(Patho)physiological regulation of adult hippocampal neurogenesis
By seizures, glucocorticoids and microRNAs
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3. Multi-omics Profile of the Mouse Dentate Gyrus after Kainic Acid-induced Status Epilepticus

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Abstract

Temporal lobe epilepsy (TLE) can develop from alterations in hippocampal structure and circuit characteristics, and can be modeled in mice by administration of kainic acid (KA). Adult neurogenesis in the hippocampal dentate gyrus (DG) contributes to hippocampal functions and has been reported to contribute to the development of TLE. Some of the phenotypical changes include neural stem and precursor cells (NPSC) apoptosis, shortly after their birth, before they produce hippocampal neurons.

Here we explored these early phenotypical changes in the DG 3 days after a systemic injection of KA inducing status epilepticus (KA-SE), in mice. Accordingly, we chose a multi-omics experimental setup and analyzed DG tissue samples using proteomics, transcriptomics and microRNA profiling techniques. We here present a description of how these data were obtained and provide them to others for further analysis and validation. This may help to further identify and characterize molecular mechanisms involved in the alterations induced shortly after KA-SE in the mouse DG.

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Background & Summary

TLE is a serious neuropathological condition hallmarked by chronic spontaneous recurrent seizures, which can originate from altered hippocampal circuit characteristics. Strong initial seizures, such as those seen during SE, have previously been proposed as a driving force in the development of TLE\(^1\). The current view on TLE development proposes a number of causal changes in hippocampal structure including DG granule cell layer dispersion\(^2\), mossy fiber sprouting\(^3\), and the induction of aberrant hippocampal neurogenesis\(^4,5\). These abnormalities can be modeled using convulsant treatments such as e.g. KA administration\(^4\). Concerning aberrant neurogenesis, multiple cellular phenotypes have been linked to the nature of newly born aberrant neurons including alterations in levels of NSPC proliferation, and subsequent changes in the levels of apoptosis, differentiation, integration and ectopic positioning of their progeny\(^4-6\).

Although the aforementioned structural alterations following KA administration have been widely documented, the molecular mechanisms involved are less well characterized. For instance, molecular events contributing to lower levels of NSPC apoptosis following KA-SE are poorly understood. Indeed, although others have previously characterized executioner caspases to be crucial mediators of SE-induced changes in apoptosis\(^7\), the upstream molecular events controlling caspase activation were unknown.

A number of previous studies have applied ‘-omics’ approaches to describe changes in DG gene expression (shortly) after KA-SE at the level of mRNA\(^8\). These results should be interpreted carefully, since previous studies have indicated a poor correlation of mRNA expression to their corresponding protein levels\(^9,10\), suggesting a significant contribution of post-transcriptional mechanisms to gene regulation. Among the many factors that could possibly contribute to post-transcriptional gene expression regulation, microRNAs (miRNAs) are a particular class of molecules whose expression could hinder target mRNA translation into protein\(^11\).

Indeed, previous studies have also approached TLE from the perspective of microRNA expression\(^12\). There were, to our knowledge, no previous records of an experimental design allowing simultaneous protein, mRNA and microRNA expression measurements shortly after KA-SE in the DG. Accordingly, we set out in such a multi-omics approach to explore in particular alterations in mitochondrial function-related protein expression, which could be affected shortly after KA-SE in the DG. We used systemic injections of KA to ensure an equal distribution of the chemo-convulsant to both hemispheres and thus both dentate gyri. In addition, to specifically identify the molecular mechanisms of KA-SE on the DG, we conducted our measurements on DG enriched samples\(^13\). To this end, we used the left DG to analyze protein expression and the right DG for RNA expression analyses. The latter included both mRNA and miRNA measurements.

Our multi-omics approach allowed us to identify a novel role for cooperative microRNA-mediated regulatory action on the expression of the pro-apoptotic protein BCL2L13 and downstream molecular events, including caspase-3 activity. The dataset described here may allow the identification and further characterization of molecular mechanisms that could help to understand the numerous phenotypical alterations taking place in the DG shortly after KA-SE.
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Methods

Animals

SE-related aberrant neurogenesis can be induced in rodents by administering chemoconvulsants, such as Pilocarpine and KA. In this particular study, SE was induced in C57BL/6j mice (n=3/group) by repeated (30 minute interval) i.p. low-dose (initial dose 24 mg/kg and subsequent doses 10 mg/kg) KA injections according to a previously described protocol by Hellier et al., modified for mice. Likewise, after each KA injection, seizure classes were scored following a modified version of Racine’s scale, and only animals scored for >5 minute long class IV-V seizures were included for further analysis. Matching quantities of saline were injected in control animals. This systemic KA exposure approach allows for a low KA distribution variability between hemispheres. Accordingly, this approach was chosen to allow for comparable KA levels to either hemisphere. The experimental workflow depicted in Figure 1 and table 1 shows how the left hippocampus of each animal was used for the analysis of protein expression and the right for RNA expression, allowing good correlation analyses. Depending on the particular phenotype studied, one can vary the time of animal sacrifice after KA-SE. Since previous reports have identified most newly born neurons to undergo selection through apoptosis within the first week after their birth, we opted for a shorter time point of 3 days after KA-SE to assess possible alterations in apoptosis-related proteins (Figure 1).

Sample preparation

To further enrich our samples with proteins and RNA originating from hippocampal neurogenic cells, we micro-dissected tissue from the DG. We excluded contribution from other brain areas to our sample content by micro-dissecting the DG using the hippocampal fissure as reference, as described before.

For proteomic analysis, the proteins of the left DG enriched tissue samples were first captured in lysis buffer and protein concentration measurements were carried before storage at -80°C until further use. Matched quantities (30 µg) of protein sample were loaded on a NuPAGE 4-12% gradient Bis-Tris gel (Invitrogen). Proteins were then digested in the gel using trypsin, which was subsequently sliced into 10 cubes per lane prior to protein purification, as previously described. Purified fractions of protein per experimental replicate were now ready to undergo mass spectrometry analysis.

For transcriptomics and microRNA profiling, total RNA was isolated from the right DG enriched tissue samples using a TRIzol based RNA extraction protocol according to the manufacturer's description. Purified RNA samples were analyzed for RNA concentrations using a Nanodrop and analyzed for RNA Integrity Numbers using a Nano Lab-on-Chip and Agilent Bioanalyzer platform. All total RNA samples had a value of >8. Total RNA sampled were then stored at -80°C until further usage.

Mass spectrometry for proteomics

Mass spectrometry spectra were acquired using a LTQ-FT hybrid mass spectrometer (Thermo Fisher), as previously described. Subsequently, lists of spectral counts per protein were generated by searching for matching spectra using the IPI mouse database version 3.59 using Sequest v27.
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Saline | Kainic acid

Figure 1. Schematic depiction of the experimental setup and subsequent workflow used to obtaining multi-omics data of DG tissue, 3 days after KA-SE.

(A) Schematic illustration showing the timeline of experimental in vivo procedures (B) Illustration on the experimental procedures, sample preparation, wet-lab workflow and statistical analysis steps carried out to generate proteomics, transcriptomics and microRNA profiles 3 days after KA-SE in the DG. The dry-lab workflow and target validation procedures are examples of how this data set was used by Schouten et al. to identify molecular mechanisms underlying alterations in NSPC apoptosis following KA-SE, not further discussed here.
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Table 1. Information on samples and their related datasets stored in online repositories.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Drug treatment</th>
<th>Tissue</th>
<th>Sample treatment</th>
<th>Data collection</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse1-3</td>
<td>Saline treatment</td>
<td>Left DG dissection</td>
<td>Protein extraction</td>
<td>Mass spectrometry</td>
<td>PXD003744</td>
</tr>
<tr>
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<td>Right DG dissection</td>
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</tr>
<tr>
<td>Mouse4-6</td>
<td>KA-SE treatment</td>
<td>Left DG dissection</td>
<td>Protein extraction</td>
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</tr>
<tr>
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<td>KA-SE treatment</td>
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</tr>
</tbody>
</table>

Illumina MouseWG-6 beadchip hybridizations for transcriptomics

500ng of each total RNA sample was processed into biotinylated cRNA using an Illumina TotalPrep RNA amplification Kit (Ambion, Life Technologies). Subsequently, 1500ng of biotinylated cRNA of each experimental replicate was hybridized onto MouseWG-6 beadchips (Illumina) according to the manufacturers’ protocol.

Mouse microRNA fluidic v3.0 cards for microRNA profiling

800ng of each total RNA sample was processed into cDNA using Taqman microRNA RT Kit and Megaplex RT primers (Applied Biosystems), according to the manufacturers’ protocol. Subsequently, cDNA samples were combined with a Taqman Universal Master Mix prior to loading onto Mouse microRNA fluidic v3.0 cards (Applied Biosystems).

Data analyses

Spectral counts were normalized against the sum spectral counts per biological sample. For the comparisons of data consisting of sample groups (n>1), various models are available to statistically test for significant differences in spectral count data, such as those obtained from mass spectrometry. Indeed, G-tests, t-tests, Fisher’s exact tests and the local-pooled-error technique have previously been used to identify differences in
spectral counts of corresponding protein levels\textsuperscript{23}. These models, however, fail to take into account variations within and in between sample groups. Accordingly, we opted to use a beta-binomial model to test for differences in protein abundances, taking these variations into account and thus resulting in an overall higher true detection rate, lower false positive rate and thus better experimental resolution\textsuperscript{23}. In addition, this method allows for the comparison of protein levels when spectral counts are detected in at least one sample group (e.g. saline treatment replicates), but not the other (e.g. KA-SE treatment replicates)\textsuperscript{23}. This software that tests, among others, spectral counts of experimental setups with multiple experimental groups composed of multiple replicates, is freely available as an R software package from https://www.oncoproteomics.nl/, and has been extensively described by Pham et al\textsuperscript{23}.

Concerning data analysis of transcriptomics, probe intensities from the Illumina mouseWG-6 beadchip arrays were analyzed in GenomeStudio. First, transcript expression detection limits were analyzed for all samples. Detection p-values were calculated based on mismatch probe intensity, negative control probe intensity and stringency. Detection p-value cut-off was set to <0.05 as a quality control and inclusion criterion for further statistical testing. Subsequently, data were background-corrected, transformed and normalized as described before\textsuperscript{24}. Permutation p-value calculation was used to determine significant differences between saline and KA-SE treatments (p < 0.01). The detection limit of each microRNA was determined by including microRNA with a raw Ct value <35 for further statistical analysis. Data were subsequently normalized against RNU6B expression levels allowing a \( \Delta \Delta \text{Ct} \)-based conversion to fold change expression levels of each microRNA between treatment groups. A 5% false discovery rate corrected t-test was used to determine statistical differences (p < 0.05 and fold change \( \geq \) 1.5).

Data Records

Data record 1
The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository\textsuperscript{25} with the dataset identifier PXD003744 (Data citation 1).

Data record 2
The microarray transcriptomics data have been deposited in NCBI’s GEO repository with the dataset identifier GSE79129 (Data citation 2).

Data record 3
The RT-QPCR microRNA profiling data have been deposited in NCBI’s GEO repository with the dataset identifier GSE79132 (Data citation 3).

Technical validation

Proteomic data

Within the saline sample group, of the total 2125 proteins identified, 1339 could be identified in all three samples resulting in an ID reproducibility of 63.0%, as depicted in the Venn diagram of Figure 2A. Likewise, within the KA-SE sample group, of the total 2113 proteins identified, 1278 could be identified in all three samples resulting in an ID reproducibility of 60.5%, as depicted in the Venn diagram of Figure 2B. These ID reproducibilities were accompanied by calculated coefficients of variance of 21.5% and
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24.6% for saline and KA-SE, respectively. These values meet the required standards for proteomic analysis. Accordingly, the Venn diagram comparison between experimental groups shows 82.1% overlapping/comparable IDs as depicted in Figure 2C. To further validate the mass spectrometry based observations western blot analysis was performed. Immunolabelling was performed on the same samples to validate the expression levels of proteins of interest (BAX and BCL2L13), normalized to beta-actin protein expression levels. As previously described by Schouten et al., we found the western blot data to support the proteomics data for the expression levels of BAX and BCL2L13 protein.

Transcriptomic data

Venn diagrams of saline group replicates (Figure 2D) and KA-SE group replicates (Figure 2E) show good (>80%) within group reproducibility percentages. Furthermore, a Venn diagram of the experimental groups with overlapping/comparable IDs is shown in Figure 2F, depicting a good between-group comparison percentage (>90%). RT-QPCR was performed on the same samples to validate expression levels of transcripts of interest (BAX and BCL2L13), normalized to alpha-tubulin mRNA expression levels. As previously described by Schouten et al., we found the RT-QPCR data to support the transcriptomics data for the expression levels of BAX and BCL2L13 mRNA.
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MicroRNA profiling data

Venn diagrams of saline group replicates (Figure 2G) and KA-SE group replicates (Figure 2H) show good (>85%) within group reproducibility percentages. Furthermore, a Venn diagram of the experimental groups with overlapping/comparable IDs is shown in Figure 2I, depicting a good between-group comparison percentage (>90%). RT-QPCR was performed on the same samples to validate expression levels of microRNA of interest (microRNA-124 and microRNA-137), normalized to RNU6B expression levels. As previously described by Schouten et al\textsuperscript{28}, we found the RT-QPCR data to support the microRNA profiling data for the expression levels of microRNA-124 and microRNA-137.

Usage notes

The beta-binomial software is freely available as an R software package from https://www.oncoproteomics.nl/, and extensively described by Pham et al\textsuperscript{23}.

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Author contributions

MS contributed to experimental design, performed experiments, analyzed data and wrote manuscript. PB performed experiments, analyzed data and wrote manuscript. SAF contributed to design, performed proteomics experiments, analyzed data and wrote manuscript. CJH performed animal experiments. SRP contributed to proteomics experiments. TVP analyzed proteomics data. RAV contributed to experimental conception and CJH supervision. PB performed experiments and analyzed data. PJL contributed to experimental design and conception and wrote manuscript. CRJ contributed to experimental conception and design experiments. CPF conceived, designed and performed experiments, analyzed data and wrote manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing financial interests.
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References

Data citations