Supplementary Material for "Digital expression profiling of the compartmentalized translatome of Purkinje neurons"

Anton Kratz[1,*,$], Pascal Beguin[2,*], Megumi Kaneko[2], Takahiko Chimura[2,3], Ana Maria Suzuki[1,$], Atsuko Matsunaga[2], Sachi Kato[1,$], Nicolas Bertin[1,4,$], Timo Lassmann[1,$], Réjan Vigot[2], Piero Carninci[1,$], Charles Plessy[1,5,$], Thomas Launey[2,5]

[1]RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Kanagawa, 230-0045 Japan

[2]RIKEN Brain Science Institute, Launey Research Unit, Wako, Saitama, 351-0198 Japan

[3]Present address: University of Tokyo, The Institute of Medical Science, Tokyo, 108-8639 Japan

[4]Present address: The Cancer Science Institute of Singapore, 12th Fl., Unit 01, MD6 Center for Translational Medicine, National University of Singapore, Yong Loo Lin School of Medicine, 14 Medical Dr., Singapore 117599

[5]Corresponding authors

Charles Plessy, RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Kanagawa, 230-0045 Japan. Tel: (+81) 45 503 9111 email: plessy@riken.jp

Thomas Launey, RIKEN Brain Science Institute, Launey Research Unit, Wako, Saitama, 351-0198 Japan. Tel: (+81) 48 467 5484, email: thomas.launey@riken.jp

[$] These members of CLST belonged to RIKEN OSC before the RIKEN reorganization of April 1st 2013

* These authors contributed equally to this work.

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1 Supplemental discussion

Beyond the minimal model, we also identified and quantified other proteins relevant for a more detailed description including neurotransmitter receptors and buffering/transport of ionic species (Fig. 5, Table S6). Several ATP-dependent trans-membrane transporters were identified, both for the Na+/K+ transport through plasma membrane (*Atp1a1* and *Atp1a3*, sharing *Atp1b1* beta subunit) and for calcium transport into the endoplasmic reticulum (SERCA2 pump: ATP2A2) and through the plasma membrane (*Atp2b1*, *Atp2b2*). In the neuron, calcium dynamics is partly controlled by calcium binding proteins that chelate free calcium in cytoplasm (*Calb1, Pvalb*) and ER/Golgi lumen (*Canx*, *Calu*, *Sdf4*), mediate calcium signaling (*Calm1, Calm3, Hpca, Clscn3, Ppp3r1, Capns1*) or calcium-dependent transport (*Myl12b*). Note that the calcium binding protein plotted in Fig. 5 only represents the most abundant transcripts, while a full list (see Table S6) shows many other proteins, with a majority of signaling-related transcripts for proteins known to be involved in regulation of synaptic transmission (*Hpcal1, Sptan1, Anxa6, Plcd4, Syt7, Cdk5r1*), with some of them being highly specific of the PC signaling network (*Hpcal1, Plcd4*).

Finally, screening our filtered transcript list for receptor function identified a full complement of excitatory glutamate receptors as well as inhibitory GABA receptor. Notably all the subunits of the AMPA ionotropic receptors mediating the bulk of synaptic transmission in PC were detected (*Gria3, Gria4, Gria2*) but not the *Gria1* subunit which is dominant in interneurons, granule neurons and Bergman glia. Interestingly, *Gria3* and *Gria4* show an almost exact 1:1 stoichiometry while *Gria2* transcripts are 16 times less abundant. This is unexpected considering that each of the tetrameric AMPA receptors should include at least one *Gria2* subunit, giving the resulting channel its characteristic impermeability to calcium ions. This result cannot be explained by an under-detection of the *Gria2* transcript however, since it is detected at a level similar to *Cacng7*, encoding a stargazing-related TARP protein, with which it forms a 1:1 complex in Purkinje cells. In addition to the AMPA-type subunits, we also identified the *Grik1* and *Grik4* subunits of the kainite-type receptor as well as the metabotropic mGluR1 receptor (*Grm1*) which plays a prominent role in synaptic plasticity in PCs. Interestingly, we only detected the GRIN1 subunit of the NMDA-type receptor which, alone, does not produce a functional receptor. This is in perfect agreement with the observation that NMDA current and

expression of other subunits become significant only after the first month post-natal (Piochon et al. 2007).

Sodium channels: Our screen identified the pore-forming alpha subunit for the fast channel Nav1.1 (*Scn1a*), and the axonal persistent channel Nav1.6 (*Scn8a*), together with their regulatory beta subunits (*Scn2b*, *Scn4b*, *Scn1b*). Literature suggests that SCN2B and SCN4B form a complex with SCN1A while *Scn1b* may associate with both alpha subunits. Together *Scn1a* and *Scn8a* represent >87% of the alpha subunit transcripts, confirming previous studies at the protein level which identified Nav1.1 and 1.6 as the predominant sodium channels in PCs (Kalume et al. 2007).

Potassium channel: Two delayed rectifiers Kv3.3 (*Kcnc3*) and Kv1.1 (*Kcna1*) were the dominant transcripts for potassium channels, with two more Kv3.1 (*Kcnc1*) and Kv1.2 (*Kcna2*) expressed at much lower (~10 fold) level. Kv3.3 and Kv1.1 were co-expressed in PCs together with their respective regulatory subunits, *Kcnc1* and *Kcnab2*. Transcripts for the large conductance, voltage and calcium dependent channel KCa1.1 (*Kcnma1*) and its associated beta protein (*Kcnmb4*) were present in near equal amount while the small-conductance Ca-activated KCa2.3 (*Kcnn3*) channel was half as abundant. Finally, the rapidly inactivating A-current and inward rectifier current known to be present in PCs (Hirano and Hagiwara 1989) were identified as Kv4.1 (*Kcnd1*) and Kir1.2 (*Kcnj10*), respectively.

Calcium channels: Both low-threshold T-Type (Cav3.1, *Cacna1g*) and P/Q type (Cav2.1, *Cacna1a*) were detected, with transcripts encoding several of the channel-associated proteins (*Cacnb2*, *Cacnb4*, *Cacna2d2*, *Cacna2d3*) regulating channel kinetics and ion conductance. We noted that the pore-forming alpha subunit (*Cacna1c*) of the L-type channel was not detected, consistent with low expression in mature PCs.

2 Supplemental methods

2.1 Virus transfection

All procedures were approved by the RIKEN Ethic Committee on Animal Research. The EYFP-RPL10A construct was built as previously described (Heiman et al. 2008) with EYFP (Miyawaki et al. 1999) instead of EGFP and then subcloned into a shuttle vector with a pCAG promoter and WPRE expression enhancer (Applied viromics LLC, Fremont, CA) for production of AAV in 293T cells, co-

expressing capsids from serotype 2 and 8. The EYFP variant of fluorescent protein was chosen here instead of the EGFP for its higher brightness and retained binding to immuno-precipitation antibodies. The resulting mosaic AAV2/2-8 virus encoding the construct were diluted zin 100 mM sodium citrate containing 20% trehalose (Saxena et al. 2012), 2% Bovine Serum Albumin (BSA) and 0.2% Fastgreen, pH 7.4, to a final titer of ~3×10^{11} gc/ml. This medium was designed to avoid aggregation and denaturation of viral particles. To increase intercellular space and facilitate virus diffusion, 200 µl of a 20% mannitol solution in 100 mM citrate buffer was injected intraperitoneally, 15 min prior to Convection Enhanced Delivery (Bankiewicz et al. 2000, Sandberg et al. 2000) of the virus solution (10 µl) into the cerebellum of P4 rat pups, using a 33 gauge needle and motor-driven injection at 2.5 µl/min. After complete recovery from anesthesia, the pups were returned to the mare and kept under observation to ensure that they received maternal care. Acute cerebellar slices (350 µm) were prepared 28-32 days later, without sex distinction among littermates. To eliminate cells damaged during sectioning, slices were incubated for 35, 60 or 90 min in oxygenated DMEM/dextran at 35°C. The three time points were later compared to detect any translatome difference that may result from cell alteration. After incubation, selected slices from the vermal region were rapidly cooled to 1-3°C in RNase-free PBS supplemented with 5 mM $MgCl_2$, 50 µM cycloheximide and protease inhibitor mix (Complete, Roche). Microdissection with a wedge-shaped razor blade fragment (0.2-0.3 mm blade length) under epifluorescence illumination allowed us to collect selectively the cerebellar lobes with Purkinje cells expressing the construct. Depending on injection area, 1-6 lobules could be collected per slice. Only lobules IV to IX were included in this study. The white matter and most of the granular layer where trimmed away to reduce contamination. Microdissection is not strictly necessary when isolating ribosome from whole PC since an affinity capture of the EYFP tag follows. However, this trimming is expected to reduce both loss and contamination of the targeted mRNA caused by non-specific interactions with immuno-precipitation antibodies/beads (Okaty et al. 2011). For specific isolation of the PC dendritic compartment, an additional section was performed to separate the 2/3 uppermost part of the molecular layer form the PC soma (Fig. 1C). The dissection of live slice under RNase-free conditions was chosen because the absence of fixation preserves RNA integrity and RNA-ribosome-L10a interaction, while allowing section of relatively thick slices for faster collection of target cells. Collected samples were immediately snap frozen by contact of the tissue fragment with the

wall of a cryotube (Nunc) pre-cooled to −196°C and conserved in liquid nitrogen until ribosome capture. For each of the three biological replicates at each time point we pooled 50 to 64 lobules from separate experiments, ensuring that the samples are homogenized against influence of sex, lobules and experiment day.

2.2 Ribosome capture

Ribosome captures were mainly carried out as already described (Heiman et al. 2008) with modifications to extract separate fractions enriched in cytosolic ribosomes or ER-bound ribosomes (Fig. 1A). Pooled cerebellar lobules with Purkinje expressing EYFP-tagged associated RPL10A were homogenized with a Teflon-glass homogenizer in ice-cold polysome extraction buffer A (10 mM HEPES pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 0.5 DTT, 100 µg/ml cycloheximide, protease and RNase inhibitors). After centrifugation for 10 min at 2000 *g* (4 °C), the supernatants and the pellet containing free and attached ribosomes, respectively were subjected to detergents solubilisation using NP40 (1%) and DHPC (diheptanoyl-sn-phosphatidylcholine, 30 mM) for 5 min (4 °C). To separate insoluble fractions, an additional centrifugation for 10 min at 13,000 *g* was performed. Both supernatants were then immunoprecipitated using a rabbit polyclonal anti-GFP antibody (Abcam, ab290) to specifically capture ribosomes present in PCs and bound to the transduced EYFP-RPL10A. GFP antibody and prewashed protein G magnetic beads (Invitrogen) were added to the supernatant and incubated for 2 h under rotation (4 °C). The supernatant were then separated from the magnetic beads/antibody/EYFP-RPL10A/RNA complexes and subjected to a second round of immunoprecipitation. The beads/proteins/RNA complexes were then washed 3 times with buffer A in which the concentration of KCl was increased from 150 mM to 350 mM. Finally, the proteins/RNA complexes were eluted from magnetic beads using nuclease-free water and TRIzol-LS reagent (1:3) (Invitrogen) before snap-freezing in liquid nitrogen.

2.3 RNA extraction

RNA was then extracted in spin columns with a silica-based membrane (PureLink RNA micro kit, Invitrogen) following the "Using TRIzol reagent with the PureLink RNA Micro kit" protocol, starting from 400 µl of TRIzol solution and eluting in 22 µl of nuclease-free water. The concentration and size profiles were then measured using 1 µl of RNA on a BioAnalyzer RNA 6000 pico kit (Fig. S2A).

215.8 ± 110.7 ng RNA was recovered per sample. RNA integrity numbers (RIN) (Schroeder et al. 2006) were significantly different between cytoplasmic and rER-bound samples 5.8 ± 0.6 and 3.3 ± 0.5 respectively ($p = 1.1 \times 10^{-5}$, paired t-test), suggesting a difference in composition rather than degradation, as cytoplasmic and rER-bound fractions were processed in parallel, and RIN is calibrated for total RNA, while our samples are ribosome-bound immunoprecipitated RNAs.

2.4 Sequence processing and alignment

The libraries were de-multiplexed using the PipelinePairedEndExtraction.pl script from the Moirai workflow system (Hasegawa et al. 2014). The first 6 bases of the second read were trimmed as they correspond to the site of annealing of the reverse-transcription primer, where sequencing errors are more frequent. Artifacts resembling empty constructs were removed using TagDust (Lassmann et al. 2009), and reads similar to the ribosomal RNA sequence V01270.1 were removed using the rRNAdust program. The version of rRNAdust used was the same as described and contained in (Harbers et al. 2013). However, in the meanwhile rRNAdust has been integrated into TagDust, and we recommend to use the newest version from http://tagdust.sourceforge.net/.

Paired reads for which one mate was removed by these filters were discarded using a custom Perl script available upon request and that is now superseded by the matchPairedEndSeq program from the Moirai workflow system. The remaining reads were aligned on the Baylor 3.4/rn4 rat genome assembly (Gibbs et al. 2004) paired end using BWA version 0.5.9 (Li 2009) with a maximum insert size of 2 Mb. Pairs that were not "properly paired", and pairs where the sum of the mapping qualities (Li et al. 2009) was lower than 20 were discarded. The alignments were then converted to BED12 format using the "pairedBamToBed12" software (https://github.com/nicolas-bertin/bedtools-pairedBamToBed12), derived from BEDTools (Quinlan and Hall 2010).

The CAGEscan 5' mates were clustered using the peak calling algorithm Paraclu (Frith et al. 2008, http://www.cbrc.jp/paraclu/) version 5 with default parameters. The algorithm identified 926,736 hierarchically organized clusters with a minimum tag count of 30. After removing the clusters which satisfy the condition (maximum density / minimum density) < 2, of which the length was shorter than 2 bp or longer than 100 bp, and which were contained within a larger cluster, 48,049 clusters remained for the analysis. The pairs starting from within each cluster are taken and all corresponding mates were

concatenated using the "CAGEscan-Clustering" software (http://fantom.gsc.riken.jp/software), yielding a sequence of start-end positions. This chain is the "CAGEscan cluster". The 48,049 CAGEscan clusters can be found in File S1.

2.5 Transcript classifier

For the cluster classification, we used the complete set of Paraclu clusters without requiring a minimum tag count. This generated 1,140,223 clusters, which were divided into those nearby (± 1000 kb) the start sites of known Ensembl genes and the remainder. The classifier splits the dataset into training and test datasets. Gaussian models are trained to capture the relative distribution of 4-mer occurrences surrounding the known TSSs in the test set using a window of 2 kb. Each sequence is scored against all models resulting in a 256 vector of values for each sequence. The latter together with the cluster label is used to derive a random decision tree ensemble model (Fan et al. 2003). Finally, the RDT model is used to classify test sequences not used in the training of any models. The entire procedure is repeated many times and predictions for each cluster averaged. The implementation is available at http://tometools.sourceforge.net/.

The specificity and sensitivity of the classifier can be visualized in the form of a Receiver Operator Characteristic (ROC) curve (Fig. S10). We chose 0.14 as the cutoff value of the classification which split the input data set into 33,865 low confidence, and 14,184 high confidence clusters.

2.6 Motif search

We used Clover (Frith et al. 2004) (version 2011-10-24) to identify those motif patterns which are statistically overrepresented in the vicinity (500 bases added in each direction) of 2,500 clusters that are the most highly enriched clusters for each respective gene, and which also have a high confidence value according to our transcript classifier. Statistical significance has been determined by Clover against a background of those clusters which are of high confidence according to the classifier, but which are not enriched in the ribosome-capture samples. Repeat sequences in the rn4 assembly, as provided by UCSC (Kent et al. 2002), have been ignored during the motif search. For the database of motif matrices to be searched, we used the non-redundant part of the JASPAR Core database for vertebrates (Sandelin et al. 2004) (downloaded 13 May 2013). The result of the motif search can be found in File S2.

2.7 Rn7SL RNA

The genomic location of the *Rn7SL* RNA in the rn4 genome were determined by taking the longest hits for human *RN7SL1* by BLAT (Kent 2002): chr6:91,323,108..91,323,406(-), chr6:91,117,284..91,117,582(+), chr12:22,851,196..22,851,493(-), and chr10:59,473,912..59,474,211(+). Expression values on these intervals were calculated as the sum of the expression values of the Paraclu clusters overlapping them.

2.8 Analysis of the dendritic clusters

To define the clusters expressed in dendrites, first all clusters with more than 1 raw tag of expression in the dendritic, rER sample plus the dendritic, cytoplasmic sample have been divided into quartiles. The clusters in the upper quartile, with expression going from 4 to 485 raw tags, are the "dendritic clusters".

To search for enriched Gene Ontology terms, we compared the 974 dendritic clusters (845 Gene Symbols) against all remaining 47,075 clusters (8,137 Gene Symbols). Gene Symbols starting with the string "Rik" or "LOC" in the identifier have been removed as they can not be recognized by GOrilla (Eden et al. 2009).

The statistically enriched terms, as identified by GOrilla, can be found in Table S3. We used REVIGO (Supek 2011) to filter, summarize and group the statistically enriched Gene Ontology terms.

To determine how many gene symbols of the dendritic clusters overlap with the genes found in neuropil by (Cajigas et al. 2012), we compared to the 2,550 gene symbols listed in their Table S10.

2.9 Comparison with other datasets

We manually inspected 6,956 *in situ* hybridizations from the Allen Brain Atlas to score the expression of genes in PCs. We used Table S5 from (Doyle et al. 2008) as a reference list of genes enriched in mouse PCs by ribosome capture. In this list, 774 gene symbols corresponded to RIKEN cDNAs (xxxxRik). Because many have been annotated since 2008, we queried NCBI's Gene database (downloaded Sept 14th, 2012) with custom scripts, to update symbols when possible. Only 186 entries kept a cDNA ID as gene symbol. This ensured an accurate comparison with our dataset (Fig. 4A, B). Table S10 from (Cajigas et al. 2012) was used as the source for the list of gene symbols for the transcripts expressed in the hippocampus neuropil. Since this list did not contain cDNA IDs, we used

the symbols without attempting to update them. We queried the February 2012 release of the CDT-DB, by selecting all entries where expression was detected at P21, and used the symbols as is. Lastly, we obtained the list of genes expressed in PCs established by Kirsch and collaborators (2012) by mining the Allen Brain Atlas, by downloading the table "purkinjeDetectorList.csv" from the authors website, and updating the gene symbols in the same manner as for (Doyle et al. 2008). Our Table S4 provides the intersection between our clusters and each of these datasets. To generate the Venn diagram in Fig. 4B we used eulerAPE 2.0.3 (http://www.eulerdiagrams.org/eulerAPE/).

2.10 Digital expression analysis

We used the Bioconductor libraries edgeR (Robinson et al. 2009) version 2.6.10 and Limma version 3.12.1 to identify clusters statistically significantly differentially represented between sets of libraries, using an adjusted *p*-value of 0.1 as a threshold. We then mined their Gene Ontology terms with GOrilla (Eden et al. 2009), and visualized the results with REVIGO (Supek et al. 2011), which clustered them according to their semantic similarity and projected them into a 2-dimensional plane based on a multidimensional scaling method. Representative terms have been chosen for each cluster of similar terms based on their enrichment p-values.

2.11 Immuno-histofluorescence quantification

To quantify the relative expression level of selected proteins in PCs and other cells, primary cerebellar cultures prepared from P19 embryonic primordium were fixed (4 % paraformaldehyde, 9.25 % sucrose) after 21-24 days *in vitro*. Triple-staining was performed overnight with anti-calbindin D28k (1/1000, mAb, Swant, SZ) and commercial antibody (1/100, rabbit and donkey anti-rabbit Alexa488-conjugated secondary antibody, A21206, Life Technologies) against one of the target protein (see Table S8), before counterstaining all nuclei with DAPI. After image acquisition (Nikon Eclipse Ti, 81 fields/coverslip), relative signal intensity in PCs and surrounding cells was computed via an ImageJ macro (supplemental file S3). For image analysis, the thresholded calbindin staining was used to define the PC regions-of-interest while the number of DAPI-stained nuclei was used as an index of local cell density. Images where PC represented less than 10 % of the imaged surface or where nuclei were too sparse (<15 %) were not analyzed. For robustness relative distributions were calculated as ratios of median fluorescence intensities between non-PC area and PCs region-of-interest.

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