Validation of Doublecortin Hippocampal Localization

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Abstract

Doublecortin plays an important role in immature neurons by helping to structure microtubules. Because it is highly prevalent in immature neurons, sites of neurogenesis, such as the hippocampus and olfactory bulbs, are frequently targeted to analyze doublecortin expression. To elucidate where doublecortin is localized in the hippocampus, a previously tested protocol was used to test its effectiveness in staining for doublecortin. As hypothesized, doublecortin was found to be located in the granule cell layer of the dentate gyrus. Additionally, the protocol proved to be effective in identifying doublecortin-labeled cells, adding support to its localization in the hippocampus.

Introduction

Doublecortin is a neuronal migration protein; it functions to bind, bundle, and stabilize microtubules (Francis et al. 1999). Due to its nearly exclusive expression in developing neurons, doublecortin has been used increasingly as a marker for neurogenesis (Couillard-Despres et al. 2005). Clinically, mutated doublecortin is part of an X-linked lissencephaly syndrome, both of which have limited viable treatment options.

To elucidate where doublecortin is localized in the hippocampus, a previously tested protocol was used to test its effectiveness in staining for doublecortin. As hypothesized, doublecortin was found to be located in the granule cell layer of the dentate gyrus. Additionally, the protocol proved to be effective in identifying doublecortin-labeled cells, adding support to its localization in the hippocampus.

In the following experiment, an immunohistochemistry protocol supplied by Denny et al. (2012) will be validated for its overall effectiveness in staining for doublecortin. As demonstrated by Denny, the anti-doublecortin stain detected neurons expressing doublecortin and did not stain for any neurons that failed to express doublecortin or that had undergone x-irradiation. This proves the protocol to be an effective measure for detecting doublecortin positive neurons, which will be confirmed in the following experiment, specifically looking for localization in the dentate gyrus region.

The following study will provide support for doublecortin’s hippocampal localization, as well as test the validity of the proposed protocol for replication. Determining subcellular localization is important for understanding protein function and is a critical step for genome annotation (Scott et al. 2005). With this knowledge, improved target identification during drug research may be facilitated, which may help find treatments for lissencephaly and double cortex syndrome, both of which have limited viable treatment options.

Methods

Collection of tissues:

Three adult male F344XBN rats were sacrificed between 28-32 months of age. Brain samples were collected through a transcerebral perfusion process using a ketamine/xylazine mixture to anesthetize the rats. Then, 4% paraformaldehyde was pushed through the cardiovascular system. Brains were incubated overnight in paraformaldehyde and 40um coronal sections were collected using a vibratome. Every 12th section was saved and placed in cryoprotectant.

Immunohistochemistry:

Doublecortin immunohistochemistry was carried out by washing sections in 1x PBST three times for ten minutes and then quenched in 0.3% hydrogen peroxide in 1x PBS at room temperature for 15 minutes. Sections were washed in 1x PBST three times for ten minutes again and then blocked in 10% normal donkey serum in 1x PBST for two hours at room temperature. Sections were incubated in primary antibody (Goat anti-doublecortin, 1:500 dilution, Santa Cruz, # SC.8066) for 48 hours at 4°C in 10% normal donkey serum. Sections were then washed in 1x PBS three times for 15 minutes at room temperature. Then, sections were incubated in secondary antibody (Jackson Immuno # 705-065-003, 1:500 dilution) for two hours at room temperature in 1x PBS. Sections were washed in 1x PBS three times for ten minutes and incubated in an ABC Elite Kit for one hour at room temperature. Sections were washed in 1x PBS three times for ten minutes and incubated overnight in 1x PBS. Then, sections were washed in 1x PBS three times for ten minutes and incubated for nine minutes in diaminobenzidine. Tissue was washed in 1x PBS three times for ten minutes and Nissl stained. Nissl staining was carried out by exposing sections to ddH2O for 30 seconds followed by incubation in 0.1% cresyl violet for 3.5 minutes. Sections were incubated in ddH2O for 30 seconds, then through an alcohol gradient of 70%, 95%, and 100% ethanol for 90 seconds, 15 seconds, and 30 seconds respectively. Sections were rehydrated in ddH2O for 30 seconds. Rat AET3AD was the only rat to undergo the Nissl staining on the first day. The next day, the remaining two rats were Nissl stained. Rat AET8LAD was exposed to the cresyl violet for four minutes, while rat AET9AD was exposed to the cresyl violet for 4 minutes and 15 seconds. Tissue was incubated in 1x PBS over the weekend. Four days later, sections were then mounted to slides and left to dry. Slides were then treated with Xylene for four minutes before being coverslipped with Permount.

Results

The diagram in Figure 1 depicts the main regions of a hippocampus. Staining was expected in the cell bodies of granule cells in the granule cell layer of the dentate gyrus (Figure 1). However, possible doublecortin staining was expected in the axonal projections of granule cells, which extend particularly to the CA3 region of the hippocampus. Analysis of doublecortin stained hippocampal sections showed positive staining in the granule cell layer of the dentate gyrus (Figure 2). However, quantification of the staining was not completed in this study. Visually, the cells appeared to be lightly stained. Upon closer inspection under the microscope, analysis verified that some limited staining was present and revealed the degree of the light staining. The limited staining made identifying doublecortin stained cells very difficult. Nevertheless, some stained granule cells in the granule cell layer (Figure 1) were found using microscopy imaging (Figure 2).

Figure 1. Hippocampus diagram. The diagram shows the outer molecular layer (mo) in lavender color, and the inner granule cell layer (sg) in magenta color, in the dentate gyrus (DG). The CA1 and CA3 regions are also shown.
Images of two different hippocampal sections were taken to verify that granule cells in the dentate gyrus were stained. The two sections from the same rat sample were chosen for imaging due to qualitatively darker staining than other sections. Figure 2A shows five doublecortin stained granule cells from the granule cell layer of the dentate gyrus. In Figure 2B, two granule cells in the granule cell layer of the dentate gyrus are shown.

Figure 2. Microscopy image of doublecortin stained cells in two different section of the same rat sample. Doublecortin-positive granule cells in the granule cell layer of the dentate gyrus are shown by the dark brown staining at 100x resolution. The black arrows point the the cell bodies of stained granule cells. A) Image of five doublecortin positive granule cells. B) Image of two doublecortin positive granule cells. Salt deposits on the slides account for the graininess of both images.

Doublecortin staining could not be quantified because of light staining and time constraints. Counting the stained cells would have been difficult and required more time than available. However, had quantification been possible, three judges would have identified the regions with doublecortin staining and counted the doublecortin-positive cells. Judges would have used the Allen Interactive Brain Atlas to become familiar with identifying regions of the brain, specifically the hippocampus. Observers would have been blind; all identifying information about the slides would have been concealed from the judges during counting. The data reported by each judge would have been compared for agreement with a Fleiss’ kappa, with a majority of votes determining placement by brain region and ties excluded.

A Kruskal-Wallis test would have been run to determine if any difference in the number of doublecortin-positive cells between the brain regions was significant. This omnibus test controls for false positives in the same way as an F-test would for parametric data. A nonparametric test was chosen because the distribution of the doublecortin-positive cells is highly skewed, with previous literature finding these cells solely in the dentate gyrus out of the three regions studied, and also due to the small sample size of this study. Large sample sizes are necessary for parametric tests, but are not necessary for nonparametric ones, which means the nonparametric test we chose offers more power under conditions which violate the normality assumptions. A Dunn’s multiple comparison test would have have been used to follow up and determine which of the differences were significant in the event of a rejected null hypothesis result from the Kruskal-Wallis. Dunn’s multiple comparison test is well suited for the hypothesized data because it is robust even to large differences in sample size, as was expected in these results.

Figure 4. An example graph shows how doublecortin (DC) staining could have been quantified. If the hypothesis were supported quantification would reveal DC-stained cells are isolated to the dentate gyrus.

Discussion

Before the experiment, the protocol was tested using one section from each rat sample. The two day test was considered successful if the counterstain done on the second day turned out well. The counterstaining was successful; therefore, the protocol was shown to successfully stain doublecortin in rat hippocampi.

Consequently, the rest of the sections proceeded to be stained. Despite slight adjustments in the protocol, all of the sections only had light staining. This made identifying doublecortin positive cells difficult. Nevertheless, microscopy revealed some successfully stained granule cells in the granule cell layer of the dentate gyrus, as hypothesized (Figure 2). Due to the difficulty in identifying stained cells, other regions of the hippocampus were not analyzed to establish the localization of doublecortin positive cells to the dentate gyrus.

The results confirmed that the immunohistochemistry protocol supplied by Denny et al. (2012) successfully stained doublecortin positive cells in adult rat hippocampal sections, specifically in the dentate gyrus. However, further adjustments, such as the time sections were in 0.1% cresyl violet, could be made for more effective, darker staining. This would allow quantification of the staining to be markedly easier and less time consuming.

These results have largely been consistent with existing literature; Rao and Shetty (2004) found doublecortin-positive cells in the granule layer of the dentate gyrus. This corresponds with where our own doublecortin-positive cells were found. Brown et al. (2003) found prominent expression of doublecortin in the dentate gyrus and along the lateral ventricle wall/olfactory bulb axis in adult neurons, but expression elsewhere decreased to levels too low to measure. Other researchers have found similar results. (Nacher et al., 2001) However, these researchers did not all use the same methodology. Brown et al., for instance, sacrificed their rats at various points in time. The similar results despite differences in methodology imply that the results are more robust than previously thought. In contrast, this study’s tissue donors were all sacrificed at approximately the same age. The results from the present study support previous findings that doublecortin is present in adult dentate gyrus cells, but not the CA3 and CA1 regions, where expression is undetectable.

Time constraints and the difficulty in identifying the lightly stained cells limited the results. Only two sections were analyzed under microscopes. Other sections may not have had positive staining in the dentate gyrus. In addition, the overall method of immunohistochemistry is limited to measuring a single point in time; it is unknown, based solely on this methodology, how doublecortin levels varied within each subject over the course of its life, as only the levels at the moment of expiration were
recorded for study.

The protocol utilized in this study, with minor modifications, has potential future use in quantifying doublecortin-positive cells in rat hippocampi. After quantifying doublecortin levels in rat hippocampi under constant conditions, further research could be done on how doublecortin levels alter under various treatments. More specific measurements of where the doublecortin-positive cells are located within subregions of the dentate gyrus could help to elucidate the role it plays in the development of immature neurons, and the role its mutated form plays in cases of lissencephaly. In addition, neurogenesis can be modeled with markers such as doublecortin; this protocol will likely find use as a method of quantifying neurogenesis in future studies.

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References


