



## Session 7.3

# The contribution of the “OMICS”-technology to the 3Rs

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## Detection of Pain and Stress by Monitoring Gene Expression

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### Summary

*One of the main concerns with regard to animal experimentation is that if animals must be used for experimental purposes, pain and distress should be abolished or reduced to an absolute minimum. Regarding the principles of the 3Rs, considerable progress has been made concerning reduction and replacement, however to implement refinement it is necessary to improve our ability to objectively recognise signs of pain and distress.*

*Here we present preliminary results indicating that gene expression analysis with DNA microarrays results in characteristic molecular signatures, which allow the identification of different pain and stress levels in mice.*

*Keywords: pain, stress, microarray, gene expression*

### Introduction and questions

The clear definition and monitoring of pain and stress is an essential aspect in the refinement of animal experimentation, but it is still one of the most contradictory topics in science. Regarding the principles of the 3Rs, considerable progress has been made concerning reduction and replacement, however to implement refinement it is necessary to improve our ability to objectively recognise signs of pain and distress. Detection of pain/stress in laboratory mice is very important, not only from the welfare point of view but also because pain and fear, together with the resulting stress, may introduce confounding variability to scientific data. However, the objective definition of pain/stress in animals is very difficult. The measurement of physiological parameters like corticosteroids, glucose, growth hormone or prolactin in plasma implicates handling of the animals by the researcher, a process that directly induces supplementary stress to the animals (Seggie and Brown, 1975; Tabata et al., 1998; Zethof et al., 1994). On the other hand, the telemetry technology allows measurements of physiological paramete-

ters like heart rate, body temperature and blood pressure for long periods of time without direct interference. This system allows the detection of changes in the circadian rhythm or in the activity of the animals. This system was for example used to show that mice housed individually have a higher heart rate compared with mice housed in pairs (Spani et al., 2003). Nevertheless, this is a very expensive technology and the implantation of the transmitter is bound to painful surgical procedures that induce pain for several days and require analgesic treatment of the animals.

From the behavioural point of view it is difficult to detect distress in the mouse. The mouse lives in constant fear of falling prey to its enemies, therefore it shows as few signs of disease, suffering or weakness as possible. Accordingly, during animal experiments, or even when an investigator is simply present in the room, the mouse will hide almost all signs of light or middle grade pain. In consequence, there are no reliable indicators to detect low and middle grade pain in the mouse. Only when an animal is almost moribund can we recognise that it is suffering pain. As a result, demands for adequate pain therapy are often



ignored or met with the anthropomorphic attitude "as long as the mouse shows no pain, it must be feeling no pain".

Pain consists of both a sensory (discriminative) and an affective (the "unpleasantness") dimension, and can be acute or chronic depending on its duration. Chronic pain states can lead to secondary effects such as anxiety, depression and stress (Price, 2000).

Nociceptive information reaches the brain from the peripheral site of injury through multiple neuronal pathways (ascending pathways). Cells in the terminal regions within the forebrain and brainstem almost invariably project back, directly or indirectly, to the areas of origin of the ascending pathways. Areas like hypothalamus and amygdala are thought to play an important role in the modulation of the affective dimensions of pain and the control of the autonomic activity (Lovick, 1996; Lumb and Lovick, 1993).

Nociception is a complicated process consisting of short and long-term responses that serve as a warning, activated in response to impending damage to the organism. The threshold for eliciting pain has to be high enough so that it does not interfere with normal activities, but low enough to prevent tissue damage. This threshold is not fixed and can be shifted up or down, and it is this plasticity of the sensory system that essentially characterises pain syndromes (Woolf and Salter, 2000). Early short-term responses following nociceptor activation are reflected in rapid changes of neuronal discharge activity in a variety of anatomically distinct systems in the central nervous system. These are followed by long-term changes that most commonly require alterations in gene expression (for a review see (Scholz and Woolf, 2002)). The activity-dependent modulation of gene expression is a characteristic feature of highly integrated systems such as pain. Different molecules are involved at different levels of this process. Neurotransmitters, peptide hormones, neurosteroids, trophic factors or/and cytokines can be released from neurons, glial cells or components of the immune system and are involved in the integration of somatosensory information. Posttranslational and transcriptional changes can drastically change the threshold, excitability and transmission properties of the nociceptors. Especially in long-term changes, activity-dependent signal transduction cascades and signalling pathways downstream of the receptors bound by cytokines and growth factors act to modify transcription in nociceptor neurons. This changes the activities of the neurons, especially their transduction, conduction and transmission properties.

A major goal of pain research at the present time is the identification of pain genes. In the past few years, molecular genetic techniques allowed the study of pain at the level of the gene. Through different techniques, like the generation and analysis of transgenic mice, antisense knockdowns, DNA microarray-based expression profiling, and linkage mapping, it was possible to identify different genes directly involved in the processing of pain. Many different strains of knockout and overexpressing mice have been evaluated for their nociceptive sensitivity, both directly as the focus of the study and indirectly as part of standard behavioural screening protocols. The findings from many of these studies have been reviewed (Mogil and Grisel, 1998; Mogil et al., 2000).

These genes are interesting targets for anti-pain treatment, but are also of outstanding interest as diagnostic markers to monitor pain. Monitoring many genes at the same time became possible with the development of DNA microarray technology. DNA microarrays are among the most powerful and versatile tools of genomics and genetics research (Fodor et al., 1993; Lockhart et al., 1996; Lockhart and Winzeler, 2000; Southern et al., 1994). DNA arrays allow us to make quantitative parallel measurements of gene expression (mRNA abundance) for tens of thousands of genes. There are two dominant types of arrays that have been used for gene expression measurements. The first are high-density oligonucleotide arrays directly synthesised on a glass surface using light-directed combinatorial synthesis (Fodor et al., 1991) containing thousands of oligonucleotide sequences. The other main array type is produced by spotting cDNAs, PCR products or oligonucleotides at specific locations on a glass slide and is called low-density array.

A diagnostic microarray that can be used to monitor pain in the mouse and is specific for stress and pain does not exist yet. Only attempts to evaluate differential gene expression profiles in animal models of pain have been published previously, but no microarrays for the direct characterisation of pain itself have been described to date (Ko et al., 2002; Saban et al., 2002; Sun et al., 2002; Wang et al., 2002). Here we present the possibility to use DNA microarrays as a rapid, reliable and objective method to assess pain and stress in mice on the molecular level.

## Results

### Design of a low-density microarray for the detection of pain in mice

As a first step in the design of a low-density microarray, we started a careful search of the available literature for all genes related to pain, stress, and anxiety. We collected about 250 genes that directly or indirectly correlate with pain/stress. Of these 250 genes, 130 were selected for spotting on a low-density microarray. We decided to spot 70-nucleotide-long oligomers on the microarrays instead of the classical PCR-amplified gene fragments. The 70mer technology allows minimisation of the secondary structure, high melting temperatures, and therefore a normalised hybridisation temperature. We designed the 70mers by choosing their sequences within the last 750 nucleotides of the genes (3'-end), all oligonucleotides were checked by blast analysis to confirm that they are representative for the specified gene.

### Definition of pain models and time points for the validation of the microarrays

In order to validate the low-density microarray, we decided to use clearly defined time points in which animals experience pain. For this we decided to use a telemetry-based postoperative pain model. Monitoring of the surgical pain by telemetry allows us not only to determine whether the animals are feeling pain, but also to determine the time window in which gene expression changes can be analysed. The pain model used consists of two different experiments. First, a moderate/strong pain is induced



by the implantation of the transmitter. During this time, heart rate, body temperature, and activity of the mouse showed strong impairment of the circadian rhythms in the animals. A tendency to reach normal values is normally detected after 1-2 weeks. After 6 weeks of recovery, the mouse was subjected to a vasectomy (mild pain). A significant increase in heart rate could be measured one day after the operation. Tissues for microarray analysis were collected five days after the transmitter implantation (moderate pain) and one day after vasectomy (mild pain). At these time points different tissues (spinal cord, brain) were collected and total RNA was purified for the analysis on microarrays.

### Labelling and hybridisation of the microarrays

Brains and spinal cords were homogenised and total RNA was isolated. Labelling was performed by incorporation of amino-allyl-UTP (aa-UTP) in antisense RNA (aRNA) and subsequent labelling with cy-hydroxysuccinimide ester. This protocol allows amplification of small amounts of sample targets and was sufficiently sensitive for the analysis of brain and spinal cord from mice after vasectomy and transmitter implantation. After comparison of the data obtained from probes after transmitter implantation and after vasectomy, we could identify about 60 genes that displayed clearly modulated expression in mice with pain (tab.1).

### Discussion

All experimental work with animals has to be monitored by careful assessment and minimisation of pain and stress. The same holds true for breeding of mutant animals. Pain and stress conditions are not only linked to the experimental procedures, but often also depend on the genetic mutations that the animals carry. However, especially in genetically modified animals, it is very difficult to correctly identify distress and pain. Often it is impossible to predict whether the animals will feel pain or will be stressed, because of insufficient knowledge on the gene that is being modified.

The assessment of pain and stress in laboratory rodents is difficult and often subject to painful investigations (e.g. telemetry studies). Telemetry is a very reliable technology, but requires

experienced personnel for the implantation of the transmitters, a long time for the analysis of the measurements and is very expensive. For these reasons it is not possible to use telemetry as a routine diagnostic method for the detection of pain.

Microarray analysis in contrast is a very powerful technique that allows the fast and reliable measurement of gene expression changes for hundreds of genes in a relatively short time.

Microarray analysis can be used alone or in parallel with behavioural observations in order to clearly define stress. By using post-mortem biopsy material, the microarray technology is – from the point of view of animal welfare – much gentler, because it does not require any surgical implantation into the animals and the animals thus do not need to recover from surgery.

The data presented here represent a preliminary analysis based on a single pain model (postoperative pain model). Further experiments with tissues from other pain models will be necessary to finally validate this diagnostic tool. In general these data suggest that it is possible to identify specific molecular signatures and that these can be considered a valuable tool for effective pain detection and control.

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**Tab. 1: Genes spotted on the low-density microarray and genes differentially expressed in a model for surgical pain. Genes were considered as representative for pain when the expression ratio between control animals and animals with pain was more than two-fold. Not listed are control genes needed for normalisation and for checking the quality of the hybridisation.**

Gene family	No. of genes present on the array	Genes involved in surgical pain
Receptors	51	31
Neuropeptides	10	6
Oxidative stress	9	4
Hormone-related genes	22	11
Neurotrophins	4	2
Heat shock	5	3
Channels	2	1
Cytokine-related genes	8	3



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