, 2001*) compared to conscious animals (*Buritova & Besson, 1998*) for NSAIDs like flurbiprofen.

Propofol has strong excitatory effects on ascending nociceptive pathways, autonomic and stress-related functions (*Kubota et al., 2007*). These excitatory effects may be related to the fact that intravenous propofol injection can be painful. Despite this observation, propofol can be used as an anaesthetic to study brain activation after heat stimulation (*Kubota et al., 2007*), although pentobarbital anaesthesia results in larger brain activation (*Kubota et al., 2007*). Propofol anaesthesia cannot be used to measure tail flick latencies (*Sawamura et al., 2004*). Only sedative dosages (6 mg/kg/hr) combined with nitrous oxide 75% result in a detectable, although still strongly increased (40% maximum possible effect), tail flick latency. Propofol can be used to measure the hindpaw withdrawal reflex in the NIWR model at low continuous infusion rates, but dose-dependently reduces this reflex at higher infusion rates or intermittent boluses (*Dirksen et al., 1997*).

Pentobarbital can be used as an anaesthetic to study heat induced c-fos activation in the brain (*Kubota et al., 2007*) at a medium dosage (60 mg/kg/hr). Tail flick latencies in mice are unaffected under pentobarbital anaesthesia. Unfortunately, the sensitivity of the tail flick test for opioids is also decreased. However, in mice latency effects of non-opioid analgesics like serotonin (5HT$_2$-receptor mediated) can be measured more sensitively in the tail flick assay under pentobarbital anaesthesia than under conscious conditions (*Banks et al., 1988a*). In the latter study, the increased sensitivity under anaesthesia can be attributed to an increased response combined with a decreased group variation.

Anaesthesia effects on functional MRI pain studies

Functional MRI is an important complementary tool to evaluate the analgesic efficacy and mechanisms of candidate drugs (*Negus et al., 2006*) for classical pain testing. An attractive feature of functional MRI experiments is the possibility of repeated measurement in the same animals. This reduces variability in longitudinal studies and reduces the number of animals required, although individual discomfort increases. This review focuses on acute pain; fMRI representations of persistent, chronic pain have been reviewed elsewhere (*Verne et al., 2004*).

The majority of experimental functional MRI studies in animals are currently performed under anaesthesia, for obvious reasons. The first studies used alpha-chloralose, but new alternatives that provide promising results include isoflurane. The number of experimental functional MRI pain/nociception studies is limited and mainly performed under anaesthesia. The number of functional human MRI pain studies is extensive and mainly under conscious conditions. Both animal and human pain studies are of interest to evaluate the effects of anaesthesia in animal experiments and the extrapolation of their results to pain in humans.

In conscious humans, functional imaging of brain responses to acute pain were reviewed in a meta-analysis (*Peyron et al., 2000*) and newer publications were recently summarized (*Borsook & Becerra,*

2006). These publications showed that frequently activated brain areas in humans fMRI and PET studies are located in the anterior cingulate, secondary somatic and insular cortical regions and with slightly less consistency in the contralateral thalamus and primary somatic area. Lateral thalamus, primary (SI) and secondary (SII) somatosensory cortex and insula are mainly involved in sensory-discriminative nociceptive processing, while the medial thalamus and the anterior cingulate cortex (ACC) are mainly involved in affective-motivational nociceptive processing (*Wiech et al., 2001*). Activation of pain control areas in the brain stem like the periaqueductal gray are not always present, but are fairly frequent.

In fMRI pain studies in laboratory animals, anaesthesia is often a mandatory prerequisite. The somatosensory cortex is still activated by stimulation under anaesthetic conditions. This is true for both noxious and non-noxious stimulations (*Masamoto et al., 2007;Chang & Shyu, 2001; Lowe et al., 2007*). The comparability of blood-oxygen-level dependent fMRI (BOLD) activation depends on the stimulation protocol used and has to be optimized for each anaesthetic (*Masamoto et al., 2007*). It is clear that BOLD responses after stimulation are attenuated by anaesthesia, e.g. the hypercapnic response is reduced under 1% and 2% isoflurane (*Sicard et al., 2003*). Whether this attenuation also influences BOLD responses to noxious stimulation is less clear. Volatile anaesthetics increase cerebral blood flow compared to opioids or pentobarbital (*Hendrich et al., 2001*). Hence, the choice of volatile anaesthetic dosage will determine the sensitivity of the fMRI measurement.

In rats, functional imaging of brain responses to acute pain stimuli is mostly performed under anaesthesia. A summary of the activated brain areas is presented in Table 2. A full comparison with human publications is not achievable, since the majority of animal publications include only particular regions of the brain in the functional imaging setup.

Alpha-chloralose is frequently used in fMRI studies. It has some negative properties (*Silverman &*

Muir, III, 1993), including poor solubility, slow pharmacokinetics, development of metabolic acidosis and hyperreactivity to auditory stimulation. Apart from these side-effects, it can only be used in terminal experiments, because it also induces peritonitis and adynamic ileus. These properties should be taken into consideration when fMRI pain studies are interpreted. Alpha-chloralose use in the capsaicin hyperalgesia model resulted in spinal dorsal horn activation after injection in the hindpaw (*Malisza et al., 2003b*) or the forepaw (*Malisza & Stroman, 2002*). Brain studies demonstrated activation of the somatosensory cortex, the motor cortex, the frontal cortex and the cingulate cortex after injection in the hindpaw (*Malisza et al., 2003a*) or the forepaw (*Malisza & Docherty, 2001*). In the latter study, subsequent morphine application resulted in decreased activity in frontal and cingulate cortex. In the formalin model, alpha-chloralose has been used to map brain activity. The same areas as in the capsaicin model demonstrated a positive BOLD response after formalin application in the forepaw and this response was reduced by pre-application of morphine (*Tuor et al., 2000*). In a second study, the nociceptive specific effects were separated from fMRI effects caused by increases in blood pressure (*Tuor et al., 2002*); especially fMRI effects during the first five minutes after formalin injection are correlated with blood pressure increases. Noxious electrical stimulation of the sciatic nerve led to a BOLD increase not only of the somatosensory and cingulate cortex, but also medial thalamic and hypothalamic activations were detected (*Chang & Shyu, 2001*); the activation in all four areas was significantly suppressed by prior morphine application. This finding of morphine suppression is in concordance with Tuor et al. (*Tuor et al., 2000; Chang & Shyu, 2001*); however, the somatosensory cortex activity did not decrease after morphine application, but activity of all regions decreased after nitrous oxide inhalation in that study.

Isoflurane has an interesting set of properties for fMRI studies compared to alpha-chloralose. It has fast pharmacokinetics in combination with an easily

Table 2. Activated spinel and brain areas in functional MRI animal nociception studies

Mode of Stimulation	Stimulation details	Reference	Anaesthesia	DH	PB	PG	HT	T	HC	A	S1	S2	M	I	C	FC	RS
Spinal studies																	
Chemical	formalin	*(Porszasz et al., 1997)*	Iso/N$_2$O 1.0/67 % insp	+													
Chemical	capsaicin	*(Malisza et al., 2003b)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV	+													
Chemical	capsaicin	*(Malisza & Stroman, 2002)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV	+													
Brain studies																	
Thermal	Peltier	*(Hess et al., 2007)*	Iso/Air 1-2 % insp			+	+	L: lateral M: medial			+	+	+	+	+		+
Chemical	capsaicin	*(Moylan Governo et al., 2006)*	Iso/N$_2$O 1.5/65 % insp	+	+	+	+	+	+								
Chemical	Capsaicin	*(Malisza & Docherty, 2001)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV								+		+		+	+	
Chemical	capsaicin	*(Malisza et al., 2003a)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV								+		+		+	+	
Chemical	formalin	*(Yu et al., 2007)*	Propofol 250 mg/kg IP							+							
Chemical	formalin	*(Shah et al., 2005)*	Halo/N$_2$O 1-2/70 % insp			+	+	+		+	+				+		
Chemical	formalin	*(Tuor et al., 2002)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV								+	+	+	+	+	+	
Chemical + Electrical	formalin	*(Tuor et al., 2000)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV								+		+		+	+	
Electrical	non+ nox-ious	*(Chang & Shyu, 2001)*	Iso/Air 1.5 % insp > α-Chloralose 80+40mg/kg IV				+	+			+		+		+		
Electrical	non+ nox-ious	*(Lowe et al., 2007; Chang & Shyu, 2001)*	Halo/? 3 > α-Chloralose 60+25 mg/kg IV					+			+		+		+	+	

DH: spinal dorsal horn; **S1, S2**: primary and secondary somatosensory cortex; **M**: motor cortex; **I**: insular cortex; **C**: cingulated cortex; **FC**: (pre)frontal cortex; **PB**: parabrachial nucleus; **T**: thalamus; **HT**: hypothalamus; **HC**: hippocampus; **PG**: periaqueductal gray; **RS**: retrosplenial cortex; **A**: amygdala

accessible administration route, provides adequate muscle relaxation, maintains the acid-base balance and is very suitable for repeated measurements. Furthermore, as its (end-) expiratory concentration can be measured on-line, it is easier to maintain constant levels of anaesthesia. At the minimal alveolar inhalation anaesthetic concentration for isoflurane (1 MAC_{50} ~1.45%), flow-metabolism coupling of the cortex persists (*Hansen et al., 1989*). Therefore, it promises to be a suitable alternative candidate for fMRI studies. Indeed, it has been used in a number of fMRI pain studies in rats in an air/oxygen or nitrous oxide/oxygen mixture. Most studies mentioned above under alpha-chloralose used isoflurane during the preparation phase of the experiment. Isoflurane (in N_2O/O_2) use in the formalin test resulted in spinal dorsal horn activation (*Porszasz et al., 1997*); this activation was blocked by local lidocaine application. In a recent article, capsaicin noxious activation in the brain was investigated (*Moylan Governo et al., 2006*); several subcortical regions responded to the noxious stimulation, including periaqueductal gray, hypothalamus and thalamus and the parabrachial and hippocampal areas. The activation of most areas increased by superimposed mechanical stimulation after capsaicin application, although periaqueductal gray activation decreased (*Moylan Governo et al., 2006*). Finally, Hess et al. used isoflurane in air. They found an activation of many cortical brain regions (*Hess et al. 2007*), including somatosensory, motor, insular, cingulate, retrosplenial, and subcortical brain regions, including lateral and medial thalamus, hypothalamus and the periaqueductal gray. Pre-treatment with zymosan in the latter study (*Hess et al., 2007*) increased noxious heat BOLD effects.

Halothane effects on fMRI are comparable with those of alpha-chloralose and isoflurane. In the formalin test, activation was found in the cortical areas, including somatosensory and cingulate cortex and subcortical areas, including thalamus, hypothalamus, periaqueductal gray and amygdala (*Shah et al., 2005*). After electrical noxious stimulation, cortical and thalamic activation was demonstrated

(*Lowe et al., 2007*).

Propofol was only used in one formalin pain study. It was applied in a non-standard way (250 mg/kg IP) and resulted in amygdala activation (*Yu et al., 2007*)

Anaesthesia recommendations for analgesiometric tests

In this review, it was demonstrated that the use of anaesthetics in pain research is feasible, especially in the first phase of preclinical analgesic assessment. This will reduce the number of animals necessary for subsequent pain testing in conscious animals. It is very likely that the use of anaesthetics in pain research will contribute significantly to the welfare of laboratory animals in analgesiometric tests. However, it should be realized that fMRI under anaesthesia provides mostly information about nociception rather than pain.

It is important to realize that there is a limited number of studies that compare both the conscious response and a response under anaesthetic conditions. Also, studies that compare two different anaesthetics within the same study are generally not available. Therefore, preliminary anaesthetic recommendations for the analgesiometric tests below should be used cautiously and further research is required to provide evidence based recommendations.

Tail flick testing can be performed under anaesthetic conditions. A low dosage of isoflurane is a good anaesthetic choice for opioid analgesia testing, since it does not influence morphine tail flick latencies (*Goto et al., 1996*). We recommend a dosage of 1.5% to minimize anaesthetic influence on analgesiometric results.

Electrical noxious stimulation can be performed under anaesthetic conditions, if noxious reflex testing is performed. For integrated, higher order behaviour like vocalisation afterdischarges or defence behaviour, conscious animals are still needed.

Formalin testing may be performed under anaesthetic conditions with the combination of halothane and nitrous oxide to reproduce flinching and licking behaviour during the second phase (*O'Connor*

& *Abram, 1995*). Thiopental is another possibility. Halothane alone only mildly influences spinal c-fos expression in the formalin test. Alternative approaches with different anaesthetics to detect neural activation are described under fMRI studies.

Anaesthesia recommendations for c-fos and fMRI nociceptive testing

The feasibility to use anaesthetics in nociceptive neural activation c-fos studies depends on the analgesic group of interest and the chosen baseline anaesthesia. Some studies have described uncoupling of behaviour and spinal Fos-IR. This requires further investigation. One solution may be to find a correlation between behaviour and neural activation in brain nuclei, to explain the observed discrepancy between c-fos and pain behaviour.

The use of anaesthetics in functional MRI pain studies depends on the research question. There may be a difference in brain activation between isoflurane and alpha-chloralose fMRI studies, both frequently used in functional MRI studies. Somatomotor, cingulate and frontal cortex activation was found in formalin studies with alpha-chloralose. Comparable studies with isoflurane are currently lacking, so it is not clear whether isoflurane in the formalin test would result in the same pattern of brain activation. From thermal and electrical stimulation studies, it is clear that under isoflurane anaesthesia, activation of these areas is possible and also of the thalamus, hypothalamus and periaqueductal gray. Activation of these latter subcortical nuclei has not yet been found under alpha-chloralose anaesthesia.

In this review, publications are referred to that demonstrate the feasibility of anaesthesia in combination with pain/nociceptive testing. However, the number of publications that compared the results of analgesiometric tests under conscious conditions with anaesthetic conditions for a broad range of analgesic agents is still very limited. Therefore, further research on analgesiometric tests under anaesthetic conditions is necessary to validate these recommendations.

Conclusions

Every anaesthetic has its particular sites of action in the central nervous system. These sites of anaesthetic action determine whether components of the pain system remain unaffected, are unresponsive or hyperresponsive and whether the application of the anaesthetic leads to meaningful results. Apart from the anaesthetic action, the interaction between anaesthetic and analgesic agents is important for the interpretation of the results. The analgesic agents also have points of analgesic action that can be influenced by the anaesthetic used. For the proper validation of the feasibility of anaesthetics in analgesiometric tests, it is therefore not only necessary to demonstrate sufficient sensitivity of the response function of the test, but also to demonstrate that analgesic effects on the response function can be measured in a reliable and sensitive way.

Reflex behaviour like the tail flick and withdrawal reflexes and nociceptive processing, including c-fos and functional MRI, can be studied best with low dosage isoflurane.

For more complex behaviour, the use of anaesthetics is not suitable because they impair higher cognitive functioning. This is mainly related to the necessity to be conscious to express e.g. aggressive behaviour and partly related to the disturbance of nociceptive processing that anaesthetics have on different supraspinal levels. However, in the future, it may become possible to predict the outcome of complex (pain) behaviour in conscious animals from the results of neural activity measurements like c-fos or fMRI in particular brain nuclei in anesthetized animals. This approach would require a large number of validation studies to prove the predictive value of neural activity for pain behaviour outcome, but would be very beneficial for the anesthetized animals in terms of discomfort reduction.

References

Banks WA, AJ Kastin, TL Trentman, HS Haynes, BG Johnson & ZH Galina. Mediation of serotonin-induced analgesia by the 5HT2 receptor in the pentobarbital anesthetized mouse model. Brain

Res. Bull., 1988a, *21*, 887-891

Banks WA, TL Trentman, AJ Kastin & ZH Galina. The general anaesthesia induced by various drugs differentially affects analgesia and its variability. Pharmacol Biochem. Behav., 1988b, *31*, 397-403

Borsook D, LR Becerra. Breaking down the barriers: fMRI applications in pain, analgesia and analgesics. Mol. Pain, 2006, *2*, 30

Borszcz GS, CP Johnson & KA Fahey. Comparison of motor reflex and vocalization thresholds following systemically administered morphine, fentanyl, and diazepam in the rat: assessment of sensory and performance variables. Pharmacol. Biochem. Behav., 1994, *49*, 827-834

Buritova J, JM Besson. Peripheral and/or central effects of racemic-, S(+)- and R(-)-flurbiprofen on inflammatory nociceptive processes: a c-Fos protein study in the rat spinal cord. Br. J Pharmacol, 1998, *125*, 87-101

Buritova J, JM Besson. Urethane anaesthesia could partly mask antinociceptive effects of non-steroidal anti-inflammatory drugs: a spinal c-Fos protein study. Brain Res., 2001, *891*, 281-284

Carroll MN, RK Lim. Observations on the neuropharmacology of morphine and morphinelike analgesia. Arch. Int. Pharmacodyn. Ther., 1960, *125*, 383-403

Chang C, BC Shyu. A fMRI study of brain activations during non-noxious and noxious electrical stimulation of the sciatic nerve of rats. Brain Res., 2001, *897*, 71-81

Charpentier J. [On a new psychophysiological method of quantitative measurement of painful reactions in the rat.]. C. R. Seances Soc. Biol. Fil., 1961, *155*, 727-731

D'Amour FE, DL Smith. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther., 1941, *72*, 74-79

Dirksen R, B Ellenbroek, J van Egmond & AR Cools. Responses to propofol in relation to GABA functionality of discrete parts of the brain of rats. Pharmacol Biochem. Behav., 1997, *57*, 727-735

Dubuisson D, SG Dennis. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 1977, *4*, 161-174

Duysens J, J Gybels. The automated measurement of hindlimb flexor reflex of the rat as a substitute for the tail-flick assay. J. Neurosci. Methods, 1988, *24*, 73-79

Farre AJ, M Colombo & B Gutierrez. Maximum tolerated temperature in the rat tail: a broadly sensitive test of analgesic activity. Methods Find. Exp Clin. Pharmacol, 1989, *11*, 303-307

Goto T, JJ Marota & G Crosby. Volatile anaesthetics antagonize nitrous oxide and morphine-induced analgesia in the rat. Br. J Anaesth., 1996, *76*, 702-706

Hansen TD, DS Warner, MM Todd & LJ Vust. The role of cerebral metabolism in determining the local cerebral blood flow effects of volatile anaesthetics: evidence for persistent flow-metabolism coupling. J. Cereb. Blood Flow Metab, 1989, *9*, 323-328

Hargreaves K, R Dubner, F Brown, C Flores & J Joris. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain, 1988, *32*, 77-88

Hendrich KS, PM Kochanek, JA Melick, JK Schiding, KD Statler, DS Williams, DW Marion & C Ho. Cerebral perfusion during anaesthesia with fentanyl, isoflurane, or pentobarbital in normal rats studied by arterial spin-labeled MRI. Magn Reson. Med., 2001, *46*, 202-206

Hess A, M Sergejeva, L Budinsky, HU Zeilhofer & K Brune. Imaging of hyperalgesia in rats by functional MRI. Eur. J Pain, 2007, *11*, 109-119

Intomart GfK / The Dutch Society for the Protection of Animals. Public opinion on animal experimentation in the Netherlands. http://www.dierenbescherming.nl/downloads/docs/opinionpoll_eng.doc; RG/YV/1.2257, 1-15. 14-5-2004. Hilversum / The Hague, The Netherlands. Ref Type: Report

Jourdan D, D Ardid, E Chapuy, A Eschalier & BD Le. Audible and ultrasonic vocalization elicited

by single electrical nociceptive stimuli to the tail in the rat. Pain, 1995, *63*, 237-249

Kubota I, Y Tsuboi, E Shoda, M Kondo, Y Masuda, J Kitagawa, Y Oi & K Iwata. Modulation of neuronal activity in CNS pain pathways following propofol administration in rats: Fos and EEG analysis. Exp Brain Res., 2007, *179*, 181-190

Lowe AS, JS Beech & SC Williams. Small animal, whole brain fMRI: innocuous and nociceptive forepaw stimulation. Neuroimage., 2007, *35*, 719-728

Malisza KL, JC Docherty. Capsaicin as a source for painful stimulation in functional MRI. J Magn Reson. Imaging, 2001, *14*, 341-347

Malisza KL, L Gregorash, A Turner, T Foniok, PW Stroman, AA Allman, R Summers & A Wright. Functional MRI involving painful stimulation of the ankle and the effect of physiotherapy joint mobilization. Magn Reson. Imaging, 2003a, *21*, 489-496

Malisza KL, PW Stroman. Functional imaging of the rat cervical spinal cord. J Magn Reson. Imaging, 2002, *16*, 553-558

Malisza KL, PW Stroman, A Turner, L Gregorash, T Foniok & A Wright. Functional MRI of the rat lumbar spinal cord involving painful stimulation and the effect of peripheral joint mobilization. J Magn Reson. Imaging, 2003b, *18*, 152-159

Masamoto K, T Kim, M Fukuda, P Wang & SG Kim. Relationship between neural, vascular, and BOLD signals in isoflurane-anesthetized rat somatosensory cortex. Cereb. Cortex, 2007, *17*, 942-950

Matsumoto M, M Inoue, A Hald, A Yamaguchi & H Ueda. Characterization of three different sensory fibers by use of neonatal capsaicin treatment, spinal antagonism and a novel electrical stimulation-induced paw flexion test. Mol. Pain, 2006, *2*, 16

MORI / Coalition for Medical Progress. The Use of Animals in Medical Research. http://www.ipsos-mori.com/polls/2002/pdf/cmp.pdf;MORI / 16141, 1-35. 2002. London, United Kingdom. Ref Type: Report

MORI/CoalitionforMedicalProgress.UseofAnimals in Medical Research. http://www.ipsos-mori.com/polls/2005/pdf/cmp.pdf;MORI / J23491, 1-9. 16-2-2005. London, United Kingdom. Ref Type: Report

MORI / Medical Research Council. Animals in Medicine and Science. http://www.ipsos-mori.com/polls/1999/pdf/mrc99.pdf;MORI / 12141, 1-54. 1999. London, United Kingdom. Ref Type: Report

Moylan Governo RJ, PG Morris, MJ Prior, CA Marsden & V Chapman. Capsaicin-evoked brain activation and central sensitization in anaesthetised rats: a functional magnetic resonance imaging study. Pain, 2006, *126*, 35-45

Murrell JC, CB Johnson. Neurophysiological techniques to assess pain in animals. J. Vet. Pharmacol. Ther., 2006, *29*, 325-335

Negus SS, TW Vanderah, MR Brandt, EJ Bilsky, L Becerra & D Borsook. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. J Pharmacol Exp Ther, 2006, *319*, 507-514

Novikova NS, TB Kazakova, V Rogers & EA Korneva. Expression of the c-fos gene in spinal cord and brain cells in rats subjected to stress in conditions of exposure to various types of halothane anaesthesia. Neurosci. Behav. Physiol, 2004, *34*, 407-412

O'Connor TC, SE Abram. Inhibition of nociception-induced spinal sensitization by anaesthetic agents. Anesthesiology, 1995, *82*, 259-266

Peyron R, B Laurent & L Garcia-Larrea. Functional imaging of brain responses to pain. A review and meta-analysis (2000). Neurophysiol. Clin., 2000, *30*, 263-288

Porszasz R, N Beckmann, K Bruttel, L Urban & M Rudin. Signal changes in the spinal cord of the rat after injection of formalin into the hindpaw: characterization using functional magnetic resonance imaging. Proc. Natl. Acad. Sci. U. S. A, 1997, *94*, 5034-5039

Russell WMS, Burch RL. The Principles of Humane Experimental Technique. London: Methuen,

1959.

Sawamura S, M Obara-Nawata, K Takeda & K Hanaoka. General anaesthetics inhibit the nitrous-oxide-induced activation of corticotropin releasing factor containing neurons in rats. Eur. J Pharmacol, 2004, *503*, 49-53

Shah YB, L Haynes, MJ Prior, CA Marsden, PG Morris & V Chapman. Functional magnetic resonance imaging studies of opioid receptor-mediated modulation of noxious-evoked BOLD contrast in rats. Psychopharmacology (Berl), 2005, *180*, 761-773

Sicard K, Q Shen, ME Brevard, R Sullivan, CF Ferris, JA King & TQ Duong. Regional cerebral blood flow and BOLD responses in conscious and anesthetized rats under basal and hypercapnic conditions: implications for functional MRI studies. J Cereb. Blood Flow Metab, 2003, *23*, 472-481

Siegmund E, R Cadmus & G Lu. A method for evaluating both non-narcotic and narcotic analgesics. Proc. Soc. Exp. Biol. Med., 1957, *95*, 729-731

Silverman J, WW Muir, III. A review of laboratory animal anaesthesia with chloral hydrate and chloralose. Lab Anim Sci., 1993, *43*, 210-216

Tuor UI, K Malisza, T Foniok, R Papadimitropoulos, M Jarmasz, R Somorjai & P Kozlowski. Functional magnetic resonance imaging in rats subjected to intense electrical and noxious chemical stimulation of the forepaw. Pain, 2000, *87*, 315-324

Tuor UI, E McKenzie & B Tomanek. Functional magnetic resonance imaging of tonic pain and vasopressor effects in rats. Magn Reson. Imaging, 2002, *20*, 707-712

van den Broek PL, CM van Rijn, J van Egmond, AM Coenen & LH Booij. An effective correlation dimension and burst suppression ratio of the EEG in rat. Correlation with sevoflurane induced anaesthetic depth. Eur. J. Anaesthesiol., 2006, *23*, 391-402

Verne GN, ME Robinson & DD Price. Representations of pain in the brain. Curr. Rheumatol. Rep., 2004, *6*, 261-265

Vierck CJ, PT Hansson & RP Yezierski. Clinical and pre-clinical pain assessment: are we measuring the same thing? Pain, 2008, *135*, 7-10

Vissers KC, F Geenen, R Biermans & TF Meert. Pharmacological correlation between the formalin test and the neuropathic pain behaviour in different species with chronic constriction injury. Pharmacol. Biochem. Behav., 2006, *84*, 479-486

Wiech K, H Preissl & N Birbaumer. [Neural networks and pain processing. New insights from imaging techniques]. Anaesthesist, 2001, *50*, 2-12

Wilder-Smith OH & L Arendt-Nielsen. Postoperative hyperalgesia: its clinical importance and relevance. Anesthesiology, 2006, *104*, 601-607

Yeomans DC, V Pirec & HK Proudfit. Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: behavioural evidence. Pain, 1996, *68*, 133-140

Yeomans DC, HK Proudfit. Characterization of the foot withdrawal response to noxious radiant heat in the rat. Pain, 1994, *59*, 85-94

Yu O, N Parizel, L Pain, B Guignard, B Eclancher, Y Mauss & D Grucker. Texture analysis of brain MRI evidences the amygdala activation by nociceptive stimuli under deep anaesthesia in the propofol-formalin rat model. Magn Reson. Imaging, 2007, *25*, 144-146

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain, 1983, *16*, 109-110.