Session 7.2
Innovative approaches for alternative methods development

RNA Interference: A Novel Alternative Approach in Nephrotoxicity Studies

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Summary

One of the main goals in functional genomics has been the development of tools that allow easy manipulation of gene expression levels that would be suitable for high throughput screening. RNA interference (RNAi) has emerged as one of the preferred approaches to achieve this goal. It is an important biological mechanism in the regulation of gene expression in animals and plants.

Here we evaluated the use of RNAi for knockdown of specific gene expression as an alternative to the production of transgenic animals. RNAi development may be in the early stages, however, the real and theoretical advantages of this system in reducing the use of animals merit further investigations.

Keywords: RNA interference, siRNA, nephrotoxicity, EMT, Rap, renal fibrosis

Introduction

RNA interference (RNAi) is a term used to describe the sequence-specific suppression of genes, initiated by double-stranded RNA (dsRNA) oligonucleotides homologous to the sequence of the silenced gene (Hammond et al., 2001). RNAi was first observed in *Caenorhabditis elegans* as a response to exogenously introduced long double-stranded RNA (dsRNA) (Fire et al., 1998). While the mechanism of RNAi has yet to be fully characterised, silencing appears to be mediated by duplexes of 21-23 nucleotides (nt), called small interfering RNAs (siRNAs), which are degradation products of longer double-stranded RNAs, cleaved by the RNAse III-like enzyme Dicer. The resulting siRNAs are incorporated into a multi-component complex known as RISC (RNA-induced silencing complex) that targets and cleaves mRNA complementary to the siRNAs (Hamilton et al., 1999; Tuschl et al., 1999; Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001; Hammond et al., 2001). An overview of the RNAi mechanism is shown in fig. 1.

More recently, short endogenous RNAs sharing similarity to siRNAs, called microRNAs (miRNAs), have been identified as key post-transcriptional regulators in such varied organisms as plants, nematodes and mammals (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2001; Reinhart et al., 2002). These miRNAs are initially transcribed as a long RNA and then processed to a pre-miRNA of ~70 nt (Lee et al., 2002). This pre-miRNA forms an imperfect hairpin structure, which is then processed by Dicer enzyme to produce the mature, single-strand ~21-23 nt miRNA (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

While this is an innate process in cells, most probably an evolutionary mechanism to protect against cellular attack from invasive viral RNAs, the mechanism provides researchers with

an excellent tool for gene silencing. Knock-out mice are traditionally the ultimate way of demonstrating gene function, however knocking out a gene that is crucial for development can be a terrible waste if the mice die before a discernable phenotype can be established. RNAi allows researchers to knock out several genes *in vitro* in a fraction of the time required to generate a knock-out mouse.

3R relevance

The relevance of this article to the goal of replacement, refinement and reduction of animal testing is due to the future ability of RNAi to possibly replace most if not all animals used in the generation of knock-out mice.

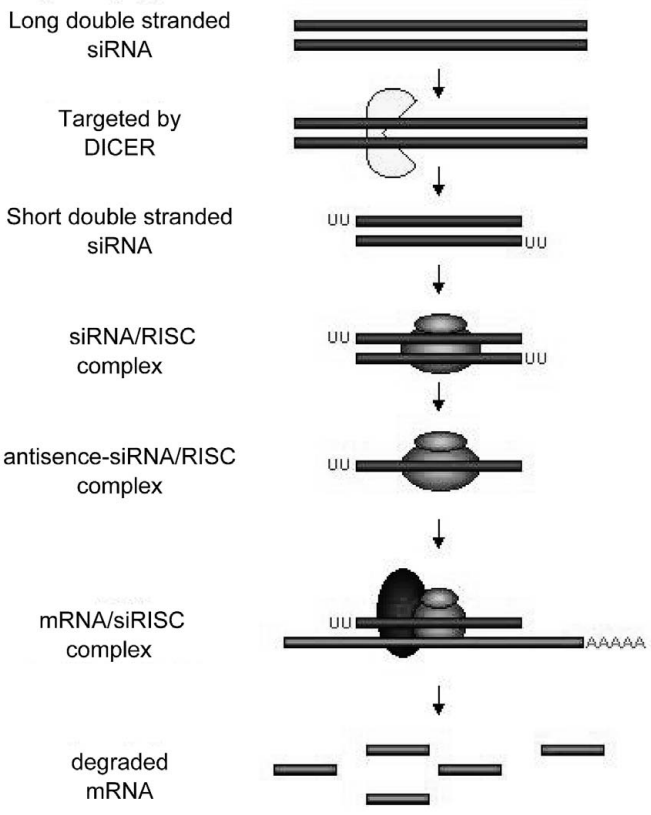

Fig. 1: Hypothesised mechanism of RNAi-mediated gene silencing
Long dsRNA molecules are targeted by the DICER enzyme and cleaved into smaller dsRNA molecules. These small dsRNAs are bound by the RISC complex, which separates and discards the non-complementary strand. The complementary strand directs the RISC complex to the target mRNA and cleaves it. Cleaved RNA is degraded by cellular proteases.

Materials and methods

Reagents

Silencer™ siRNA Construction Kit was obtained from Ambion Inc. Lipofectamine 2000 transfection kit was obtained from Invitrogen. siPORT amine, siPORT lipid transfection kits were obtained from Ambion Inc. Antibodies were obtained from BD Biosciences. PCR primers were supplied by Sigma-Genosys (UK). Dulbecco's Modified Eagles Medium / Nutrient Mix F12 (DMEM/F12) was obtained from Gibco BRL Ltd (UK). Cell culture dishes and slides were purchased from Falcon (UK).

Cell culture

HK-2 cells were maintained in Dulbecco's Modified Eagles Medium/Nutrient Mix F12 (DMEM/F12) (Gibco BRL Ltd, UK) containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 pg/ml triiodo-l-thyronine, 10 ng/ml epidermal growth factor, 50 U/ml penicillin, 50 µg/ml streptomycin and 2mM glutamine. Culture medium was changed every second day. Cells were grown to confluency in 75 cm^2 Falcon flasks and maintained at 37ºC in a humidified atmosphere containing 95% air, 5% CO_2.

siRNA design

Sequences targeted for RNAi were scanned for occurrences of AA, using Target Finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). This online tool identifies occurrences of AA in a target sequence and lists it with the subsequent 19 downstream nucleotides. Target siRNAs that had a low GC content (30-50%) and, where possible, were not within 50 bases of the ATG start codon were considered. Targets were analysed by BLAST search and only siRNAs that had no significant homology with other genes were selected.

siRNA synthesis and transfection

Small interfering RNAs (siRNAs) were synthesised with the Silencer™ siRNA Construction Kit (Ambion) as directed by the manufacturer, with the exception that half reaction steps were used. Template siRNA oligonucleotides were designed and DNA oligonucleotides corresponding to the sense and antisense strands of the siRNAs were synthesised and desalted (Sigma-Genosys). In separate reactions the sense and antisense DNA oligos were annealed to the T7 promoter primer and subsequently filled in with the Klenow fragment of DNA polymerase. RNA was transcribed from these double-stranded DNA templates using T7 RNA polymerase. The sense and antisense RNA reactions were hybridised and digested with RNase to leave double-stranded 21-mer RNA duplexes with UU overhangs. siRNAs were purified by column chromatography and quantified by UV spectrophotometry. siRNA corresponding to 30 nM was mixed with Lipofectamine 2000 (Invitrogen) as directed by the manufacturer and the mixture was used for transfection of the cells.

RT-PCR analyses

Total RNA was extracted from cells using TRIzol reagent (Life Technologies-BRL). Briefly, a 5 µg sample of total RNA from each treatment group was used to generate cDNA with the

Gibco-BRL RT kit. 2 µl of the cDNA was taken from each sample and used in a PCR reaction containing 1 µl of forward primer (100 ng/µl), 1 µl of reverse primer (100 ng/µl), 2 µl of 10 mM dNTPs, 5 µl of 10X buffer, 3 µl of 25 mM MgCl2, 0.25 µl of Taq Polymerase (Promega). The number of PCR cycles performed was determined to be within the linear range of the reactions.

Western blot analyses

Whole cell extracts of control and treated cells were obtained in radioimmune precipitation assay buffer (50mM Tris-HCl, 150mM NaCl, pH 7.5), and analysed for the indicated molecules by Western blot and enhanced chemiluminescence detection as described previously (Slattery et al., 2005).

Results

RNAi optimisation

To optimise RNAi in HK-2 cells, siRNAs directed against GAPDH were synthesised and delivered using various transfection reagents (siPORT amine, siPORT lipid, Lipofectamine 2000) and concentrations (1nM-100 nM). Initially, higher concentrations (50 and 100 nM) of siRNA were used to identify the most suitable transfection reagent. RT-PCR was performed on RNA isolated from HK-2 cells transfected with 50 or 100 nM siRNA using 3 different transfection reagents. It was determined that Lipofectamine 2000 was the most effective transfection reagent for siRNA transfection of HK-2 cells. However high concentrations (50-100 nM) of siRNA can mediate non-specific gene silencing (Elbashir et al., 2001), therefore it was necessary to further optimise conditions using lower concentrations (10 or 30 nM) of siRNA with Lipofectamine 2000. Using RT-PCR for GAPDH, 30 nM of GAPDH siRNA was established to have approximately 50% silencing efficiency. β-Actin was used as a loading control. A similar decrease was detectable by Western blot analysis.

Specific gene silencing using RNA interference

Rap genes were identified as being differentially expressed in *in vitro* models of nephrotoxicity, therefore Rap protein expression was silenced using gene-specific siRNAs. In an *in vitro* model of nephrotoxicity, siRNAs directed against either Rap1 or Rap2 were investigated. In treated cells transfected with Rap1 siRNA there was a decrease in Rap1 protein levels, but not in Rap2 levels. Concurrently, TECs treated with Rap2 siRNA demonstrated a decrease in Rap2, but not Rap1 protein levels.

Discussion and conclusion

Functional investigation of the complex regulation of molecular switches and their effectors are key to understanding organ toxicity, including nephrotoxicity. Nephrotoxicity is caused by several drugs, such as immunosuppressive agents, and is associated with the development of renal fibrosis. We have established several *in vitro* models of nephrotoxicity and have identified key genes, using both microarray and differential gene expression (SSH) technology, which we believe are involved in this process. We have used RNAi to analyse the role of these genes in the development of nephrotoxicity and we are currently examining the possible involvement of endogenous miRNA regulation of candidate genes. A number of interesting differentially expressed genes are being examined further as potential therapeutic targets.

In conclusion we have demonstrated that silencing of key genes with RNAi has helped to elucidate their role in the development of renal fibrosis and nephrotoxicity. These results not only indicate the specificity and sensitivity of the siRNAs used, but also show the potential of RNAi as a replacement for animal studies. Finally, we believe that the potential role of RNAi as an alternative to animal models is just beginning to be realised.

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Detecting Neurotoxicity Through Electrical Activity Changes of Neuronal Networks on Multielectrode Neurochips*

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Summary

The innovative alternative method of multielectrode neurochip recording, which offers improvements compared to standard animal experimentation, has been further optimised by advanced multi-parametric data analysis and used to study toxicity patterns of known toxicants. Neuronal networks on 64-electrode neurochips develop long-term stable spontaneous electrical activity. Changes in spatio-temporal electrical activity were quantified and subjected to pattern analysis to evaluate the effects of selected neurotoxic compounds. Several examples underline the suitability of this broadband biosensor system for functional neurotoxicity testing and safety pharmacology. Our results demonstrate that neuronal networks retain tissue-specificity and respond to transmitter receptor blockers and other neurotoxic compounds in a substance-specific, dose-dependent manner.

Keywords: functional neurotoxicity, multielectrode arrays, multiparametric data analysis, primary neuronal networks

Abbreviations: TMT, trimethyltin chloride; CV, coefficient of variation; GABA, γ-aminobutyric acid

Introduction

Spontaneously active networks in culture have been proposed as a sensitive and efficient model system to study the neurotoxic properties of chemicals, as biosensors, and to accelerate drug development (Nelson, 1978; Gross, 1994; Gross et al., 1997). In contrast to patch-clamp and imaging methods, the neurochip technology allows online and real-time analysis of up to 256 neurons in short- and long-term studies.

Primary cultures of central nervous tissue on multielectrode neurochips offer the potential to study neuroactive properties of compounds that are technically difficult or impossible to obtain *in vivo*. *In vitro* studies can replace animal experiments that would often deliver only qualitative data. Neuronal networks represent the functional units of information processing in the brain. Due to their high level of complexity they reflect major aspects of neuronal function in mammals. Multielectrode neurochip recordings allow detection of functional deviations in developing, adult and ageing brain regions and provide insights into the mechanisms of action of neuroactive compounds.

Here we investigated whether primary neuronal networks from frontal cortex and spinal cord retain the specific receptor endowments of their different parent tissues by applying toxins acting as antagonists at the γ-aminobutyric acid (GABA) and glycine receptors. The antifouling agent trimethyltin chloride (TMT) was chosen as a representative neurotoxicant in animal models (Wenger et al., 1982; Chang and Dyer, 1983; Aschner and Aschner, 1992) to demonstrate that our method is suitable to determine the neurotoxic potential of exogenous substances (Gramowski et al., 2000). Additionally, the neurotoxic potential of human blood preparations from hepato-encephalopathic coma patients containing complex mixtures of endogenous neurotoxic metabolites (Loock et al., 2004) was studied in order to detect their influence on network electrical activity.

Materials and methods

Chemicals and test samples

The chemicals bicuculline methiodide, 5-fluoro-2'-deoxyuridine + uridine (FDU), picrotoxin, poly-D-lysine, strychnine hydrochloride and trimethyltin chloride were from Sigma-Aldrich Chemical GmbH (Taufkirchen, Germany). DNase I (from bovine pancreas), laminin and papain were purchased from Roche (Mannheim, Germany), foetal bovine serum from Pan Biotech GmbH (Aidenbach, Germany). Horse serum, Minimum Essential Medium (MEM) and Dulbecco's Modified Essential Medium (DMEM) were from GIBCO BRL (Paisley, UK). Blood plasma samples from hepato-encephalopathic patients were obtained during an FDA approval study and were provided by Teraklin AG (Rostock, Germany).

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Multielectrode neurochips

Multielectrode neurochips were provided by the Center for Network Neuroscience (CNNS) at the University of North Texas. The fabrication techniques and the culture methods have been described previously (Gross et al., 1985; Gross, 1994). Briefly, commercially available, sputtered indium tin oxide (ITO) plates were photoetched, cut into 5x5 cm^2 plates, and spin-insulated with polysiloxane. The electrode tips were de-insulated and electroplated with a thin layer of gold to lower the interface impedance. Flaming through a stainless steel mask created a confined adhesive region (1-4 mm diameter islands centred on the electrode array), which was coated with poly-D-lysine (25 µg/ml; 30-70kD) and laminin (16 µg/ml).

Cell culture

Cortical and spinal cord tissues were harvested from embryonic day 16 and 14 NMRI mice (Charles River Inc., Sulzfeld, Germany), respectively. The mice were sacrificed by cervical dislocation in compliance with the German Animal Protection Act. The tissue was cultured according to the method of Ransom et al. (1977) with minor modifications that included the use of DNase I (8000 units/ml) and papain (10 U/ml) for tissue dissociation. The tissue was dissociated enzymatically with papain and mechanically with transfer pipettes, and seeded in DMEM (Auditory and Frontal Cortex) or MEM (Spinal Cord) 10/10 (10% horse and 10% foetal calf serum) at a density of $0.5x10^6$ cells/cm^2 onto neurochips (Gross et al., 1993; Gramowski et al., 2004). Cultures were incubated at 37°C in a 10% CO_2 atmosphere until ready for use, usually three weeks to three months after seeding (fig. 1). Culture media were replenished three times a week with DMEM or MEM containing 10% horse serum. The networks develop from a mixture of different types of postmitotic neurons and glia cells. The glia cells have important auxiliary functions regarding metabolism and supply the neurons with ions and nutrients. The co-culture of neurons with glia cells ensures stability over many months. Within the first week (usually after 3–4 days *in vitro*), the developing cultures were treated with 5-fluoro-2'-deoxyuridine (25 µM) and uridine (63 µM) for 48 h to prevent further glia proliferation. Primary spinal cord, frontal and auditory cortex networks cultured on multielectrode neurochips routinely develop spontaneous electrical activity. Activity starts after approximately three to four days *in vitro* in the form of random spiking which, after 3 weeks in culture, stabilises into complex activity patterns, composed of coordinated bursting and interburst spiking (Gramowski et al., 2004). Such networks can remain spontaneously active and pharmacologically responsive for more than six months (Gross, 1994). For this study, cultures between 26 and 113 days *in vitro* were used.

Microscopy

Networks were studied by DIC Nomarski, conventional and confocal scanning immunofluorescence microscopy and diaminobenzidine (DAB) staining for neuronal or glial markers with an Eclipse 800 from Nikon (Düsseldorf, Germany) and TCS-SP2 from Leica (Bensheim, Germany) at the Rostock University Live Cell Imaging Center. Scanning electron microscopy was performed with a Zeiss DSM 960A (Oberkochen, Germany) at the Rostock University Electron-Microscopy Center (EMZ).

Extracellular recording

The multielectrode neurochips were maintained in a recording chamber (Gross, 1994) at 37°C under humidified 10% CO_2 atmosphere to ensure a constant pH of 7.4. Recording was performed with the Multichannel Acquisition Processor System, a computer-controlled 64-channel amplifier system from Plexon Inc. (Dallas, TX, USA) providing programmable amplification, filtering, switching, and digital signal processing of multielectrode signals. The total system gain used was 10 K with a simultaneous 40 kHz sampling rate. The multichannel signal acquisition system delivered single neuron spike data. Spike identification and separation were accomplished with a template-matching algorithm in real time (fig. 2). This was also used to determine action potential shape and amplitude changes during the course of an experiment.

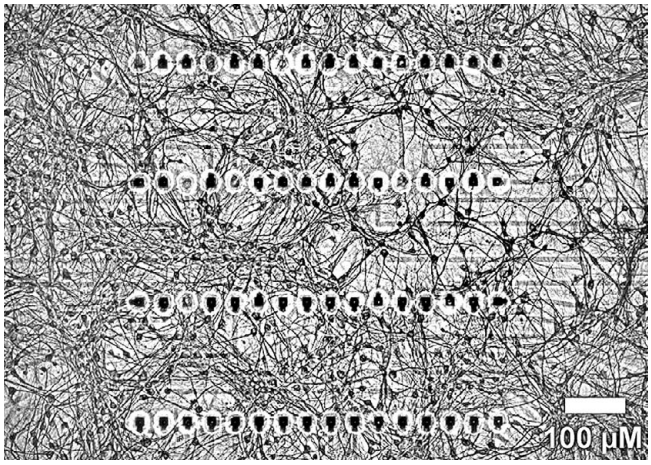

Fig. 1: Dissociated frontal cortex network on a multielectrode neurochip after 21 days *in vitro*. The culture was stained with DAB against neurofilament and MAP2.

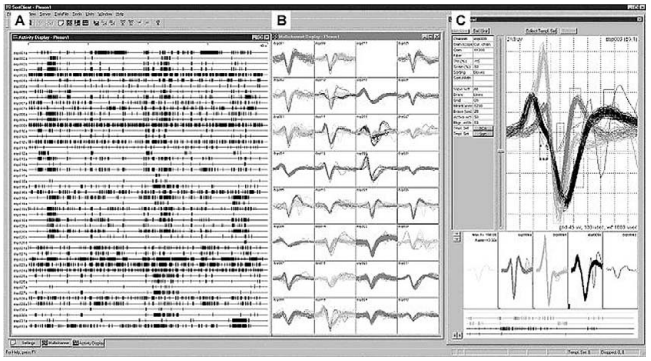

Fig. 2: Graphical user interface displaying online the native electrical activity of a spinal cord culture. (A) 40 seconds of neuronal activity. Each action potential is represented by a vertical tick. (B) Overlaid action potentials at active electrodes. (C) Separation of multiple neurons (bottom) from one electrode (top), which captures the signals from several neurons.

Data analysis

Spikes in spike trains are clustered in so-called bursts. These represent the ubiquitous and prominent feature of network activity and are an essential aspect of the neuronal code (Gross, 1994; Lisman, 1997). We extracted a total of 67 activity describing parameters to quantify the substance specific activity changes: 31 parameters derived from spike train data, their derived 31 normalised values to compensate for the variability of the activity in different cultures and five parameters derived from the dose-response curves. Bursts were determined by spike train analysis based on spike intervals with the software NeuroEXplorer (Plexon Inc., Dallas, TX, USA). Spike rate and burst rate values were derived from 60 s bin data. The dose response curves were fitted to the Hill equation, determining the effective concentration causing 50% of the maximal response (EC_{50}). The spike and burst parameters were additionally processed to derive temporal and network coefficients of variation (CV_{TIME} and $CV_{NETWORK}$) for various activity features. These CVs were used to describe the spatiotemporal behaviour of the network activity (Keefer et al., 2001; Gramowski et al., 2004). CV_{TIME} reflects the periodic behaviour of a single neuron's activity pattern and was already used in earlier studies, analysing single channel recording activity (Bracci et al., 1996). $CV_{NETWORK}$ reveals the coordination between different neurons in a specific activity state and is a measure of synchronicity.

Statistics

Results are expressed as series means ± SEM. The features' distributions were tested for normality. The level of significance after compound application was assessed using Student's paired t-test. For assessing the difference between sets of data, Student's unpaired t-test was used. $P < 0.05$ was considered statistically significant.

Results

In vivo like tissue specificity

We compared the spiking and bursting activity of spinal cord and frontal cortex networks to demonstrate that *in vitro* networks are pharmacologically histiotypic representations of their parent tissues. Neurotoxins with known site of action, namely the inhibitory $GABA_A$ and glycin receptor systems were used. Disinhibition of the electrical activity was achieved by blocking the $GABA_A$ receptors with 40 µM bicuculline ("BCC" activity) or the glycine receptors with 1 µM strychnine ("STR" activity), while complete disinhibition was reached with both compounds ("BCC+STR" activity).

Blocking the inhibitory circuits with bicuculline and/or strychnine elicited rhythmic and synchronised activity with clear-cut differences between both tissues. Strychnine increased the burst rate of spinal cord networks to 815% of native activity, while in frontal cortex networks it increased only to 127%. Bicuculline induced an increase in spike rate to 457% for spinal cord networks, compared to 204% in the case of frontal cortex networks (fig. 3). Thus, inhibition in frontal cortex cultures is mainly mediated by $GABA_A$ receptors, but in spinal cord cultures by glycine receptors. This reflects the *in vivo* situation and demonstrates that important aspects of tissue specificity are retained *in vitro*. The 7-fold increase in spike rate in spinal cord networks after complete blockade of inhibitory receptors indicates that spinal cord is more mediated by inhibitory circuits than frontal cortex with a twofold increase.

Blocking specific receptors

To demonstrate the high sensitivity of the network activity changes to specific receptor blockers we chose the $GABA_A$ receptor antagonists bicuculline and picrotoxin. Both inhibit

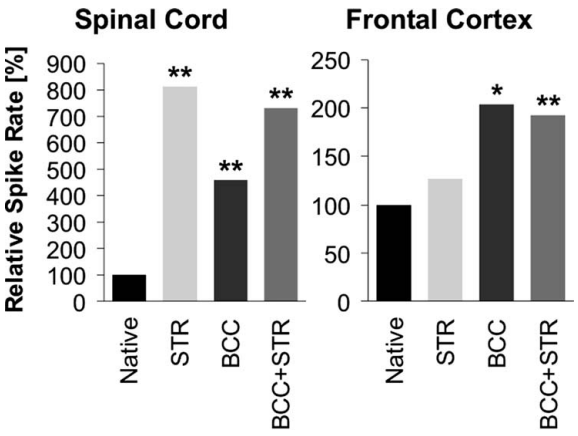

Fig. 3: Tissue-specific responses of spinal cord and frontal cortex networks. Spiking activity increases after blockade of the inhibitory $GABA_A$ receptors with 40 µM bicuculline (BCC) and/or glycine receptors with 1µM strychnine (STR). There is a stronger influence of GABA in the frontal cortex in contrast to a stronger influence of glycine on the spinal cord. (*, $p<0.05$; **, $p<0.01$).

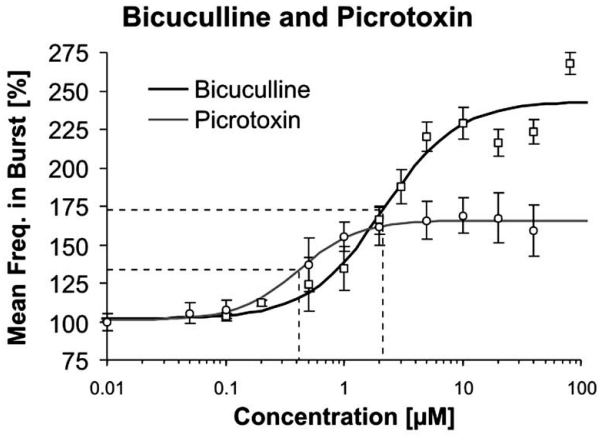

Fig. 4: Dose response curves for the two $GABA_A$ receptor antagonists bicuculline (n=6) and picrotoxin (n=7). Although in both cases $GABA_A$ receptor mediated inhibition is blocked, the different modes of action cause different responses. The changes of the mean frequency of spikes in bursts distinguish the two neurotoxins.

$GABA_A$ receptor transmission. Bicuculline blocks the GABA binding site, whereas picrotoxin clogs the Cl^- channel. Both compounds caused an increase in overall network activity with EC_{50} values of 2.1 µM for bicuculline and 0.4 µM for picrotoxin (fig. 4). They also increased network activity synchronisation and oscillation and enhanced bursting by increasing spike frequency in bursts. However, bicuculline made the burst shape significantly more uniform than picrotoxin. This was revealed by a lower CV_{TIME} of spikes in bursts. Since these severe deviations from native activity patterns were caused by the neurotoxins, such disturbances are indicators of functional neurotoxicity.

Tissue-specific neurotoxicity

Two different tissues of the mouse CNS (spinal cord and auditory cortex) exhibited characteristic and dose-dependent changes of their electrophysiological activity patterns after treatment with trimethyltin chloride (TMT), a standard neurotoxicant. Spinal cord networks began to respond at 1-2 µM, and shut off activity at 4-7 µM TMT. Auditory cortex cultures first responded at 2-3 µM; shut-off occurred at 7-8 µM TMT. Repeated applications of low doses of TMT always influenced the electrical activity in a reversible manner, with no overt cytotoxic effects. The EC_{50} values were 1.5 ± 0.5 µM (spinal cord) and 4.3 ± 0.9 µM (auditory cortex), indicating a relatively low variability among different networks of one tissue type (fig. 5). The non-overlapping EC_{50} range for cortical and spinal cord cultures suggests tissue specificity for network responses to TMT. Shut-off concentrations are within a factor of two of the lethal concentrations reported for mice *in vivo*. Action potential amplitude and shape did not change even when complete cessation of activity was approached, suggesting that acute TMT applications did not affect neuronal metabolism that may lead to a lowering of membrane potentials (fig. 6).

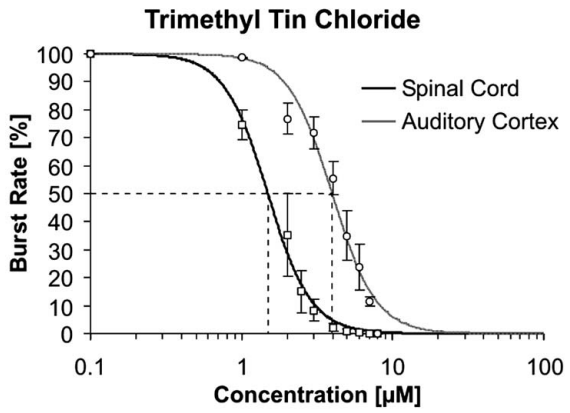

Fig. 5: Dose response curves of trimethyltin chloride on 5 spinal cord and 3 auditory cortex networks. EC_{50} values are 1.5 ± 0.5 µM for spinal cord and 4.3 ± 0.9 µM for auditory cortex, demonstrating tissue-specific sensitivity to this neurotoxicant.

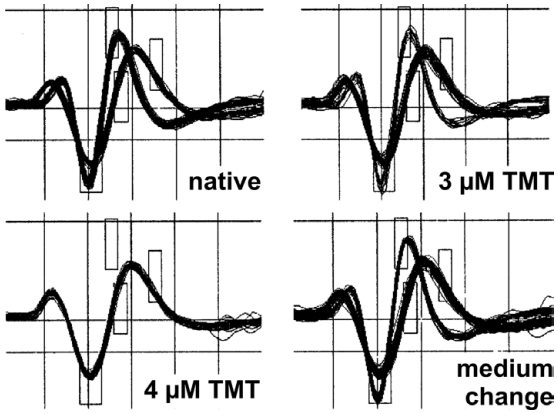

Fig. 6: Multiple superimposed action potential traces from two spinal cord neurons. They were simultaneously recorded from one electrode showing action potential stability despite exposure to 3 and 4 µM trimethyltin chloride (TMT) as well as reversal of neuronal shut-off after adding fresh medium (lower panels). These signals measured 405 and 309 µV peak-to-peak with a duration of 1.5 ms.

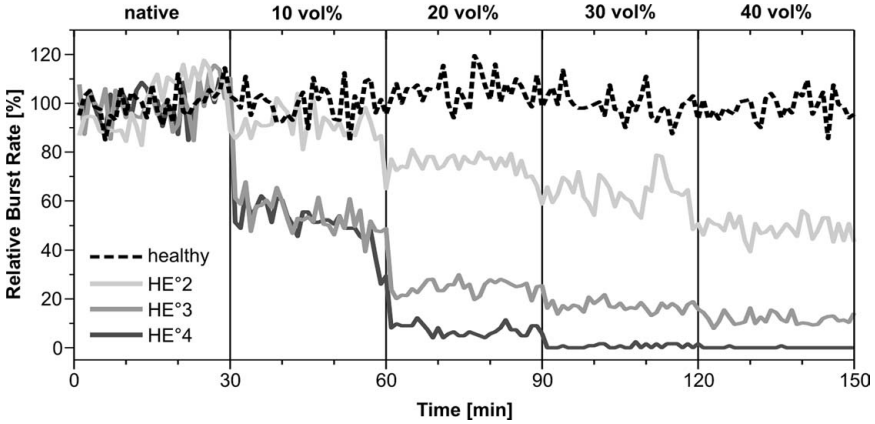

Fig. 7: Burst rate from four networks with application of blood ultrafiltrate from healthy volunteers and patients with grades of hepatic encephalopathy from HE grade 2 to 4. The burst rate decreases with increasing hepatic coma stage and volume percentage of ultrafiltrate in the culture medium.

Endogenous neurotoxins

Our system was used to monitor the CNS intoxication in hepatic encephalopathy patients. In liver failure, water-soluble metabolites as well as predominantly protein-bound compounds accumulate in the blood plasma and promote the development of encephalopathy and coma. Plasma ultrafiltrates contain the water-soluble compounds, while plasma extracts contain the protein-bound compounds. Therefore, both blood preparations were tested for their influence on network activity due to neurotoxic compounds. Plasma ultrafiltrates and extracts from healthy subjects caused only minor changes to the network activity. In contrast, ultrafiltrates from coma patients with hepatic encephalopathy (stage II-IV) predominantly decreased spike rate and burst rate (fig. 7) in a dose-dependent manner. Extracts of plasma from such patients altered network activity in a similar manner and additionally increased burst duration (data not shown).

Discussion and conclusion

Nervous tissues express patterns of electrical activity as part of their normal function. Any major interference with these patterns can generate behavioural and/or autonomic malfunctions in response to a toxic agent. The cessation of electrical activity, even if not associated with cell death ("functional neurotoxicity"), can lead to the death of the organism. Also, major changes in pattern generation can severely alter the performance of organisms without necessarily threatening survival. This is generally categorised as "behavioural toxicity" (Fiedler et al., 1996). Our studies primarily deal with functional neurotoxicity during acute exposure. The detected similarity in concentration ranges for the *in vivo* and *in vitro* situation suggests that neuronal networks *in vitro* allow a quantitative assessment of functional neurotoxicity. Whole network responses are closer to the animal situation than data from single cell studies.

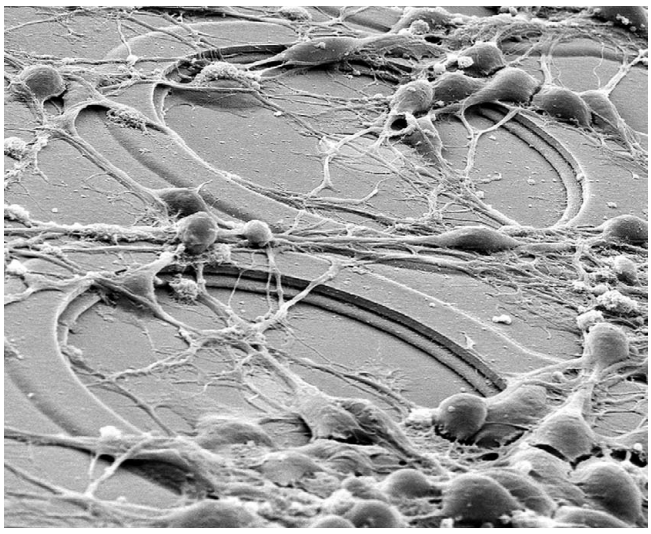

Fig. 8: SEM picture of a neuronal network growing on palladium electrodes of a CMOS silicon neurochip two days after seeding.

The multielectrode neurochip recording from functional neuronal networks is the experimental approach of choice when modifications of activity patterns by compounds are to be detected. A refined approach that we use for data analysis is performed with methods of pattern recognition, which allows the analysis of the significance and relevance of the multitude of computed features (up to 200) that are used to quantitatively describe the activity patterns. In this way a better understanding of features that are significant to describe toxicity is possible by using feature score and feature selection methods well known from pattern recognition. In this manner evidence of several burst features such as shut-off, changes in bursting patterns, in rhythmicity, overexcitation, EC_{50} values or reversibility are assigned to known toxins to characterise significant changes. In a second step the toxicity of substances is classified by their spike train responses and used to judge the toxic potential of unknown substances.

A quantitative database of neuroactive effects in specific nerve tissues *in vitro* provides a tool to classify known and unknown neuroactive compounds. Detailed knowledge of the *in vitro* effects will greatly contribute to risk assessment, although it is difficult to predict which specific symptoms in whole animals may be correlated with the different features of network activity. However, we have previously discussed how this system can be used to generate databases of well-characterised substance "fingerprints", which allow detailed comparisons of the activity spectra of neurotoxic substances (Gramowski et al., 2004).

A new generation of multi-sensor neurochips in silicon CMOS (Complementary Metal Oxide Semiconductor) technology will provide a further considerable improvement of this approach (fig. 8). It allows, in addition, online monitoring of the metabolic state of the cell system by integrated physiological sensors for temperature, oxygen consumption and pH value (Baumann et al., 2004; Krause et al., 2006).

3Rs

The multielectrode neurochip technology offers a *refinement* by giving multiparametric, fingerprint-like descriptions of the action of toxic compounds on nervous tissues. In addition, it reduces the number of animals required for neurotoxicity testing. From one parent mouse, neuronal tissue for 40-50 neurochips can be derived. Further, with each of these neurochips, dose response series with 10 to 15 concentration steps, single dose repeats, or chronic experiments can be carried out. This will reduce the number of animal experiments needed for the study of toxic compounds by a factor of up to 500 and at the same time yield much more detailed information on the mode of action of the compounds. Additionally, cultures from knock-out mice can *replace in vivo* animal disease models.

Different from patch clamp and brain slice techniques, this approach allows long-term studies (for weeks and more) as well as the testing of complex mixtures and unknown compounds. It is closer to the real situation in the nervous system, because it makes accessible the complexity level of multicellular functional ensembles, and not only that of single cells or single channels. This is also a reason why this technique is expected to give fewer false negative results than other *in vitro* techniques.

Other applications, especially in pharmacology and drug development, are presently also studied and they will, in addition to toxicity testing, open additional avenues of improved drug research and animal experiment reduction (Gramowski et al., 2004; Stüwe et al., 2005). Therefore, the application of the multielectrode neurochip technology will certainly contribute to the 3Rs of alternative methods for animal experiments and at the same time improve the quality of risk assessment and consumer protection.

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