A proteomic and functional analysis reveals that 5-HT$_6$ receptors modulate neuronal differentiation by recruitment of Cyclin-dependent kinase 5

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Abstract

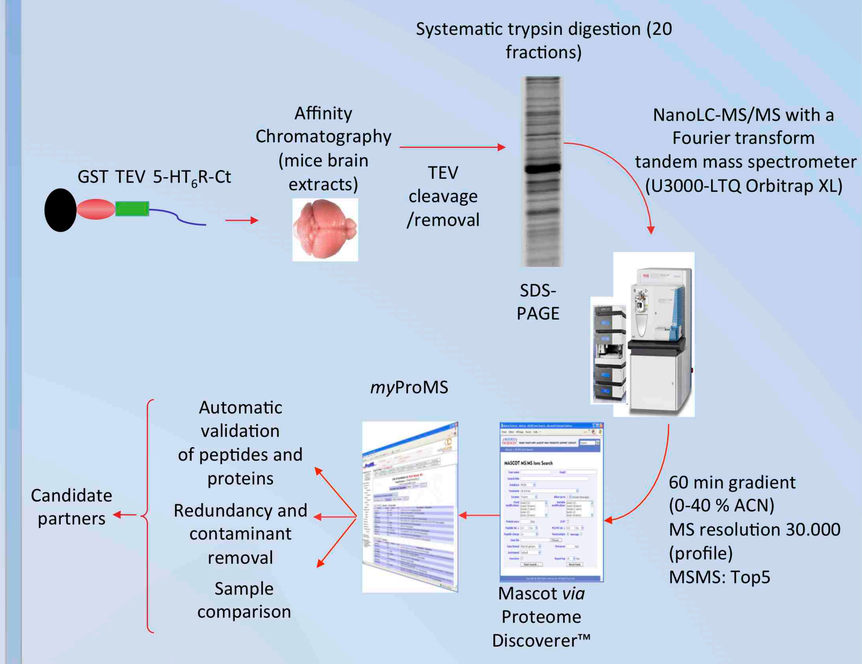
The serotonin 5-HT$_6$ receptor is expressed in CNS regions involved in the pathogenesis of disorders like Alzheimer's disease and schizophrenia. 5-HT$_6$ receptors are a promising target for treatment of the accompanying cognitive deficits, since their blockade consistently enhances mnemonic performance in rodents [1]. Paradoxically, **still little is known about 5-HT$_6$ receptor-associated signaling pathways, an issue we have addressed by a proteomic approach**. Previously, we showed physical association of the 5-HT$_6$ receptor with several members of the mTOR pathway and demonstrated that mTOR recruitment by prefrontal 5-HT$_6$ receptors perturbs cognition in schizophrenia [2]. Here we show that **5-HT$_6$ receptors interact with Cyclin-dependent kinase (Cdk)5, a protein which controls actin cytoskeleton dynamics and modulates neurodevelopmental processes such as neuron migration, neurite outgrowth, synaptogenesis and dendritic spine morphogenesis**.

The present study explored the role of Cdk5, under the control of 5-HT$_6$ receptors, in the differentiation of NG108-15 neuroblastoma cells and rat embryonic hippocampal and striatal neurons in primary culture, assessed by neurite outgrowth. Expression of voltage-gated Ca^{2+} channels was also analyzed in NG108-15 cells using Fura2 imaging.

Expressing 5-HT$_6$ receptors in NG108-15 neuroblastoma triggered neurite outgrowth and induced expression of functional voltage-gated Ca^{2+} channels. **These effects were not further enhanced by an agonist (WAY181187, 1 µM) but were prevented by SB258585 (10 µM), a selective 5-HT$_6$ antagonist**. Thus, upon SB258585 treatment, cells showed a decrease in neurite length of 48.4% compared to untreated cells. Expression of a dominant-negative (DN) Cdk5 or treating cells with roscovitine (pharmacological inhibitor of Cdk5) likewise inhibited NG108-15 cells differentiation induced by 5-HT$_6$ receptor expression. SB258585 also impaired the association of 5-HT$_6$ receptor with Cdk5 in NG108-15 cells, as determined by co-immunoprecipitation, suggesting that this interaction was necessary for induction of differentiation. **Treating striatal neurons with either SB258585 or roscovitine immediately after seeding decreased neurite length, as determined 24 hrs later. Conversely, exposure of neurons to WAY181187 did not significantly affect neurite.** Further supporting a role of endogenously expressed receptors in differentiation of striatal neurons, **silencing 5-HT$_6$ receptor expression in cultured neurons significantly reduced neurite length**.

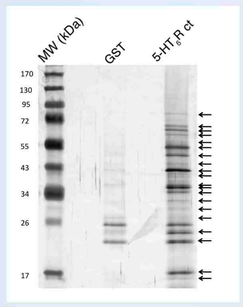
Complementing work indicating that 5-HT$_6$ receptors modulate neuronal migration [3], the present data show that 5-HT$_6$ receptors developmentally promote neuronal differentiation and reveal a critical role for Cdk5 in this process. These novel insights into molecular substrates underlying neurodevelopmental effects of 5-HT$_6$ receptors are of potential importance to the pathophysiology and treatment of early-onset CNS conditions like autism-spectrum disorder and schizophrenia.

Experimental Layout

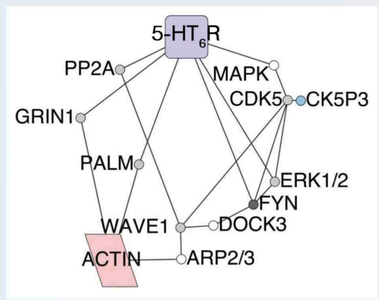


Characterization of brain proteins interacting with 5-HT$_6$ receptor C-terminus

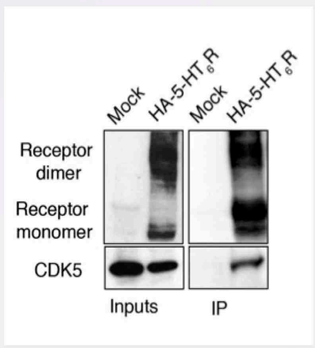
Our experimental approach allowed us to demonstrate **association of the 5-HT$_6$ receptor C-terminus with a network of proteins involved in the actin cytoskeleton dynamics**, which is crucial for neurite growth, dendritic spines formation and neuronal migration. Among those, we find the **Cyclin-dependent kinase 5 (Cdk5)**.



A SDS-PAGE analysis of mouse brain proteins obtained after pull-down with a GST labelled 5-HT$_6$ C-terminal tail. Control pull-down was performed with GST alone. A colloidal coomassie blue stained gel obtained in a typical experiment is shown . Arrows indicate bands corresponding to specific interactors.



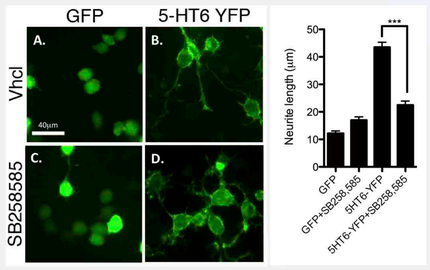
A Simple Interaction File has been designed and imported in Cytoscape (v 2.8.0) to graphically show the interactions between 5-HT$_6$ and a network of protein involved in cytoskeleton regulation. Light grey nodes show proteins found in our screen and the dark grey node shows a protein already described as a 5-HT$_6$ partner, not found in our screen, but belonging to the same network.

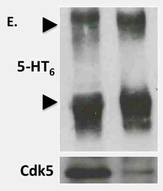


Co-immunoprecipitation of native Cdk5 and HA tagged 5-HT$_6$ receptor from NG108-15 cells shows an interaction between the receptor and endogenous Cdk5.

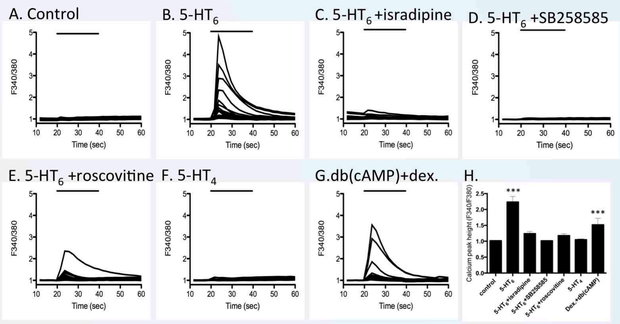
The 5-HT$_6$ receptor induces the differentiation of NG108-15 cells

Transfection of the 5-HT$_6$ receptor in NG108-15 neuroblastoma cells induces **neurite growth and the expression of calcium channels**, two hallmarks of NG108-15 differentiation.





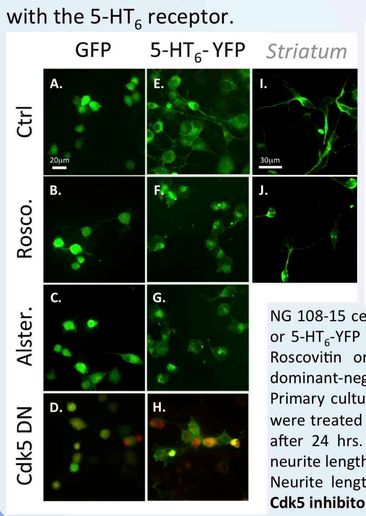
NG108-15 cells transfected for 48h with either GFP (A, C) or 5-HT$_6$-YFP (B, D). When indicated, cells were treated with 10µM SB258585.The histogram shows the quantification of neurite length in each condition. The co-immunoprecipitation (E) shows that SB258585 disrupts the interaction between 5-HT$_6$ and Cdk5.

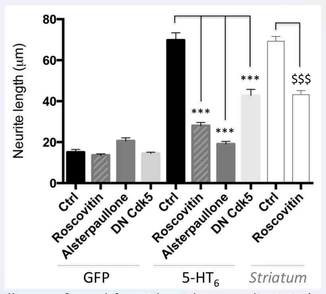


Representative traces for intracellular calcium release induced by potassium depolarization (black line) in NG108-15 cells transfected for 48 hours with cytosolic GFP (A, G), 5-HT$_6$-YFP (B-E) or 5-HT$_4$-GFP (F). Cells in C were treated with isradipine (3µM) just prior to depolarization, cells in D and E were treated 4 hours after transfection with either SB258585 (10µM) (D) or roscovitine (25µM) (E). Cells in G were differentiated with db(cAMP) and dexamethasone for 48 hours. The histogram in H shows the quantification of the calcium peak height for three independant fields of view with at least 10 transfected cells measured in each field of view.

The differentiation induced by 5-HT$_6$ requires Cdk5 signaling

To see if the differentiation induced by the 5-HT$_6$ receptor requires the activity of Cdk5, we used two approaches: **a pharmacological approach** in which neuroblastoma cells and cultured striatal neurons were treated with two Cdk5 inhibitors, and **a genetic approach** where a dominant negative of Cdk5 (Cdk5D144N) was coexpressed in NG108-15 cells along with the 5-HT$_6$ receptor.





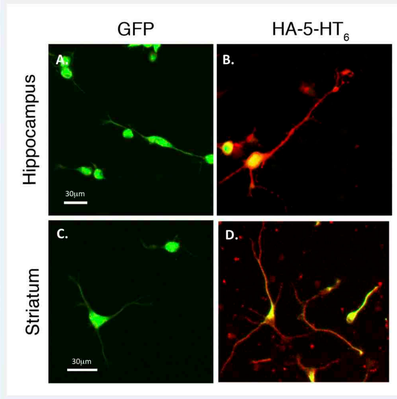
NG 108-15 cells transfected for 48h with cytosolic GFP (A-D) or 5-HT$_6$-YFP (E-H). When indicated, cells were treated with Roscovitin or Alsterpaullone, or co-transfected with the dominant-negative mutant Cdk5D144N-mCherry.

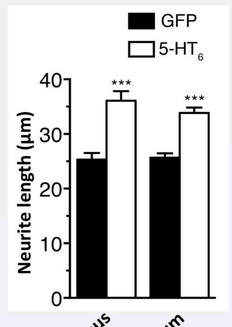
Primary culture of striatal neurons from E15,5 rat pups (I-J) were treated with roscovitin just after seeding and observed after 24 hrs. Neuronal MAP2 labeling was used to assess neurite length, nuclei were stained with Hoechst.

Neurite length quantification shows **shorter neurites with Cdk5 inhibitors and Cdk5 dominant negative**.

5-HT$_6$ receptor overexpression promotes neurite growth in cultured hippocampal and striatal neurons

Cultured hippocampal and striatal neurons were infected with a sindbis virus coding for either GFP or an HA-tagged 5-HT$_6$ receptor. Here we show that **over-expressing 5-HT$_6$ significantly increases neurite length.**

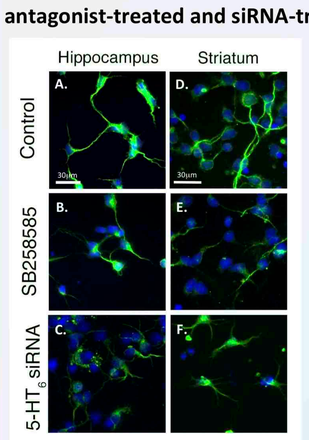


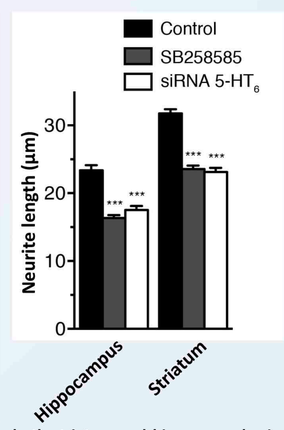


Primary culture of hippocampal (A and B) and striatal (C and D) neurons from E17,5 and E15,5 rat embryos, respectively, infected with GFP (A and C) or HA-5HT$_6$ receptor (B and D). MAP2 labeling was used to measure neurite length, and HA labeling (red) shows 5-HT$_6$ receptor expression. The histogram shows neurite length quantification. **5-HT$_6$ expressing neurons show longer neurites.**

Endogenous 5-HT$_6$ receptor is involved in neurite growth in striatal and hippocampal neurons in culture

We used the 5-HT$_6$ receptor antagonist SB258585 and a 5-HT$_6$ receptor targeting siRNA to block endogenous 5-HT$_6$ receptor in rat striatal and hippocampal neurons. We found that **neurite length is significantly reduced in both antagonist-treated and siRNA-transfected cultures**.





Primary culture of hippocampal (A-C) and striatal (D-F) neurons from E17,5 and E15,5 rat embryos, respectively.
MAP2 labeling (green) was used to measure neurite length, and Hoechst staining shows nuclei. Neurons were either treated with SB258585 (B and E), or transfected with a 5-HT$_6$ targeting siRNA (C and F), 4 hours after plating.

Inhibiting endogenous 5-HT$_6$ receptor in both striatum and hippocampal primary cultures significantly decreases neurite length, as shown on the histogram.

Conclusion

Expressed almost exclusively in the CNS, highest densities of 5-HT$_6$ receptor are present in striatum and nucleus accumbens, and to a lesser extent, in several regions of the hippocampus and the cerebral cortex, *i.e.* structures that are involved in the control of cognitive functions and implicated in the pathogenesis of numerous neurological and psychiatric diseases ([4];[5]). The 5-HT$_6$ receptor has recently emerged as one of the most promising targets for treating cognitive dysfunction in both Alzheimer's disease and schizophrenia ([1];[2];[6]), and although in the last few years, we've seen many new compounds aimed at modulating 5-HT$_6$ receptor activity, the mechanisms involved in the regulation of their functional activity remain paradoxically largely unexplored. Therefore, we decided to use a non-biased approach to get a global picture of signalling proteins physically connected to the receptor. Interestingly, this approach has revealed an interaction with a network of proteins controlling the actin cytoskeleton dynamics, notably **Cyclin-dependent kinase 5** (Cdk5). Our study shows that through this interaction, the 5-HT$_6$ receptor is indeed involved in the control of cell differentiation and neurogenesis, in neuroblastoma cell lines as well as neurons in culture. Since this network has been identified as a key regulator of neuro-developmental mechanisms such as neuronal migration, neurite outgrowth and dendrite spine morphogenesis [7] we believe it could explain the **regulation of neuro-developmental processes by 5-HT$_6$ receptors**.

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The authors declare that they have no conflict of interest.

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