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Full-length transcriptome profiling of the rat hippocampal neurons in primary culture at different time points after activation using Nanopore sequencing

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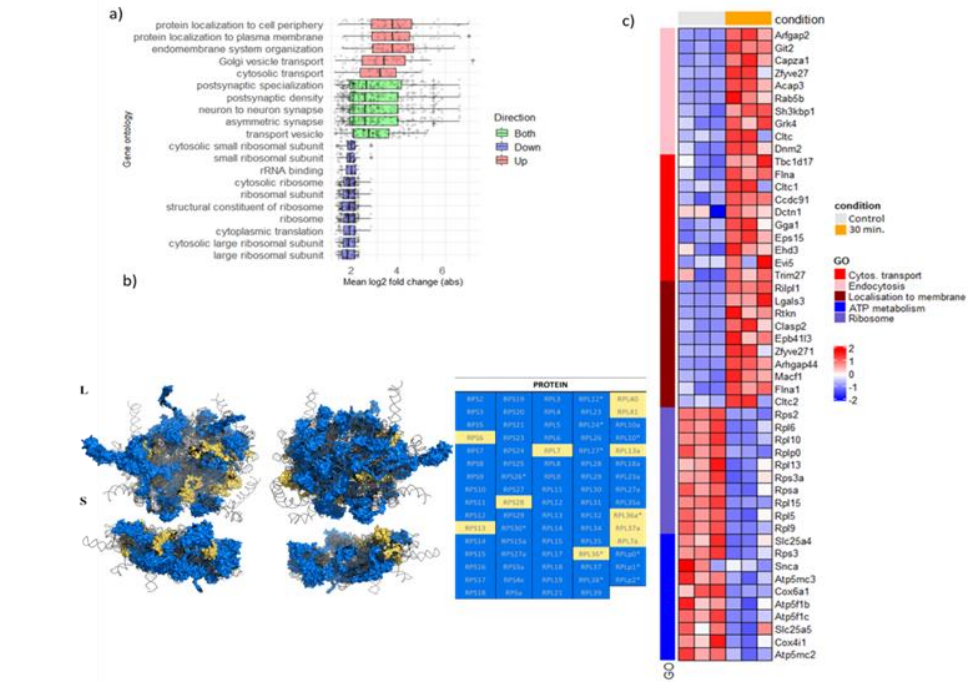
Motivation and Aim: Neurons underpin cognitive ensemble processes as they are responsible for transmitting, processing, and storing information. The functional attributes are mirrored in the cell's morphology: neuronal cells are highly compartmentalized, with axons, dendrites, and the soma potentially containing distinct sets of RNA. Consequently, the transcriptional intensity and translational status of an mRNA pool can vary based on neuronal activation, extracellular conditions, and numerous other factors [1]. Neuronal stimulation can initiate the ultimate step of mRNA maturation, ensuring that mRNA synthesis is finalized just prior to the commencement of protein synthesis. The processes of transcription and translation can be separated, with various strategies in place for preserving mRNA [2]. Additionally, mRNA serves as a fundamental precursor to the protein machinery and occupies a crucial role in the execution of neuronal functions.The central objective of this study was to determine if the read count acquired through long-read sequencing with the Oxford Nanopore Technologies (ONT) MinION device is adequate to detail the transcriptomic alterations in primary neuronal cultures (PNC) following their stimulation by PTX for durations of 30 minutes, 1 hour, and 5 hours. This process is akin to the usual occurrences in a functioning brain, whereas alternative methods of neuronal activation *in vitro* tend to be less nuanced.

Methods and Algorithms: The hippocampal regions from P0 Wistar pups were prepared into primary neuronal cultures following a conventional protocol [3]. Neurons were cultured at a concentration of 250,000 cells per well. On the 15th day *in vitro* (DIV15), a standard phenol-chloroform method was used to extract RNA. Subsequently, 150 ng of total RNA was reverse transcribed with random decamer primers. In total, 20 cDNA libraries derived from poly(A)+ RNA underwent reverse transcription and were amplified into full-length cDNA using the TeloPrime Full-Length cDNA Amplification Kit V2, following the instructions provided by the manufacturer. Long-read sequencing was conducted using the MinION platform, and data production was facilitated by the ONT MinKNOW software (version 3.4.12). For the alignment of reads to the reference genome (mRatBN7), the minimap2 tool was employed. Reads aligned to the genome were quantified with FeatureCounts. The statistics for primary versus secondary alignments, as well as the proportion of reads at different coverage levels, were calculated from BAM files using the pysam library and the GenomicFeatures package in R, along with bespoke scripts. Annotation of protein domains was carried out using

the Conserved Domain Database (CDD), with a focus on PFAM domains exclusively. The coding potential was assessed using the CPAT tool. The analysis of differential expression at the gene level was conducted utilizing the DESeq2 package in R, based on transcript counts and abundances derived from Salmon. For the 30-minute mark, adjusted p-values were set at less than 0.05, and for the 1-hour and 5-hour marks, the threshold was less than 0.1. Searches within Gene Ontology and KEGG categories were carried out using the clusterProfiler package in R, applying an adjusted p-value criterion of less than 0.05. The ribosomal and endocytic pathways from the KEGG database were depicted using the Pathview online tool. The evaluation of differential transcript usage (DTU) was performed using the IsoformSwitchAnalyzer software. Four distinct approaches for assembling long-read transcriptomes were analyzed: Flair (in both relaxed and stringent modes), FLAMES, Stringtie2, and Bambu, all utilizing their respective default settings. The performance of these techniques in reconstructing transcripts at the locus level was evaluated using the software gffcompare version 0.11.7.
Results: In our study, we identified a total of 23,652 new transcripts when compared to the reference annotations, approximately 6,000 of which were completely new, predominantly originating from transposon-related locations. The examination of differentially expressed genes (DEGs) revealed that 3,046 genes exhibited differential expression; within this group, 2,037 genes showed increased expression and 1,009 showed decreased expression 30 minutes following PTX treatment. At subsequent time points of 1 hour and 5 hours, the numbers of differentially expressed genes were significantly lower, with 446 and 13 genes respectively (see the Fig. 1a–c).
Conclusion: Gene Ontology and KEGG pathway analyses indicated a direct correlation between both upregulated and downregulated genes with protein metabolism processes. Significantly, genes involved in protein transport and localization saw an increase in regulation, whereas numerous genes responsible for ribosomal proteins, which normally have a high expression level, were downregulated following a 30-minute incubation with PTX. This suggests a reallocation of transcriptional resources in favor of genes activated by the treatment. For the ribosome-associated proteins Rpl15 and Rps8, a marked decrease in expression was observed in neuronal cultures after a 30-minute exposure to PTX, as verified by digital PCR (dPCR; Fig.1d).
We observed a significant reduction in the expression of genes involved in ATP metabolism, predominantly those associated with mitochondrial genes that are typically highly expressed. Additionally, there was a substantial alteration in the expression levels of genes within the endocytosis pathway, with the majority of these genes experiencing upregulation. A 30-minute incubation with PTX did not result in a significant increase in alternative splicing events. Surprisingly, only slight variations were observed at each time point of the experiment (with 16, 16, and 4 isoforms affected after 30 minutes, 60 minutes, and 5 hours, respectively), according to Differential Transcript Usage (DTU) analysis. Nevertheless, there was a prominent switch in the transcripts of the Gabrb1 gene, which is responsible for encoding the β1 subunit of the GABA receptor, a known direct target of PTX.



d)

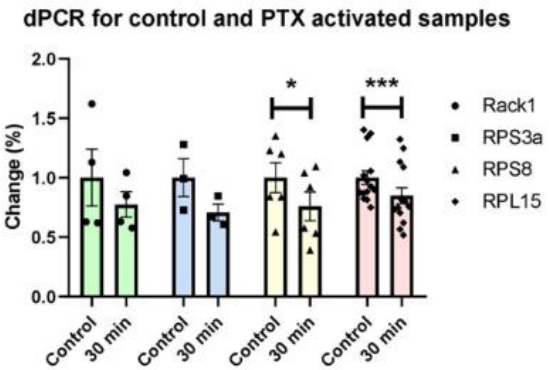


Fig. 1. a) Top 10 down- and up-regulated Gene Ontology categories for DEGs identified at 30 min after the PTX application. Y axis represents GO category, X axis – log2 fold change, averaged across all genes in the corresponding category. b) Visualization of the ribosomal pathway genes that are differentially expressed at 30 min after the PTX application. Structure of the mammalian ribosome, two views each for the large (L) and small (S) subunits. Grey color represents rRNA. Protein products of downregulated genes are shown in blue, protein products of non-DE genes are shown in yellow. c) Heatmaps showing top 10 DEG (sorted by log 2 fold change) per GO category. d) dPCR results for selected genes encoding ribosome and ribosome-associated proteins. Control group: $n = 4$ for Rack1; $n = 3$ for Rps3a; $n = 6$ for Rps8; $n = 15$ for Rpl15; "30 min" PTX activation group: $n = 4$ for Rack1; $n = 3$ for Rps3a; $n = 6$ for Rps8; $n = 15$ for Rpl15 biological replicates. Expression levels of genes of interest were normalized to the average Hprt expression in the same sample. The results are present as proportional change in gene expression ± SEM normalized to the control group value; * – $p < 0.05$, *** – $p < 0.001$, Wilcoxon signed rank test

The discovery of new loci and isoforms in this research could enhance our comprehension of the functional mRNA array involved in neuronal plasticity. Concurrently, the gene clusters annotated by Gene Ontology (GO) could provide deeper insights into the specific molecular mechanisms that explain the absence of inhibitory signals from interneurons, which is a result of the blockade of GABA receptors during normal physiological functions.

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