NEUROTOXICITY OF THE OLFACTORY TOXICANT 2,6-DICHLOROPHENYL METHYLSULPHONE IN THE OLFACTORY BULB: IMPAIRED EXPRESSION OF GENES RELATING TO NEURODEGENERATIVE DISEASE

Bergström, U[1*], Olsson, JA[1*], Hvidsten, TR[2], Komorowski, J[2], Brandt, I[1#]

[1]Dept of Environmental Toxicology, and [2]The Linnaeus Centre for Bioinformatics, Uppsala University, Sweden
* These authors contributed equally to this work.

Abstract:
2,6-Dichlorophenyl methylsulphone and a number of structurally related chemicals are CYP-activated toxicants in the olfactory mucosa in mice and rats. This toxicity involves both the olfactory neuroepithelium and its subepithelial nerves. In addition, 2,6-dichlorophenyl methylsulphone induces glial acidic fibrillary protein expression (Gfap, a biomarker for gliosis) in the olfactory bulb, as well as long-lasting learning deficits and changes in spontaneous behavior in mice and rats. In the present study recently reported in part by Bergström et al[1], we used 15k cDNA gene arrays and real-time RT-PCR to determine 2,6-dichlorophenyl methylsulphone induced effects on gene expression in the olfactory bulb in mice. Seven days following a single ip dose of 2,6-dichlorophenyl methylsulphone, 56 genes were found to be differentially expressed in the olfactory bulb. Forty-one of these genes clustered into specific processes regulating or associated with, for instance, cell differentiation, cell migration, apoptosis and Alzheimer's disease. The genes selected for real-time RT-PCR were chosen to cover the range of B-values in the cDNA array analysis. Altered expression of Gfap, Rnr2, Ncor1 and Olfml3 were confirmed. Nine of the differentially expressed genes have been associated with Alzheimer's disease.

Introduction

2,6-Dichlorophenyl methylsulphone and the structurally related herbicide dichlobenil are CYP-activated olfactory toxicant in rodents. The toxic lesions induced by these compounds involve both the olfactory neuroepithelium and the subepithelial olfactory nerves. In the olfactory bulb, a strong and persistent induction of glial acidic fibrillary protein (GFAP, a biomarker for gliosis) is observed.[2,3] In addition 2,6-dichlorophenyl methylsulphone induces long-lasting learning deficits and changes in spontaneous behaviour (hyperactivity-like) in mice and rats.[4] As revealed by autoradiography, 2,6-dichlorophenyl methyl-(^{14}C)sulphone is transferred to the olfactory bulb (unpublished information).
As a first step to elucidate the mechanism of persistent neurotoxicity of 2,6-dichlorophenyl methylsulphone, we employed 15K cDNA gene arrays and quantitative RT-PCR to determine the effects on gene expression in the olfactory bulb in mice.

Materials and methods

Chemicals
2,6-dichlorophenyl methylsulphone (97% purity) was purchased from Synthelec AB, Lund, Sweden.
Animals
Female NMRI mice were acclimatized for 2 weeks before start of the experiment. They were housed at 23°C with a 12/12-light/dark cycle.
Dosing and tissue preparation
Groups of 16 mice received an ip injection of 2,6-dichlorophenyl methylsulphone (32 mg/kg), dissolved in corn oil. Thirty-two control mice received corn oil only. Seven days after administration, the animals were killed by cervical dislocation. The olfactory bulbs were rapidly dissected, immersed in RNAlater (Ambion, Huntingdon, UK), and stored at –20°C until further processing.
RNA preparation
Total RNA was extracted from the olfactory bulbs using Micro to Midi total RNA purification system (Invitrogen, Paisley, UK). Possible traces of DNA were removed from the samples by using DNA-free (Ambion). Total olfactory bulb RNA from 2,6-dichlorophenyl methylsulphone treated mice was pooled into four groups each, with RNA from four animals in each pool. For vehicle treated animals, a single reference pool of RNA was produced.

Microarray experiments

Gene expression in the olfactory bulb of vehicle treated mice was compared to the expression in the olfactory bulb of 2,6-dichlorophenyl methylsulphone treated mice. A reference design including dye reversal was used for hybridizations to cDNA-spotted 15k mouse arrays with genes spotted in duplicates (University Health Network Microarray Centre, Toronto, Ontario, Canada). The probes were prepared in according to the labelling protocol of the Genisphere 3DNA Array 350 kit (Genisphere, Hatfield, PA, USA). In brief, cDNA was synthesized from 4µg pooled RNA with the use of SuperScript III first-strand synthesis system for RT-PCR, with either the Cy3- or Cy5-specific RT-primer provided in the labelling kit. The Cy3 and Cy5 reactions were combined and concentrated. The combined Cy3/Cy5 sample was loaded onto the microarray and incubated overnight in a humidified Corning hybridization chamber at 65°C. After hybridization, the microarrays were washed. The slides were then immediately dried and scanned at 10µm resolution with a Perkin-Elmer/GSI Lumonics ScanArray 4000 scanner, using the QuantArray (GSI Lumonics). Spots on the images were identified and quantified using the GenePix®Pro 5.1.0.11 software (Axon Instruments, Union City, CA, USA). The results from the arrays were loaded into the Linnaeus Centre for Bioinformatics Datawarehouse (www.lcb.uu.se/lcbdw.php, Uppsala University, Sweden).[5] Statistical analysis of the mean spot intensity was performed using R packages from Bioconductor (www.bioconductor.org/) in the Linnaeus Centre for Bioinformatics Datawarehouse. Differentially expressed genes were identified after a print-tip loess normalization within the arrays followed by merge of duplicate spots. Hypothesis testing was performed using B-statistics v4.1.4 (Bioconductor) and genes with a B-value above 1 were considered to be differentially expressed.[6]

Real-Time RT-PCR

Total RNA (3µg) of the pooled samples was transcribed into cDNA with SuperScript III First strand synthesis system for RT-PCR (Invitrogen) using random hexamer primers according to the manufacturer's protocol. The sequences for real-time RT-PCR spanned over an exon-exon boundary. The microarray analysis indicated that β-actin was not differentially expressed and this gene was therefore used as a reference gene. Real-time RT-PCR was performed using the Quantitect SybrGreen PCR kit according to the manufacturer (Qiagen, Valencia, CA, USA), and a thermal cycler Rotorgene 3000 (Corbett Research, Mortlake, NSW, Australia). Melting point curves were included at the end of the program. All samples were run in triplicates. In addition, in all real-time RT-PCR runs, duplicate reverse template controls (RTC; i.e. Superscript III was omitted for a mixture of the samples), and duplicate no-template controls (NTC) were run. These controls did not result in any amplification or had C_T levels well over the most diluted samples. The relative gene expression was calculated as a quotient of the target gene and the β-actin reference gene, using the excel-based program DART v1.0.[6] The data was statistically tested in GraphRad Prism 4 with column statistic (vehicle versus treated). Further details of the methods are found in Bergström et al 2007.[1]

Results and discussion

Seven days following a single ip dose of 2,6-dichlorophenyl methylsulphone, 56 genes were found to be differentially expressed in the olfactory bulb. Twenty-two of these genes clustered into processes regulating, cell cycle control (10 genes), cell differentiation (6 genes), cell migration (4 genes), apoptosis (10 genes), signal transduction (9 genes) and Alzheimer's disease (9 genes). Genes selected for quantitative RT-PCR were chosen to range genes differentially expressed with various significance. Altered expression of Gfap, Rnr2, NcorR1 and Olfml3 were confirmed. Table 1 lists 10 genes identified in microarray experiment relevant to investigate with quantitative RT-PCR.

Alzheimer's disease (AD) represents two thirds of all cases of dementia. The symptoms are well investigated and the degenerative lesions are characterized by peptide β-amyloid containing plaques and neurofibrillary tangles in the brain with a focus on the limbic system. Early signs of the disease are often found in the olfactory bulb.[8,9] The biological causes behind AD are not very well understood.

In Table 1, the third column from the right shows differentially expressed genes that are associated with AD; the genes are shortly commented on in the right column. Neutral sphingomyelinase (Nsmaf) was up regulated in 2,6-dichlorophenyl methylsulphone-treated mice. Amyloid-β peptide mediates neural apoptosis and cell death via neutral sphingomyelinase pathway.[10,11] Casein Kinase 1 isoform alpha (Csnk1a1) is co localized with neurofibrillary tangles in AD tissue, Csnk1a1 was downregulated in our study.[12,13]. Cyklin B1 (Ccnb1) was upregulated in 2,6-dichlorophenyl methylsulphone-treated mice. Cyklin B1 is required for cells to enter mitosis. Cell cycle events play a major role in the loss of neurons at all stages of AD.

Table 1
Some genes differentially expressed in 2,6-dichlorophenyl methylsulphone treated mice.

NIA EST ID[a]	Gene symbol[b]	log_2 fold change from micro array studies	Cell cycle	Cell differentiation	Cell migration	Apoptosis	Signal transduction	Cellular metabolism	Alzheimer disease	Confirmed with q RT-PCR	comments
H3128A01	Ptger2	-0.75	*				*		*		Ptger2, synonyms are prostaglandin E receptor 2 (subtype EP2), PGE2 receptor EP2 or Prostaglandin EP2 receptor. Ptger2 protects neurons against oxidative stress and cell death
H3105B10	Ccnb1	0.84	*			*			*		Is required when the cell shall enter mitosis; over-expressed in all stages of AD
H3028G09	Ets2	0.98	*		*	*	*		*		Duplicated at chromosome 21, inducing AD in Down´s syndrome patients.
H3150G09	Grlf1	0.93	*		*		*	*	*		Mediates Src-dependent signalling for neurite outgrowth. Blocks transcription of glucocorticoid receptors.
H3096C05	NcoR1	-0.74		*			*			Yes	Binds Gfap-promotor, NcoR1; prevents ligand binding to the RXR/RAR
H3109A05	CD24a	1.26		*	*	*					Postmitotic expression, stimulates cell migration
H3154A11	Flna	-1.86			*						Regulates actin cytoskeleton; FlnA is required for migration of many cell types including neurons
H3055E05	Tax1bp1	-0.98				*					Inhibits TNF-mediated apoptosis
H3138F10	Nsmaf	1.31				*	*		*		Death receptor binding protein, involved in Aβ mediated neuronal apoptosis and cell death, Implications for AD
H3130C08	Csnk1a1	-0.76				*	*		*		Casein Kinase 1 is tightly associated with Paired-Helical Filaments isolated from AD Brain
H3055B01	Rnr2	-1.46						*	*	Yes	RNR2 in humans encodes humanin which protects from neuronal cell death
H3144B05	Asnsd1	0.77						*	*		Associated with prenylation which is required for release of ApoE, which stimulates development of AD
	GFAP								*	Yes (Up)	Activated astrocytes expressing glial fibrillary acidic protein (GFAP) are closely associated with AD pathology, such as tangles, neuritic plaques and amyloid depositions

[a]NIA EST ID at http://lgsun.grc.nia.nih.gov/cDNA/15k.html; [b]Gene symbols from the NCBI web site UniGene (www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=10090)

Ccnb1 is reported to be expressed in neurons in the proximity to neurons with neurofibrillary tangles.[14,15] Rnr2, encoding 16S ribosomal RNA, was down-regulated in 2,6-diClPh-$MeSO_2$ treated mice. The human homolog to Rnr2 encodes also a small protein named Humanin that has been shown to suppress neural cell death in an AD model.[16] The rat homolog for humanin is called rattin.[17] To our knowledge, humanin has not previously been detected and studied in mice. The decreased memory ability induced by 2,6-dichlorophenyl methylsulphone indicates that the olfactory toxicant could be an interesting model for inducing an AD-like condition in rodents. We hypothesize that an AD-like condition can be induced via the olfactory mucosa and olfactory bulb in rodents. A chemically induced rodent model for AD would be a valuable tool in Alzheimer's disease research. There are numerous rodent models for Alzheimer's disease based on different mutations. The inheritable factor in AD is, however, minor. The major numbers of AD cases have an idiopathic origin. It would therefore be important to develop an AD model without specific mutations. The olfactory toxicant 2,6-dichlorophenyl methylsulphone might be a candidate compound in the development of such a chemical AD model. Further investigation whether 2,6-dichlorophenyl methylsulphone affects the same genes as in AD in the olfactory bulb and other brain areas are needed.

Acknowledgements
Economic support was given by the Commission of the European Communities, specific RTD program Quality of Life and Management of Living Resources, QLKI-2000-00261 entitled "Comparison of exposure-effect pathways to improve the assessment of human health risks of complex environmental mixtures of

organohalogens". It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

References

1. Bergström, U., Olsson, J.A., Hvidsten, T.R., Komorowski, J. and Brandt, I. Differntial gene expression in the olfactory bulb following exposure to the olfactory toxicant 2,6-dichlorphenyl methylsulphone and its 2,5-dichloroinated isomer in mice.*Neurotoxicology*, in press. 2007
2. Bahrami, F., Brittebo, E. B., Bergman, A., Larsson, C., and Brandt, I. Localization and comparative toxicity of methylsulfonyl-2,5- and 2,6-dichlorobenzene in the olfactory mucosa of mice. *Toxicol Sci* 1999; 49: 116-123.
3. Bahrami, F., Bergman, U., Brittebo, E. B., and Brandt, I. Persistent olfactory mucosal metaplasia and increased olfactory bulb glial fibrillary acidic protein levels following a single dose of methylsulfonyl-dichlorobenzene in mice: comparison of the 2,5- and 2, 6-dichlorinated isomers. *Toxicol Appl Pharmacol* 2000; 162: 49-59.
4. Carlsson, C., Fredriksson, A., and Brandt, I. 2,6-dichlorophenyl methylsulphone induced behavioural impairments in rats and mice in relation to olfactory mucosal metaplasia. *Pharmacol Toxicol* 2003; 93: 156-168.
5. Ameur A, Yankovski V, Enroth S, Spjuth O, Komorowski J. The LCB Data Warehouse. *Bioinformatics* 2004; 22: 1024-6
6. Lönnstedt, I. and Speed, T.P. Replicated microarray data. *Stat. Sinica*, 2002; 12: 31–46.v
7. Peirson SN, Butler JN, Foster RG. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 2003;31: e73
8. Braak, H., and Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol (Berl)* 1991; 82: 239-259.
9. Kovacs, T., Cairns, N. J., and Lantos, P. L. beta-amyloid deposition and neurofibrillary tangle formation in the olfactory bulb in ageing and Alzheimer's disease. *Neuropathol Appl Neurobiol* 1999; 25: 481-491.
10. Jana A and Pahan K. Fibrillar amyloid-beta peptides kill human primary neurons via NADPH oxidase-mediated activation of neutral sphingomyelinase. Implications for Alzheimer's disease. *J Biol Chem*. 2004; 279(49):51451-9
11. Lee JT, Xu J, Lee JM, Ku G, Han X, Yang DI, Chen S, Hsu CY. Amyloid-beta peptide induces oligodendrocyte death by activating the neutral sphingomyelinase-ceramide pathway. *J Cell Biol*. 2004; 164:123-31
12. Kannanayakal TJ, Tao H, Vandre DD, Kuret J. Casein kinase-1 isoforms differentially associate with neurofibrillary and granulovacuolar degeneration lesions. *Acta Neuropathol (Berl)*. 2006; 111(5):413-21
13. Kuret J, Johnson GS, Cha D, Christenson ER, DeMaggio AJ, Hoekstra MF. Casein kinase 1 is tightly associated with paired-helical filaments isolated from Alzheimer's disease brain. *J Neurochem*. 1997; 69(6):2506-15
14. Yang Y, Mufson EJ, Herrup K. Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease. *J Neurosci*. 2003; 23(7):2557-63
15. Smith MZ, Nagy Z, Esiri MM. Cell cycle-related protein expression in vascular dementia and Alzheimer's disease. *Neurosci Lett*. 1999; 271(1):45-8.
16. Hashimoto, Y., Ito, Y., Niikura, T., Shao, Z., Hata, M., Oyama, F., and Nishimoto, I. Mechanisms of neuroprotection by a novel rescue factor humanin from Swedish mutant amyloid precursor protein. *Biochem Biophys Res Commun* 2001; 283, 460-468.
17. Caricasole, A., Bruno, V., Cappuccio, I., Melchiorri, D., Copani, A., and Nicoletti, F. A novel rat gene encoding a Humanin-like peptide endowed with broad neuroprotective activity. *FASEB J*. 2002; 16, 1331-1333.