Refinement and Reduction in Animal Experimentation: Options for New Imaging Techniques

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Summary

Attempts to substitute animal experiments with in vitro or in silico methods were of limited success when complex (regulatory) processes, e.g. of the cardiovascular, metabolic or neuronal system, were to be analysed. Consequently, strategies to reduce the number of and the burden placed on experimental animals in these fields of research are required. One option consists in the application of non-invasive imaging techniques like (functional) magnetic resonance imaging ((f)MRI), positron emission tomography (PET), and optical imaging (OI). All these methods allow for the observation of functional changes within the body of e.g. genetically modified animals without pain, suffering or (premature) termination. The use of these methods has now reached new dimensions of resolution and precision.

With this article we would like to demonstrate a few options of these techniques. We hope that our enthusiasm becomes contagious, thus motivating more scientists to make use of the still expensive equipment which has become available in "small animal imaging" centres.

On the basis of four examples – three from our group – we would like to highlight some merits of the new technologies.

Zusammenfassung: „Reduction" und „Refinement" von Tierversuchen kann durch bildgebende Verfahren erzielt werden

Bildgebende Verfahren – (funktionelle) Magnet Resonanz Tomographie (fMRT), Positronenemissionstomographie (PET) und optische Bildgebung (OI) – werden vermehrt in der experimentellen Forschung eingesetzt. Sie haben sich als nützlich erwiesen, um die Anzahl der Versuchstiere und deren Leiden zu reduzieren.

In dem vorliegenden Artikel wird an Beispielen aus der Grundlagenforschung gezeigt, wie unterschiedliche nichtinvasive Bildgebungsverfahren zu erfolgreichen Methoden auch im Sinne des Tierschutzes werden können. Ihr Einsatz verspricht eine deutliche Verminderung der Anzahl von Tieren und ihrer Belastung. Mit Hilfe dieser Verfahren kann die Kontrolle und Regulation komplexer Organfunktionen, etwa des kardiovaskulären oder des neuronalen Systems, in Narkose analysiert werden, ohne die Tiere zu töten oder wesentlich zu belasten. Derartige, medizinisch relevante und wissenschaftlich hochwertige Ergebnisse können zurzeit mit in vitro- oder in silico-Methoden bei komplexen Fragestellungen nicht erzielt werden.

Besondere Anerkennung verdient daher die Unterstützung dieser Forschungsrichtung durch das BMBF (Bundesministerium für Bildung und Forschung) und verschiedene Tierschutzorganisationen.

Keywords: functional imaging, magnetic resonance imaging, positron emission tomography, optical imaging

1 Introduction

Much devotion and many funds have been invested in developing *in vitro* methods supplementing animal experimentation in scientific research and industrial development. Regrettably, the expected perfect solution was not discovered. Even moderate hopes, e.g. for substituting the unacceptable Draize test with tissue culture methods, could not be fulfilled with confidence (Eskes et al., 2007; Spielmann et al., 2007). Only few of the *in vitro* methods that have been proposed have found approval by the OECD (cf. OECD Guidelines in the internet concerning chemical safety and biosafety). Still, *in vitro* methods for predicting skin corrosion (Grindon et al., 2007; Kidd et al., 2007), phototoxicity (Lelièvre et al., 2007) and measuring skin penetration (Chilcott et al., 2000) allow for some substitution of whole animal testing (Thasler et al., 2006). Regrettably, *in vitro* evaluation of putative genotoxicity has shown that "the required battery of these sensitive *in vitro* genotoxicity assays has a low specificity, i.e. high percentage of false positive results for non-carcinogens" (Paul Carmichael at the recent ECOPA meeting, Brussels, 2007). *In vitro* technologies describing the complex cell-cell interactions, as e.g. in the complex central nervous system or the well regulated cardiovascular system, are not feasible as yet. Therefore, the investigation of drugs or toxic chemicals in animals is still unavoidable in order to

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provide safety and improved therapeutic means for mankind.

Consequently, all attempts to *reduce* animal use and to *refine* experimentation to spare animals pain and suffering deserve more attention – even if these two R's do not comprise the ideal solution in view of those who put their hopes entirely on complete substitution of animal experimentation with *in vitro* or *in silico* techniques (Goldberg and Hartung, 2007). In this context it appears noteworthy that the German Federal Ministry of Education and Research (BMBF) has started to support projects attempting to make use of modern imaging techniques to reduce and replace animal use. Altogether, the BMBF will provide more than 10 million € (for 3 years) to further explore and develop these tools (for futher details see BMBF: Bildgebende Verfahren als spezielle Beiträge zur Reduktion von Tierversuchen und zur Verminderung der Belastungen von Versuchstieren" within the framework "Biotechnologie – Chancen nutzen und gestalten" – see BMBF, 2007). Particularly, the combination of modern genomics (knock-out, conditioned knock-out, multiple knock-out, knock-in, conditioned knock-in mice) with non-invasive technology allows for the evaluation of otherwise healthy animals under anaesthesia to answer questions about, for example, the involvement of certain genes (receptors) in acute, subacute and chronic pain (including hyperalgesia and allodynia) (Lacroix-Fralish et al., 2007), cardiac regulation and dysfunction (Mangoni et al., 2003; Wehrens et al., 2003).

It is gratifying to see that the initial sponsoring of this approach (our group and others by e.g. the Doerenkamp-Zbinden Foundation and SET) and the presentation of results at meetings organized by DFG, BMBF, FFVFF, SET, ZEBET and others have faciliated this major andertaking.

2 MRI in experimental research at the Doerenkamp chair in Erlangen

We here report on our efforts to apply imaging techniques for the purpose of reducing animal use and suffering and of refining scientific results for obtaining unmatched data quality. Two examples prove that MRI does indeed provide a tool for substantial reduction and refinement in animal experimentation. They are taken from original publications (where a description of the methodological details can be found).

a) Imaging of the beating heart

Cardiac infarction is the major cause of death in Western, industrialized nations. In the future it is hoped to replenish the damaged and scarified heart with new, functioning cardiac tissue, engineered from embryonic cells or – depending on the scientific progress – later on by autologous heart cells derived from the patient's own adult stem cells (Mummery et al., 2007). A model which evaluates this option is the infarcted rat heart, which, after a period of healing, receives a patch of functional, active heart cells produced *in vitro* from embryonic heart cells (Zimmermann et al., 2006). This model is widely used, and our collaborators in Hamburg were awarded the "Forschungspreis zur Förderung von methodischen Arbeiten mit dem Ziel der Einschränkung und des Ersatzes von Tierversuchen" by the BMBF for developing techniques to engineer these heart cell patches *in vitro*. Still, in order to learn if the patches that are implanted on the scarified heart integrate and function in their new environment synchronously with the genuine, surviving cells, the animals had to be sacrificed, the hearts removed and investigated *in vitro* – functionally and histologically. With the help of MRI technology (alternatively by ultrasonic imaging) we could show day by day to what extent the heart cell patch was integrated and functioning (Zimmermann et al., 2006; and Fig. 1a, 1b). No animal had to be sacrificed at any point in time for this analysis, but all

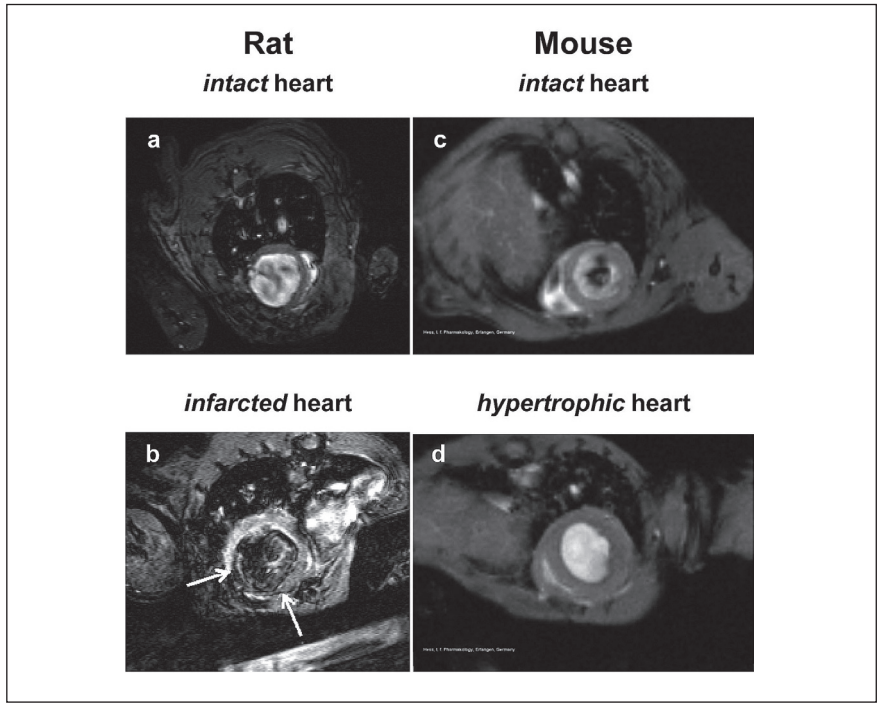

Fig. 1: Dynamic heart imaging in rats and mice
Time resolved imaging of the beating heart allows for comparing hearts in distinct beating phases under experimental conditions. (a) Normal rat heart compared to (b) infarcted heart (ventricle is dilated, infarcted area is flaccid, thin and lacks water, i.e. grey instead of normal white heart muscle). Arrows mark the region of the infarct, muscle free area.
(c) Normal mouse heart and (d) hypertrophic heart after aortic constriction. In both cases a clear enlargement of the heart, i.e. hypertrophy, is visible (for details (rat figures) comp. Zimmermann et al., 2006). The mouse pictures were produced under similar conditions. The development of heart hypertrophy is described in Herrmann et al., 2007.

could be observed "throughout", and the functional parameters could be defined. It is obvious that animal lives were saved by this procedure and the experimental animals did not suffer much, because the (non-invasive) MRI scans of the beating hearts were taken under anaesthesia. Current technological developments allow tto apply this scanning procedure also to (genetically modified) mice. We recently started to investigate knock-out mice with defects of cation channels (Herrmann et al., 2007) that may lead to cardiac hypertrophy. Again, this process can be monitored with MRI methods as can the effect of preventative therapy (Fig. 1c, 1d).

b) Pain under anaesthesia

Modern pain research has shown that pain (nociception, as it is called in animals) results from peripheral stimulation of nociceptors, transmission of nociceptive neuronal impulses into the spinal cord and activation of several cortical areas involved in pain perception, pain recognition and emotional tinting. We could show recently that the activation of typical brain areas happened under anaesthesia, too, producing similar activation patterns as observed in conscious man (Hess et al., 2007). Different aspects of pain processing can be investigated by fMRI in rats as in man (Apkarian et al., 2005; Borsook and Becerra, 2006; Sergejeva et al., in press), but with fewer ethical constraints. Of course, the effect of, for example, unapproved new drugs can only be evaluated in animals. As in man, mild heat stimuli, when applied to irritated skin areas, led to much more activation of brain centres than when the same stimulus was applied to non-irritated skin (hyperalgesia). Moreover, the imaging approach allows for daily application of painful stimuli to animals under anaesthesia, monitoring the changes of quantitative and qualitative reactions in different brain areas, which may reflect mechanisms contributing to pain chronification in man.

This approach could recently be used to define a new target of analgesic drug action. It could be shown (as given in Fig. 2) that a new model substance specifically reduces significantly correlates of nociceptor mediated (hyperalgesic) pain and of neuropathic pain (Knabl et al., 2008).

3 Optical Imaging (OI):

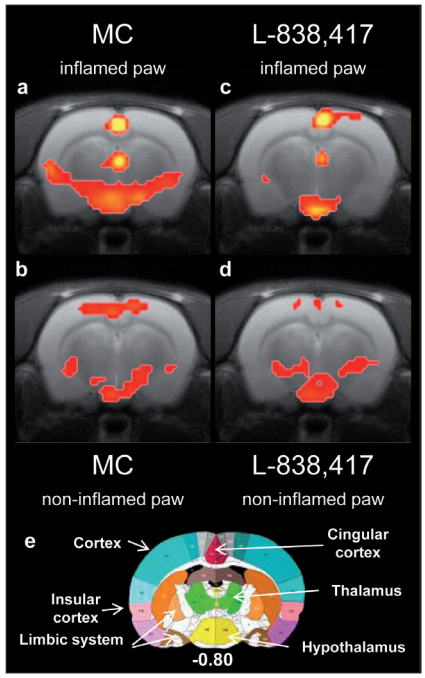

Fig. 2: Functional brain imaging, demonstration of the effect of a novel analgesic compound (L-838,417)
fMRI is able to demonstrate nociception-induced cerebral activation patterns despite isoflurane anaesthesia
The figure depicts in false colour images brain activation measured by the so-called BOLD (blood oxygenation level dependent) effect. Brain activation (O_2 extraction) results from the application of a short heat burst to either inflamed (a, c) or non-inflamed paws (b, d). The figure demonstrates that the analgesic compound (L-838,417) reduces the brain activation resulting from the stimulation of the inflamed paw (c) to the normal level (b). The analgesic compound did not measurably change the brain activation following application of heat bursts to non-inflamed paw skin (d). To identify the involved brain areas, major brain structures are depicted in (e). The results displayed are comparable to the data shown in Knabl et al., 2008. Inflammation was initiated 6 hours before the first measurement by the injection of zymosan A subcutaneously into the left hind paw.

an additional, promising, new technique

This technique is by far not as advanced as MRI and PET. It does, however, offer promising aspects. This can be seen in Figure 3. The present method for measuring inflammation in animals (defining the (inflamed) paw volume with a calliper or by fluid displacement) is painful. In search of a less stressing method, we tried OI. Using a new optical marker (OsteoSense®, VisEn Medical), the degree of inflammation in animals overexpressing TNFα and – along with that – with inflammatory reactions in the hip and the mandibular joints could be visualised and evaluated quantitatively. We injected the fluorescent tracer into the tail vein of the animals (some paravenous tracer is nicely visible in Fig. 3a and 3b). Employing a highly sensitive *in vivo* imaging system (real-time PhotonImager, Raytest®), we could show that high tracer accumulation occurred in the inflamed paws and jaw joints of mice overexpressing the TNFα gene. We are presently investigating different options for quantitative analysis, which would allow earlier disease detection as well as measuring the degree of inflammation with higher sensitivity and dynamic range. (A full publication of these data is in preparation.) Moreover, the anti-inflammatory activity of drugs or compounds could be detected at a much earlier disease stage, and the animals would suffer less. It is obvious that measuring inflammation in a quantitative manner in these animals without dissecting the paws or using callipers in order to measure the thickness or – as has been done formerly – cutting off the paws and weighing them. Thus reduces both, the number of animals needed and the experimental burden inflicted on these animals.

4 Positron Emission Tomography (PET)

PET investigation in animals offers interesting options. This is exemplified in Figure 4. Of course, the requirement of radioactive tracers, which in addition must be produced acutely (requiring a cyclotron), limits the use of this approach.

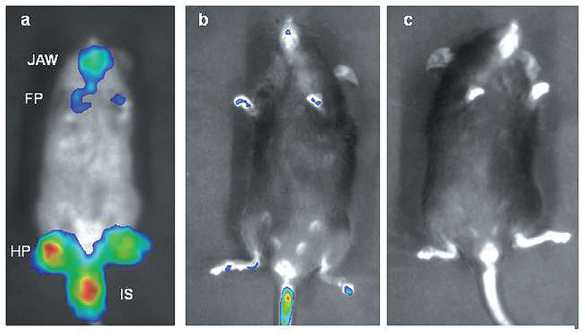

Fig. 3: Optical imaging of inflammation
Non-invasive visualisation of bone/joint inflammation in TNFα-overexpressing transgenic mice with Osteosense® (a); control mouse (b); TNFα transgenic mouse without Osteosense® (c). TNFα-overexpressing mice display spontaneous joint inflammation, comparable to patients with rheumatoid arthritis. Recording time per scan was 1 min with 740 nm excitation and 780 nm emission wavelengths. High concentrations of the dye were found at the injection site (IS) for TNFα (a) and WT mice (b), but only for TNFα transgenic animals at the inflamed hindpaws (HP), forepaws (FP) and the jaw joint (JAW) (a). No specific inflammatory signal could be obtained without the dye (c). Publication in preparation.

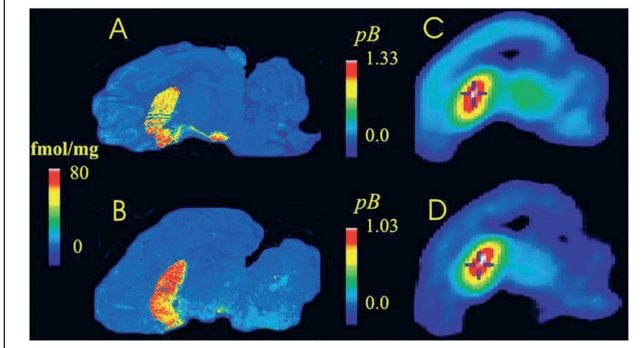

Fig. 4: Imaging of dopamine receptors (D_1 and $D_{2/3}$) in the corpus striatum of the pig brain (red area)
Conventional autoradiograms (A, B) require injection of radioactive drug, sacrificing and physical slicing of the brain, whereas non-invasive PET imaging (C,D) can be performed on the living animal. Dopamine receptors are essential targets in neurodegenerative processes, as Parkinson's disease and other diseases, and depression or schizophrenia. They also play a major role in regulating attention and physical activity. PET with its high sensitivity is used to describe the spatial receptor distribution with complete brain coverage (C, D). PET imaging also allows investigating the specific binding of drugs to these receptors. Many important drugs exert their activity as inhibitors or activators of these receptors. The method also serves as a non-invasive tool for detecting brain abnormalities (Minuzzi et al. (2006) *Synapse 59*, 211-219; with permission).

Still, with the help of drugs carrying an emitting tracer, e.g. [C-11]diprenorphine, it is possible to investigate the binding of neurotransmitters or drugs *vice versa* the displacement from receptors, the distribution of receptors and (NS-transmitters and morphologically/genetically determined) derivations, etc. The change of receptor density and the specificity of the blockade of pharmacologically relevant receptors are presently used also in drug research (Tietze et al., 2008). This imaging technique is well established in humans, but as yet not widely used in small animals, e.g. rodents. Application in humans is limited to approved drugs and constraints resulting from radioactive tracers. The alternative, however, would be to apply radioactive tracers to animals, sacrifice different groups of animals at different time intervals and expose slices of their bodies for autoradiography. It is obvious that much more animals would be required to be able to follow receptor binding and/or disappearance (displacement) from receptor over time.

5 Conclusions

Following pioneering activities, sponsored by concerned lay persons, demands from researchers and concerns of politicians. A major effort of the German research supporting organisation BMBF has led to the funding of research groups that attempt to develop imaging techniques for *reducing* the number and the suffering of animals (*refinement*) in research which cannot be pursued with *in vitro* or *in silico* technology. We expect that this funding will allow a rapid development of advanced technologies which will continue reducing the number of animals sacrificed in neurobiological, pharmacological, toxicological and cardiovascular research and at the same time increase the scientific quality of the research results. We believe that refinement and reduction are at present very promising avenues towards more and better animal protection.

References

Apkarian, A. V., Bushnell, M. C., Treede, R. D. et al. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *Eur. J. Pain 9*, 463-484.

BMBFannouncementofthefunding:http://www.bmbf.de/_search/searchresult.php?URL=http%3A%2F%2Fwww.

bmbf.de%2Ffoerderungen%2F10498.php&QUERY=bildgebende+und+verfahren

Borsook, D. and Becerra, L. (2006). Breaking down the barriers: fMRI applications in pain, analgesia and analgesics. *Mol. Pain. 18 (2)*, 30.

Chilcott, R. P., Jenner, J., Carrick, W. et al. (2000). Human skin absorption of Bis-2-(chloroethyl)sulphide (sulphur mustard) in vitro. *J. Appl. Toxicol. 20(5)*, 349-355.

Eskes, C., Cole, T., Hoffmann, S. et al. (2007). The ECVAM international validation study on in vitro tests for acute skin irritation: Selection of test chemicals. *ATLA 35(6)*, 603-619.

Goldberg, A. M. and Hartung, T. (2007). Ersatz für Tierversuche – nicht nur zum Tierschutz. *Spektrum der Wissenschaft*, (Feb) 60-68.

Grindon, C., Combes, R., Cronin, M. et al. (2007). Integrated decision-tree testing strategies for skin corrosion and irritation with respect to the requirements of the EU REACH legislation. *ATLA 35(6)*, 673-682.

Herrmann, S., Stieber, J., and Ludwig, A. (2007). Pathophysiology of HCN channels. Pflugers Arch. 454(4), 517-522.

Hess, A., Sergejeva, M., Budinsky, L. et al. (2007). Imaging of hyperalgesia in rats by functional MRI. *Eur. J. Pain. 11(1)*, 109-119.

Kidd, D. A., Johnson, M., and Clements, J. (2007). Development of an in vitro corrosion/irritation prediction assay using the EpiDerm skin model. *Toxicol. In Vitro 21(7)*, 1292-1297.

Knabl, J., Witschi, R., Hösl, K. et al. (2008). Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature 451(7176)*, 330-334.

Lacroix-Fralish, M. L., Ledoux, J. B., and Mogil, J. S. (2007). The Pain Genes Database: An interactive web browser of pain-related transgenic knockout studies. *Pain 131(1-2)*, e1-4.

Lelièvre, D., Justine, P., Christiaens, F. et al. (2007). The EpiSkin phototoxicity assay (EPA): development of an in vitro tiered strategy using 17 reference chemicals to predict phototoxic potency. Toxicol. *In Vitro 21(6)*, 977-995.

Mangoni, M. E., Couette, B., Bourinet, E. et al. (2003). Functional role of L-type Cav1.3 Ca2+ channels in cardiac pacemaker activity. *Proc. Natl. Acad. Sci. U S A. 100(9)*, 5543-5548.

Minuzzi, L., Nomikos, G. G., Wade, M. R. et al. (2005). Interaction between LSD and dopamine D2/3 binding sites in pig brain. *Synapse 56(4)*, 198-204.

Mummery, C., van der Heyden, M. A., de Boer, T. P. et al. (2007). Cardiomyocytes from human and mouse embryonic stem cells. *Methods Mol. Med. 140*, 249-272.

OECD Guidelines: http://www.oecd.org/department/0,3355,en_2649_34377_1_1_1_1_1,00.html, http://www.oecd.org/department/0,3355,en_2649_34365_1_1_1_1_1,00.html, http://www.oecd.org/department/0,3355,en_2649_34385_1_1_1_1_1,00.html.

Sergejeva, M., Brune, K., and Hess, A. (2008). Cerebral representation of hyperalgesia: evidence from functional imaging in humans and animals. *J. Neuroimaging* (in press).

Spielmann, H., Hoffmann, S., Liebsch, M. et al. (2007). The ECVAM international validation study on in vitro tests for acute skin irritation: Report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. *ATLA 35(6)*, 559-601.

Thasler, W. E., Schlott, T., Kalkuhl, A. et al. (2006). Human tissue for in vitro research as an alternative to animal experiments: a charitable "honest broker" model to fulfil ethical and legal regulations and to protect research participants. *ATLA 34(4)*, 387-392.

Tietze, R., Löber, S., Hübner, H. et al. (2008). Discovery of a dopamine D4 selective PET ligand candidate taking advantage of a click chemistry based REM linker. *Bioorg. Med. Chem. Lett. 18(3),* 983-988.

Wehrens, X. H., Lehnart, S. E., Huang, F. et al. (2003). FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell 113(7)*, 829-840.

Zimmermann, W. H., Melnychenko, I., Wasmeier, G. et al. (2006). Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat. Med. 12(4)*, 452-458.

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