Quantitative 3D imaging of orexin and orexin receptor distribution in the intact mouse brain

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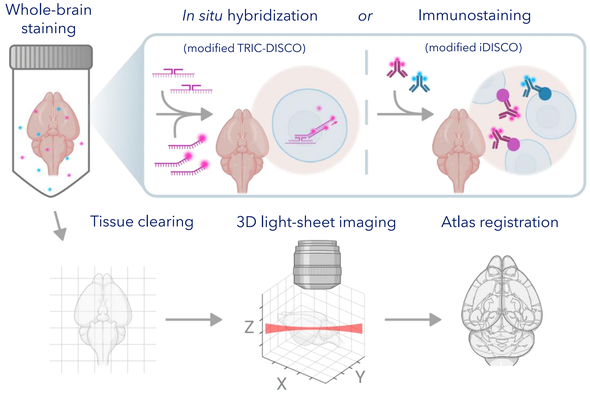
Background & Aim

Orexins (Ox, also termed hypocretins) and their cognate receptors, Ox1R (Hcrtr1) and Ox2R (Hcrtr2), play an important role in controlling several fundamental physiological functions such as sleep/wakefulness, appetite regulation, energy homeostasis, reward and reproduction. Hence, disturbances in the orexin system are implicated in several diseases, notably narcolepsy, for which there is a considerable interest in developing more effective treatments. To improve circuit-level understanding of the orexin receptor system, the present study aimed to generate a complete 3D map of the orexin system in the intact mouse brain at single-cell resolution using whole-brain immunohistochemistry (IHC) and in situ hybridization (ISH).

Methods

Male C57BL/6J mice (8-9 weeks old) were maintained under a reversed light/dark cycle (lights off 3 AM, lights on 3 PM) and terminated 6 hrs into the dark phase (9 AM). Brains were perfusion-fixed and processed for whole-brain IHC (Hcrt, n=8 mice) and ISH (Hcrt, n=1). Upon clearing, whole-brains were scanned using light sheet fluorescence microscopy (LSFM) followed by AI-based, automated 3D quantitative image analysis.

1 Experimental design: Whole-brain *in situ* hybridization and immunostaining



2 Whole-brain mapping of Orexinergic neurons and their downstream projection targets

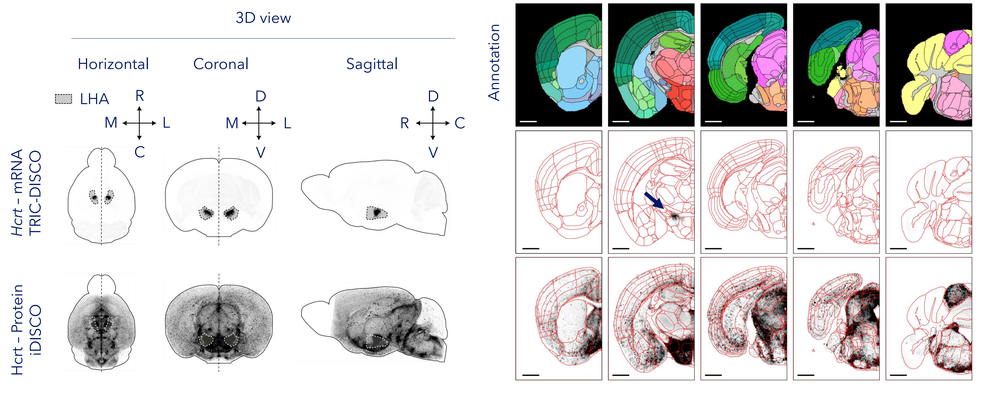


Figure 1. Experimental design.
Whole mouse brains were stained for *Hcrt* mRNA (n=1) or protein (n=8) and cleared using modified TRIC-DISCO or iDISCO protocols, respectively. The samples were scanned using either the LCS-SPIM (*Bruker*) or UM2 (*Miltenyi*) systems. Subsequently, the raw mRNA data or averaged protein data were transformed to the Gubra multimodal atlas, enabling comprehensive analysis of regional information across the entire brain. Abbreviations: *Hcrt*: Hypocretin neuropeptide precursor; TRIC-DISCO. Tris-mediated retention of *in situ* hybridization signal during clearing; iDISCO: Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs.

Figure 2. Whole-brain mapping of Orexinergic neurons and their downstream projection targets.
The figure illustrates the subcellular localization of *Hcrt* mRNA and protein across the entire mouse brain. *Hcrt* mRNA predominantly stains the soma of *Hcrt*-positive cells, emphasizing their restricted localization in the central (rostro-caudal) region of the LHA (Arrow). In contrast, protein staining reveals not only the soma but also the downstream projections of these neurons, highlighting their extensive reach across various regions of the hypothalamus, midbrain, and brainstem. The boundaries of the LHA are indicated by the gray dashed area. Abbreviations: D: Dorsal; V: Ventral; L: Lateral; M: Medial; R: Rostral; C: Caudal; LHA: Lateral hypothalamic area; TRIC-DISCO. Tris-mediated retention of *in situ* hybridization signal during clearing; iDISCO: Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs. The scalebar is 1mm.

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3 Pipeline to map gene expression across the whole-mouse brain

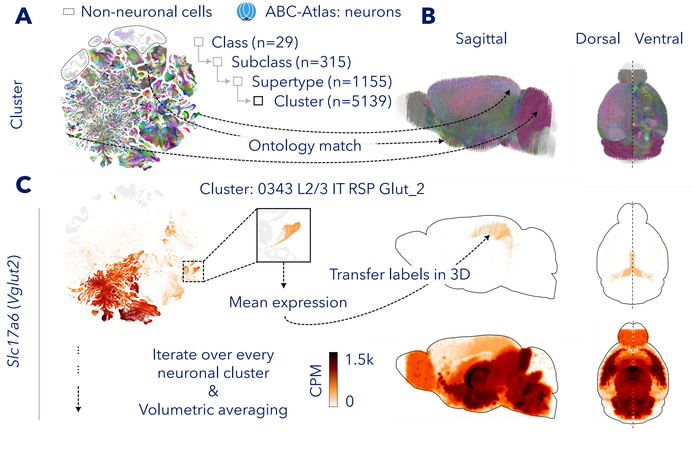

Figure 3. Utilizing the ABC-Atlas to map gene expression across the whole mouse brain in 3D.
(A) UMAP representation of the 10X snRNAseq dataset from the ABC-Atlas, where each color denotes a distinct neuronal subtype. **(B)**: Merged MERFISH data (n=5 animals), transformed onto the Gubra LSFM template, with each cell labeled by its transcriptional identity. **(C)** Since both datasets share the same ontological framework, average gene expression levels can be calculated for each neuronal subtype and projected back into 3D space. For each neuronal cluster, volumetric averaging and smoothing are applied to generate a whole-brain map of predicted gene expression for a specific gene of interest.
Abbreviations: UMAP, Uniform manifold approximation and projection; Slc17a6, Solute carrier family 17 member 6 (*Vglut2*); CPM, Counts per million.

4 Spatial mapping of Orexin receptor expression and peptide co-localization domains

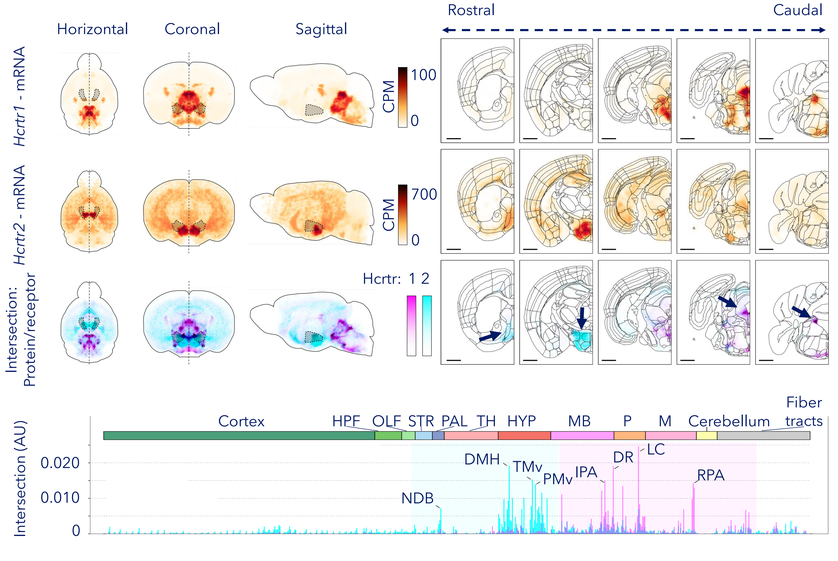

Figure 4. Spatial mapping of Orexin receptor expression and peptide co-localization domains.
(A) Predicted expression patterns of *Hcrtr1* and *Hcrtr2* across the entire mouse brain. *Hcrtr1* is predominantly localized in the caudal regions (midbrain and brainstem), whereas *Hcrtr2* shows a more rostral bias (hypothalamus). **(B)** Whole-brain map and **(C)** region-specific quantification illustrate the co-localization of Hcrt protein (Orexinergic projections) with the respective *Hcrtr1* (magenta) and *Hcrtr2* (cyan) receptors. This mapping suggests that Orexin may primarily signal through *Hcrtr2* in rostral areas and through *Hcrtr1* in caudal regions. The scalebars are 1mm. Abbreviations: CPM: Counts per million. HPF: Hippocampal formation; OLF: Olfactory areas; STR: Striatum; PAL: Pallidum; TH: Thalamus; HYP: Hypothalamus; MB: Midbrain; P: Pons; M: Medulla; NDB: Diagonal band nucleus; DMH: Dorsomedial nucleus of the hypothalamus; TMv: Tuberomammillary nucleus, ventral part; DR: Dorsal nucleus raphe; LC: Locus ceruleus; RPA: Nucleus raphe pallidus.

Conclusion

- We have developed a pipeline enabling automated whole-brain mapping and quantification of *Orexin* mRNA and protein expression.
- By integrating *Ox1r* and *Ox2r* mRNA data from the ABC-Atlas, we provide a comparative anatomical map of Orexin vs. Orexin receptor expressing brain regions.
- We confirm the *Ox1r* and *Ox2r* mRNA expression in key brain regions controlling wakefulness.
- Whole-brain IHC and ISH is instrumental for increasing circuit-level understanding of the Orexinergic system.