New insights into developmental neurotoxic effects in zebrafish at protein level using Tandem Mass Tags®.

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Special attention has been given to the substances causing developmental neurotoxicity as the developing central nervous system (CNS) is more vulnerable to chemicals than the adult CNS. Due to the physiological and morphological complexity of the CNS, it is a major challenge to test substances for their neurotoxic potential. The neurotoxic potency of chemicals has mainly been determined with neurobehavioral and neuropathological *in vivo* tests. As these tests are time consuming and expensive new strategies are required to cope with the anatomical and physiological complexities of the CNS.

The zebrafish (*Danio rerio*) is an emerging model in behavioural and neurological studies. Zebrafish larvae display numerous behavioural patterns highly similar to rodents and humans. Neurotoxicity is mostly studied in zebrafish using behavioural tests. Little knowledge exists on protein regulation for neurotoxicity testing. Prefractionation of samples allows deeper insights into the proteome using MS approaches by reducing the complexity. The prefraction always represents a trade-off between number of identified proteins and instrument time and therefore increased instrument costs. Isobaric Tandem Mass Tags® (TMT®) reagents with their ability of multiplexing up to 10 samples in a single experiment (TMT10plex) are a time-effective solution and still allow for prefractionation techniques without increasing MS instrument time tremendous.

Here, zebrafish were exposed to neurotoxic substances (Chlorpyrifos (CPF); Chlorpyrifos-oxon (CPF-oxon), and Methylmercury (MeHg)) from 0 till 5 days post fertilization. CPF must be bioactivated into CPF-oxon whereas CPF-oxon and MeHg do not need bioactivation. The applied concentrations did induced behavioural alterations but no phenotypic malformations.

For proteomics analysis, the heads of 100 animals per exposure group were pooled, with four biological replicates. These samples were compared with two reference samples included per analytical TMT10plex. The samples were pre-fractionated using basic RPLC and further analysed by LC-MS using MS3 acquisition for accurate quantitation.

In total, 2,395 protein groups were identified, represented by 8,291 peptide sequences. Statistical analysis (PCA, PLS, LIMMA) revealed that the protein regulation by CPF is nearly symmetrical (101 down- and 126 up-regulated) while protein regulation was asymmetrical by MeHg (65 down- and 132 up-regulated) and CPF-oxon (39 down- and 126 up-regulated). CPF reveals the highest regulation fold changes for individual proteins, while CPF-oxon and MeHg exhibit a higher fold change considering the entity of all significantly regulated proteins. The effect of CPF-oxon and MeHg on protein level is essentially similar. Clustering by Gene Ontology annotation grouped 17 proteins to three different clusters related to proteolysis, proteases and peptidase activity. We identified a couple of protein candidates which are significantly regulated for the various treatments and will be validated in further experiments using orthogonal methods.

With this study we gain new insights into developmental neurotoxic effects on protein level. Our study reveals a high similarity of compounds being effective during early development vs. compounds needed to be bioactivated. We detected a high number of proteomic changes at exposure levels where only behavioral alterations where observed.