ALTERATION OF NEURONAL GROWTH AND MIGRATION IN OLFACTORY STRUCTURES

A Major Qualifying Project Report

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ABSTRACT

Typically, new neurons migrate randomly out of the RMS into the olfactory bulb. This experiment seeks to view the effect of the release of GABA from active granule cells, which is hypothesized to cause directed migration of neurons in the olfactory bulb. Mouse granule cells were experimentally altered through use of lentiviral vectors delivering genes for ion channel Nachbac. Then, fluorescence microscopy, and the Neurolucida computer module were used to investigate the behavior and distribution of new neurons in the olfactory bulb. Data analysis and experiments in the UMASS Lab show that experimental group TRPA1 works to silence granule cell activity. It is shown that TRPA1-treated GCs work to repel new neurons from the area.

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BACKGROUND

The overall goal of this experiment is to investigate how pre-existing neurons work to affect the behavior of migrating new neurons in the olfactory bulb. The reason for research into this area is to better understand how neurons move and integrate into existing brain circuits. Major brain repair strategies that focus on cell replacement have stalled due to a major difficulty, which is that transplanted neurons do not properly integrate into existing brain circuits. This is due to a number of failures with transplanted neurons which do not allow them to connect and integrate like natural neurons. Most transplanted neurons cannot disperse throughout the mature brain to reach their targets, and often remain in the graft location. Transplanted neurons also cannot extend dendrites and axons like natural neurons, which is a severe problem with attempting to establish synaptic connections with other neurons. These issues make it so that stem cells grafted into the mature brain in attempts to treat diseases such as Huntington's Disease (Benraiss and Goldman, 2011) fail to completely and properly treat the condition.

Thus, there is a great need to further understand how neurons move and integrate into brain circuits, and it is a great issue for human health. With greater understanding of neuronal distribution and integration, it is believed that steps can be made in researching clinical neuronal repair and use of stem cells in brain repair. There are currently no truly effective treatments to replace neurons which have been lost, so understanding the migration and integration of neurons in the brain is critically important to developing neuronal repair strategies.

The main focus of this MQP was to test the hypothesis that the activity of pre-existing neurons regulates the migration of new interneurons and directs them to their final location, where they form synapses and integrate into new neuronal circuits. Particularly, insight into how

neurons reach their final destination was sought. In order to research this, the olfactory bulb is used as the major model of the brain.

THE OLFACTORY BULB

In this experiment, the olfactory bulb is the model system used to study neuronal migration, assembly of brain circuits, and the effects of mature cells on new neurons. The olfactory bulb is a structure found in the forebrain of many vertebrates including mammals such as mice and humans. This structure typically deals with the perception of odors and smells in animals. The olfactory system is well known and studied often, and is considered an extremely useful model for studying the assembly of brain circuits.

The olfactory bulb is a very valuable and practical model for studying neuronal systems for a number of reasons. The first major reason is that the olfactory bulb is very simple structurally, especially when compared to other areas of the brain. There has been extensive study on the anatomy and physiology of the olfactory bulb, and the structure and layout of the olfactory bulb is not very complex (Scott et al., 1993), thus making it an attractive model to use for testing of neurons and neural circuits (Sassoe-Pognetto, 2011). Additionally, the olfactory bulb is useful because neurons which are generated in the subventricular zone (SVZ) continuously migrate and integrate into the olfactory bulb through the pathway known as the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994; Lois et al., 1996). Once these immature cells migrate into the olfactory bulb through the RMS, they differentiate into specific neurons (Lois et al., 1996). These neurons integrate into the olfactory bulb through this mechanism throughout the life of the animal (Hinds, 1968).

Due to the simplicity of the anatomy in the olfactory bulb, as well as the continual production and migration of new neurons into the area, the olfactory bulb is the model chosen to

investigate integration and migration of neurons in the brain. Particularly, in this MQP, the olfactory structure in mice is used as the brain model for study.

Despite the large wealth of knowledge about the structure and function of the olfactory bulb, there are still a number of mysteries regarding the mechanism of neuronal movement out of the RMS and into the olfactory bulb. Generally new neurons in the RMS of the olfactory bulb will either leave the RMS and migrate radically to the granule cell layer and will differentiate, or they will continue to migrate tangentially in the RMS (Scott et al., 1993). It is not fully understood the mechanism by which this decision is made. Although there is research that indicates that signaling with the neurotransmitter GABA can affect behavior of migrating neurons (Bolteus and Bordey, 2004), the mechanism by which this may act *in vivo* is not fully understood. However, preliminary research prior to this MQP has resulted in the hypothesis that pre-existing granule cells may regulate the behavior of migratory cells through actions located in their basal dendrites. The hypothesis is that mature granule cells regulate this transition of new neurons out of the RMS and into the granule cell layer.

GRANULE CELLS

Granule cells are a type of neuron located in the brain in a number of different locations, including the olfactory bulb. Although small in size, there are a large number of these cells present in the brain, and they are the target of the experimental procedure. Preliminary results at UMASS Medical have resulted in two important observations being made about granule cells in the olfactory bulb. It has been revealed that the basal dendrites of granule cells extend into the RMS where new migrating neurons are present. This is shown in **Figure 1**, which was received from the Lois Lab as part of the research design briefing.

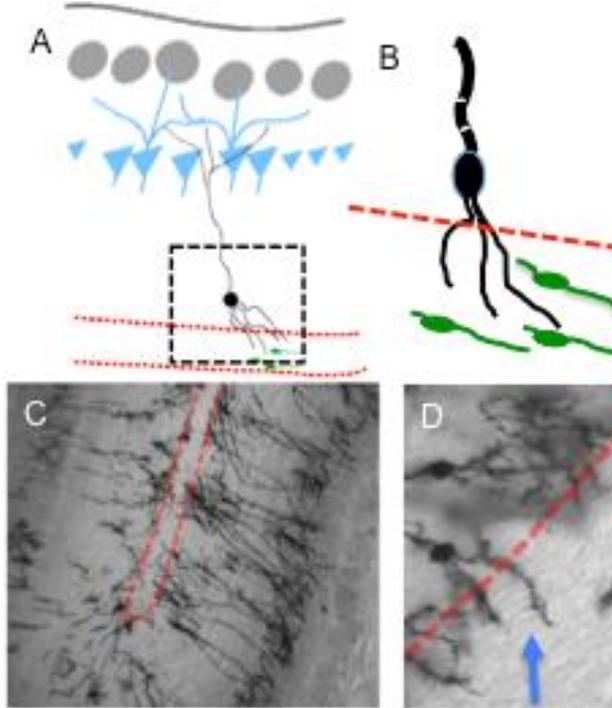# Figure 1. Relationship Between Granule Cell Basal Dendrites and the RMS

Figure 1 shows both diagrams as well as microscopic views of the presence and position of granule cells in the olfactory bulb. Figures A and B are diagrams which illustrate the organization of cells in the olfactory bulb. Granule cells are marked as black, new migratory cells are marked as green, and the RMS is labeled with a dotted red line. Mitral cells are blue and glomeruli are grey. Figure 1A shows an extended view of the olfactory bulb, while B illustrates a magnification on the granule cell. Particularly, 1B shows that the basal dendrites of the granule cell extend into the RMS, near migrating cells. Figures 1C and D are magnifications of olfactory bulb samples which are immunostained for granule cells. Again, the dotted red line illustrates the RMS, and the dark black stains reveal granule cells. Figure 1D is a magnification of the area which shows that the basal dendrites extend into the RMS (labelled by the blue arrow), and are shown to be near a migrating cell.

The second major preliminary observation made in the Lois lab is that, in studying granule cells with electron microscopy, synaptic vesicles can be seen in the basal dendrites. This is illustrated in **Figure 2**.

Figure 2. Presence of Synaptic Vesicles in the Basal Dendrites of Granule Cells
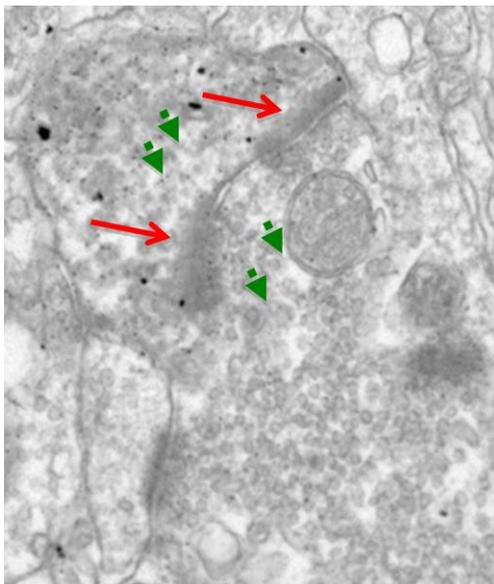

Figure 2 shows the presence of both neurotransmitter vesicles as well as synaptic sites present in the basal dendrites of granule cells. The green arrows point to neurotransmitter vesicles, while the red arrows show dense synaptic structures. As with Figure 1, this image is received from Professor Lois' lab to describe their preliminary results from experiments in the olfactory system.

The distal dendrites of granule cells have been studied in depth, as they heavily interact with the mitral cells present in the olfactory bulb. This interaction can be seen in Figure 1A. The distal dendrites of the granule cells work to release the neurotransmitter GABA onto the dendrites of the mitral cells (Schoppa and Urban, 2003). The mechanism is that mitral cells activate these granule cells by releasing glutamate onto them, and then the granule cells release GABA onto the cells which inhibit mitral activity (Schoppa and Urban, 2003). Although this is the typical mode of action, it is seen that when action potentials fire in granule cells, they do not always require input from mitral cells, and can be synaptically activated at local domains causing a release of GABA (Schoppa and Urban, 2003). Thus it is shown that the distal dendrites of granule cells work to release neurotransmitter when an action potential is fired, and these dendrites directly interact with other cells in the olfactory bulb.

Initial observations and results point to the idea that the basal dendrites work in a similar way; that they are also neurotransmitter release sites. This is important, because as stated and shown in Figure 1, these basal dendrites extend into the RMS, where new migrating neurons are found. If the synaptic vesicles in granule cells do indeed work to release neurotransmitter into the RMS, it could provide a mechanism for directed migration of the neurons out of the RMS and into the olfactory bulb, towards the granule cells. This knowledge and evidence leads to the hypothesis that neurotransmitter release by mature granule cells regulates the migration of immature cells in the RMS.

GABA

The major neurotransmitter which is studied in this experiment is the neurotransmitter GABA. GABA (full name gamma-Aminnobutyric acid) is a common neurotransmitter in mammalian systems, generally serving as an inhibitor. It has been seen that *in vitro*, GABA has

shown a major effect on the behavior of migrating neurons in the RMS. Particularly, the effects of GABA on neurons has been shown to have a significant effect on their continued migration through the RMS. Experiments and data have shown that many migrating neurons have receptors for the GABA neurotransmitter, and that inducing these receptors results in a depolarization of GABAergic interneurons (Bortone and Polleux, 2009; Inada et al., 2011). This upregulation of the GABA receptors causes depolarization of the interneurons, which works to stimulate motility of the new and migrating neurons. (Bortone and Polleux, 2009; Inada et al. 2011). This activation of the GABA receptor leads to an acceleration of multidirectional motility in vivo, pointing to the fact that GABA may influence the movement of neurons the RMS of the olfactory bulb as well (Bortone and Polleux, 2009; Inada et al. 2011).

It is hypothesized that GABA released from the basal dendrites of the granule cells directs migratory movement of new neurons in the RMS. The action of these granule cells through firing and release of neurotransmitter is believed to instruct migrating neurons to leave the RMS and instead directionally move toward the granule cell layer, where they will differentiate. As the release of GABA from the granule cells through synaptic vesicles is based on the firing rate of the neurons, an experimental method must be used in order to increase the firing rate of a cluster of granule cells; this is done through delivery of ion channels.

NACHBAC

Nachbac is an ion channel which serves a critical role in this experiment. It is known that Nachbac is a voltage-gated prokaryotic ion channel, which is selective for sodium (Ren et al., 2001). This ion channel is present in prokaryotic systems, particularly *Bacillus halodurans* (Ren et al., 2001; Yue et al., 2002). This specific ion channel can be expressed in mammalian systems (Yue et al., 2002), and voltage-gated channels often function in higher order cell actions. These

functions include neurosecretion, muscle contraction, excitability, and synaptic transmission

(Ren et al., 2001; Yue et al., 2002). The sequence and structure of Nachbac is shown in **Figure-3**, in an image which is present in the Yue et al. paper from 2002.
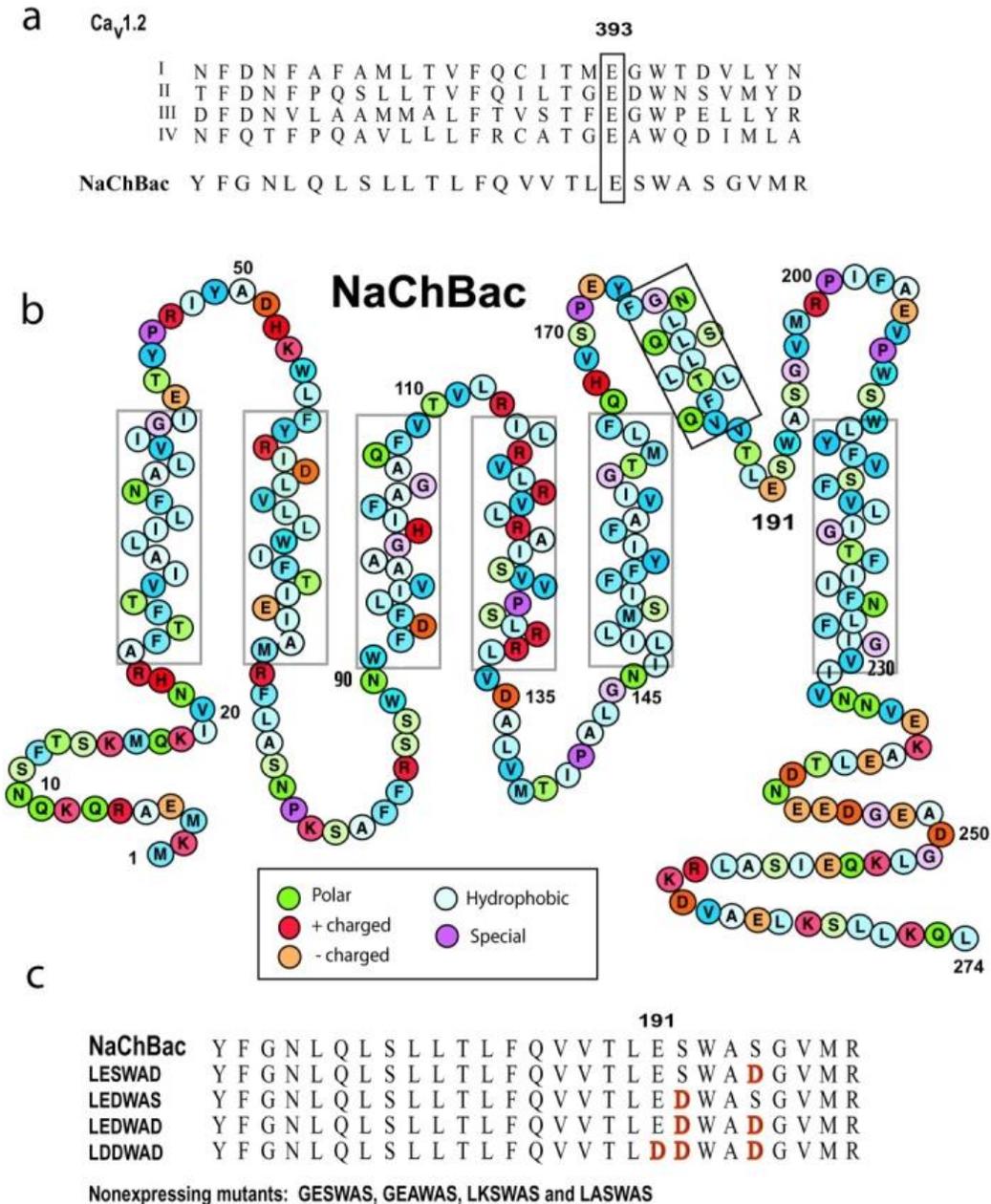
Figure 3. Sequence and Structure of Sodium-gated Ion Channel Nachbac

This figure, particularly part 3b, shows the amino acid structure of Nachbac. It is shown that there are roughly 6 transmembrane regions in the protein. These regions are labelled by vertical boxes. There are 274 amino acid residues in total. The expressed sequence shown here is very similar to voltage-gated calcium channels found in mammalian systems, and is considered an orthologue (Yue et al., 2002).

The Nachbac channel is specifically studied in this experiment due to the results of previous experiments and observations. It has been observed that the addition of Nachbac to mature granule cells increases the firing rate of these cells *in vivo*. This is due to two main factors; the first is that the activation threshold of Nachbac is approximately 15 mV more negative than the native sodium channels in granules (Lin et al., 2011). The secondary factor which causes this is that it inactivates on the order of hundreds of milliseconds, which is much longer than typical mammalian channels (Lin et al., 2011). Thus, neurons and cells treated with Nachbac are expected to have increased neuronal firing, and fire with a much lower input requirement than in wildtype mammalian granule cells.

However, it is also known that the firing rate of granule cells treated with Nachbac is still relatively modest (Lin et al., 2011), which means it likely will provide an extreme enough reaction to test the hypothesis. In this case, the experimental ion channel that is tested is instead dtrpa1 (labeled simply as TRPA1), which is a *Drosophila* cation channel. Nachbac will still be used in order to create the control group for the experiment.

TRPA1

TRPA1 (specifically dtrpA1) is an ion channel which works similarly to Nachbac; it works to induce neurons to fire action potentials at a high rate (Rosenzweig, et al., 2005). TRPA1 works in much the same way as Nachbac, but fires at a higher rate, and is thus a better candidate for the experimental ion channel. Thus, it was chosen for the final experiment over Nachbac in order to produce a more significant result through greater hyperactivity of granule cells.

In preliminary experiments, TRPA1 was seen to depolarize mammalian cells in vitro. However, during the time of this experiment, through concurrently running experiments in the Lois Lab, it was discovered that this ion channel does not work as expected. Contrary to preliminary experiments, this ion channel does not work to make granule cells hyperactive; instead, it works to make them silent. TRPA1 actually works to reduce the firing rate of granule cells, and thus reduce the amount of GABA released from the granule cell basal dendrites. Although this is not as expected, it still provides a method for studying the effect of granule cells on neuronal motility in the RMS. This experiment proceeded with the purpose of seeing how a reduction in granule cell activity (and thus a reduction in GABA release) affected the motility of RMS neurons.

LENTIVIRUS VECTORS AND IMMUNOSTAINING

Lentiviruses are vectors which can be used to deliver genes to cells and neurons *in vivo*. Two major lentiviruses are used in this experiment, as they are able to deliver the genes of interest into the neurons of lab mice. Specifically, lentiviruses can infect postmitotic neurons, and can be used to deliver genes to mature neurons, which in this case refers to the mature granule cells of mice. Lentiviruses are shown to have great success infecting terminally differentiated neurons (Naldini et al., 1996; Blomer et al., 1997), and thus are an ideal candidate for delivering the control and experimental ion channels to the granule cells of interest. In addition, these lentiviruses can encode for GFP (green fluorescence protein), which is used for immunostaining and fluorescence microscopy. One of the lentiviruses used in this experiment is FUGW, which encodes for the control group. This vector encodes for an inactive form of Nachbac fused with GFP, which is delivered stereotaxically into the granule cell layer of the olfactory bulb in mice. Using this method, it is possible to label a discrete cluster of mature

granule cells, which allows for identification of the granule cells of interest (**Figure-4**). This

identification is done by staining the olfactory structure for GFP; the granule cells which contain

this GFP will be highlighted during fluorescence microscopy. For the control, these cells are

unaltered, but the process for the experimental group and labeling is similar.

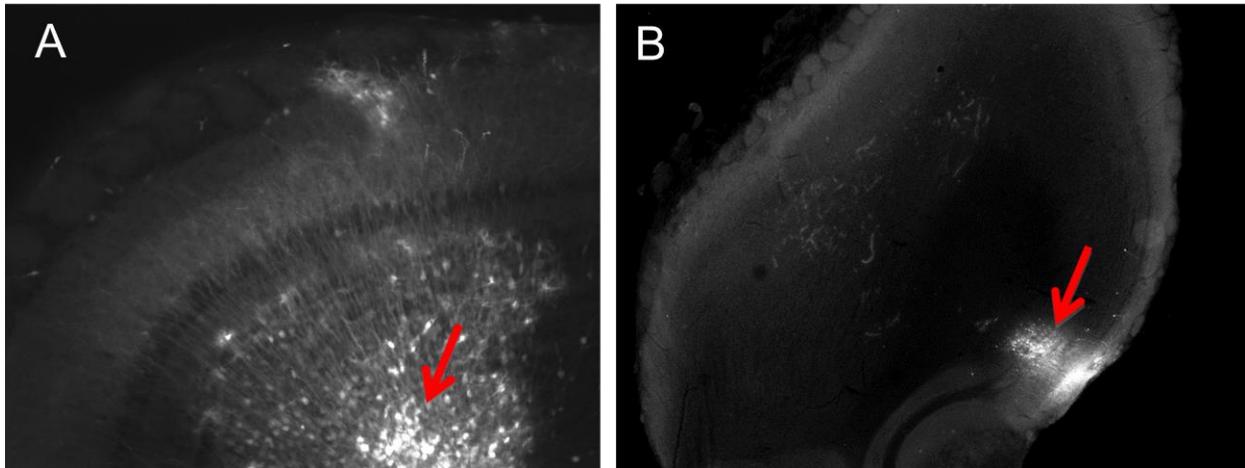**Figure 4. Labeled Clusters of Granule Cells**

Figure 4 shows two different views of labeled granule cells. Figure 4A shows a zoomed in view of granule cells
labeled, and was taken for the research design paper. This figure shows a specific, discrete cluster of granule cells
which have been labeled with GFP. The cluster is indicated with a red arrow. Figure 4B is a 4x shot of an olfactory
bulb structure that also clearly shows labeled granule cells. This picture was taken of a control olfactory structure
during data collection with Neurolucida. Again, the labeled granule cells are indicated with a red arrow.

A similar method was used for the experimental vector. For TRPA1, a vector was devised

which coded for the ion channel as well as for TRP and GFP. Thus, the lentivirus allowed for

identification of the experimentally altered granule cells through immonostaining for GFP, as

was done for the control group. Using this method, both the experimental and control granule

cells can be altered and tagged in one method using the lentiviruses, when they are delivered

stereotaxically into the granule cell layer.

In addition to labeling the granule cells, the new neurons which are of interest will also

be immunostained in order to see them with fluorescence microscopy. Oncoretroviruses

encoding Palmmcherry will be injected into the SVZ of mice in order to label granule cell progenitors. This injection labels the new neurons. Immunostaining can be done on the olfactory system for BrdU, which is a common tag for immunostaining. BrdU is typically used to detect proliferating cells and cells which have replicating DNA; it is used in that way here as well. New neurons will be stained for BrdU, and when fluoresced, will show up under microscopy. In this way, the new and migrating neurons which have replicating DNA and are not differentiated yet can be illuminated and looked at with respect to position in the olfactory system.

NEUROLUCIDA

Neurolucida is a software module produced by MBF Bioscience. It is an advanced system which allows for things like neuron tracing, neuron analysis, and neuronal and brain mapping. Neurolucida can be used to trace and mark neurons, brain structures, and when used in conjunction with fluorescence microscopy, it can be used to create a map of the neuron with the stained cells labeled and marked. In this experiment, there is heavy use of the Neruolucida module in order to analyze the olfactory bulb structures. Through Neurolucida, a map of the olfactory bulb showing the locations of the granule cells as well as the progenitor cells can be created, and the NeuroExplorer program in this module can provide quantification of the neuronal morphology as well as the position of the neurons in the overall structure relative to both the olfactory bulb itself, and to each other. Thus, this is an extremely important computer module to make use of for this experiment.

PROJECT PURPOSE

It is believed that GABA released from the basal dendrites of mature granule cells regulates migration of new neurons in the RMS, influencing them to migrate toward the granule cell layer. To test this, a discrete cluster of mouse granule cells are genetically altered to render them hyperactive using vector TRPA1. Once this is done, the mouse is killed and the brain is cross sectioned and immunostained for the granule cells (GFP/TRP) and new neurons (BrdU). Using the Neurolucida software, the migration and distribution of new-forming neuronal cells are studied, to study the effect of GABA release on migration of new neurons in relation to the position of granule cells. The overall aim of this experiment is to investigate how the activity of pre-existing neurons affects the behavior of migrating cells, and specifically, the goal is to understand how the basal dendrites in pre-existing granule cells may regulate the behavior of migratory cells within the RMS.

METHODS

PREPARATION AND INJECTION OF LENTIVIRUS

This stage of the experiment was handled primarily by Ting-Hao Huang of the Lois Lab. Mr. Huang works in the Neurobiology department of UMASS Medical School under Professor Carlos Lois. Lentiviruses and retroviruses as described in the background were prepared in the lab, with the intention of stereotaxically injecting them into the SVZ of a mouse brain. This process has been performed previously in order to mark cells within the SVZ (Kelsch et al., 2007). This process was performed a number of times, with different directed injections. There were two major groups of injections; injections into the anterior SVZ, and injections into the posterior SVZ. For both types of injections, lentiviruses containing the experimental (TRPA1) or control (FUGW) vectors were inserted, as were injections for the new neurons (BrdU). The process for this involved two distinct injections in the mice. The first process involved injecting a small volume (20 nl) of ion channel lentivirus (TRPA1 or FUGW) into the granule cell layer, thus delivering the ion channel as well as labeling the granule cell cluster of interest. The second injection took place 10 days later, and involved injecting oncoretroviruses encoding Palmmcherry into the SVZ to label the progenitors.

There were three groups of injections in the anterior SVZ - TRPA1, FUGW, and another FUGW group which involved a differential survival time for the mice. For posterior injections, there were only two main groups, TRPA1 and FUGW. For the anterior injections, there were 6 mice injected with TRPA1, three with FUGW, and three with the differential FUGW/BrdU injection. For posterior injections, there were five TRPA1 mice and six FUGW mice. Due to the action of the lentiviruses, all cells of interest were either tagged with GFP (mature granule cells) or BrdU (immature progenitors).

After the mice were prepared with the injections, they were killed between a period of 4 to 10 days, as it is known that during this period, most new neurons born in the SVZ will have migrated to the RMS (Lois and Alvarez-Buylla, 1994). Once killed, the mice were perfused with paraformaldehyde for preservation, and the brain tissues were cut into 50 µm thick sections using a vibratome, which is also a process that was previously performed (Kelsch et al., 2007). With the sections prepared, microscopy was be used to analyze the placement, structure, and morphology of the cells present in the olfactory bulb.

FLUORESCENCE MICROSCOPY AND NEUROLUCIDA

Using a confocal microscope specialized for fluorescence, the prepared slides of olfactory structures were studied. This microscope was hooked up to a specific lab computer which contains the Neurolucida module in order to study and map the olfactory structures. By fluorescing the slides, the different cells can be seen. The fluorescence for GFP/TRP highlighted the location and presence of the mature granule cells of interest (either control or experimental), and the fluorescence for BrdU showed new migrating neurons and their location in the olfactory bulb.

Once these locations were determined, the Neurolucida program was used to mark the positions and locations of the overall structure of the olfactory bulb, the mature granule cells, and the new neuronal cells. In this way, a data file was prepared which effectively mapped out the olfactory bulb and showed the locations of both the granule cells and the new neurons in one image. This process was performed for all olfactory structures of interest; both the control groups and experimental groups for the anterior and posterior injections. Please see the Results section for example images of the fluorescence microscopy and olfactory bulb maps prepared by Neurolucida.

It should be noted that only new neurons which could clearly be seen were considered and marked on the neuronal map. This is because the slides were a two-dimensional cross section, so an attempt had to be made to limit the influence of the three-dimensional brain structure on the results. By only considering the new neurons which were tagged clearly and showed up distinctly, it was believed that one would only consider neurons which were present on the same plane as the mature granule cells.

NEUROEXPLORER ANALYSIS

The NeuroExplorer program is another computer program which is part of the Neurolucida module. This program makes use of Neurolucida maps and allows for analysis of the data files prepared in Neurolucida. Thus, using the maps of the olfactory bulbs produced in Neurolucida, NeuroExplorer was used to gain insight into the positions of granule cells and neurons in the olfactory bulb. This program produced images (Fig 5, Results) based on the data files it received from Neurolucida, and the bulb structures were analyzed using this program. The most important form of analysis that this program ran was that it determined the distance between any marker for progenitor neurons and the mature granule cells. Thus the average distance of progenitors to the granule cells was calculated and produced by NeuroExplorer. This was the major form of data produced in this MQP.

Once this data was produced by Neuroexplorer, it was written down and tabulated in Microsoft Excel. This allowed for data analysis, particularly calculation of the average distances of marker to granule cell area for each injection type. In this way, the effect of the TRPA1 injection on new neuron migration could be seen by comparing the results of the NeuroExplorer analysis of the TRPA1 injected olfactory bulbs to that of the control. Please see Tables 1 and 2 in the Results section for the data.

RESULTS

The purpose of this project was to study the effects of the release of neurotransmitter GABA on new migrating neurons in the RMS of the olfactory bulb. It is known that the release of GABA affects taxis of new neurons, but the mechanism by which it acts in the olfactory bulb is not fully understood. It is hypothesized that mature granule cells fire and release neurotransmitter from their basal dendrites into the RMS, which causes directed migration of the new neurons out of the RMS and into the granule cell layer, where they differentiate. Thus, the goal of this experiment was to render a discrete cluster of granule cells hyperactive, and view the effects it has on neuronal migration in the olfactory bulb. It is hypothesized that rendering the mature granule cells hyperactive will cause migration of new neurons to the area through release of neurotransmitters through the basal dendrites.

Figure 5. Hypothesis of Hyperactive Granule Cells on Migrating Neurons
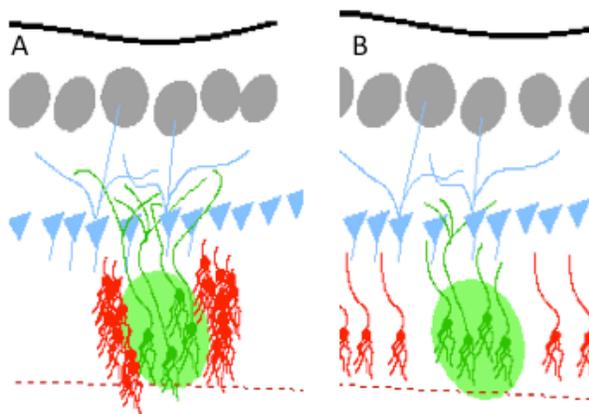

Figure 5 shows the hypothesized effects of hyperactivity (A) vs. wild type (B) in granule cells. Hyperactive granule cells (green) are hypothesized to work to recruit new cells (red) to its location through release of the GABA neurotransmitter. This is not expected to be seen in the wildtype control (B). Comparatively, it is believed that the hyperactive cells will show a greater density of new neurons near the area of interest than the wildtype.

Using the Neurolucida computer module, the results of the experiment can be seen. Combining techniques of fluorescence microscopy with computer use results in images of the stained olfactory structure being seen on the computer. With the differential staining for GFP/TRP and BrdU, the computer produced two distinct images when the fluorescence was

changed. One image showed the GFP/TRP fluorescence, indicating the position of the mature

granule cells. The other image shows the position of new neurons present in the olfactory bulb

through fluorescence of BrdU. **Figure 6** shows an example of the fluorescence microscopy as

seen on the computer.

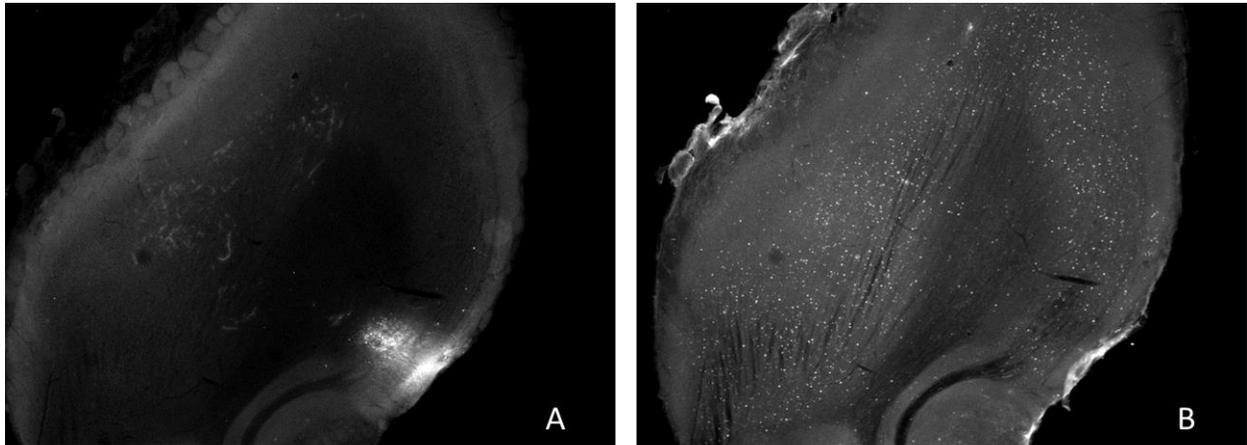**Figure 6. 4x Fluoresced Images of Posterior Injection**

Figure 6 shows two different views of fluorescence microscopy for the olfactory bulb. Figure A shows the
fluorescence of the granule cells of interest, which were tagged with the control lentavirus; thus this is fluorescence
for GFP. Figure B is fluorescence for new neurons, and thus is tagged for BrdU. As can be seen, the location and
presence of both mature granule cells (A) and new migrating neurons (B).

Using the Neurolucida program, a map can be made of the olfactory bulb and the cells present.

The first major step was to draw contours around the entire olfactory structure, and then draw a

contour around the highlighted granule cell area. An example of this is seen in **Figure 7**.

Figure 7. Overall structure and Outline of GFP Stained Area at 10x Objective
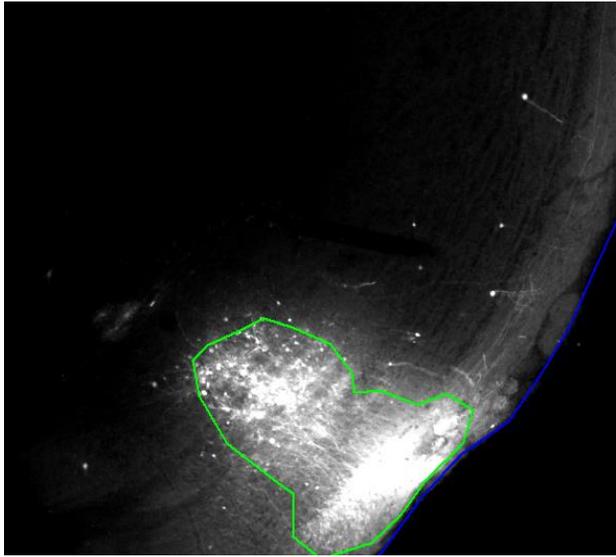
As shown in Figure 7, there are two contours drawn around the structures present in the olfactory bulb. The blue contour illustrates the edge of the overall brain structure, while the green contour outlines the GFP-stained granule cells. In this way, the location of the mature granule cells is tracked with Neurolucida.

Once this process is performed, the second half of the olfactory bulb mapping takes place. The fluorescence changes so that BrdU-stained cells are highlighted. Then, each individual cell is marked using Neurolucida. The marker chosen is a red X, which indicates the position of the new neuronal cell in the overall structure. An example of this marking is shown in **Figure 8**.

Figure 8. Individual Neuron Marking in Neurolucida at 10x
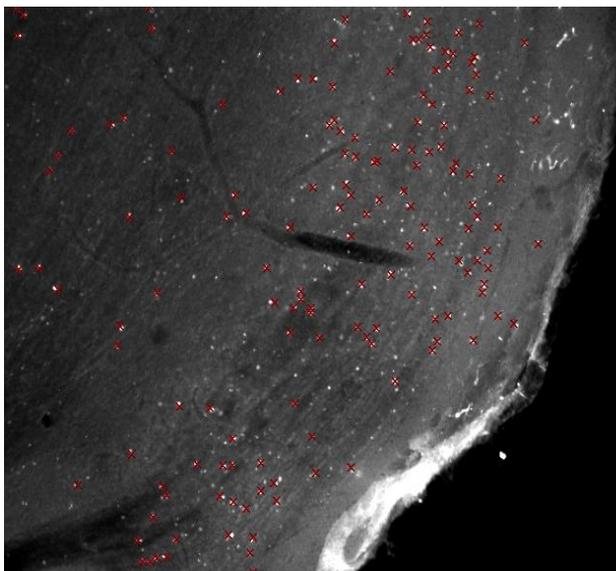

Figure 8 shows the marking for individual new neurons as shown in the Neurolucida program. As can be seen, the fluorescence has been changed to show BrdU-marked cells, which are marked with a red X wherever they appear. As stated in the Methods, some cells are not marked as they may not be entirely clear, in an attempt to only mark cells which are on the same plane as the granule cells.

Once both the contours and markers have been mapped in Neurolucida, a final image and data

file of the olfactory bulb can be produced. This final image is saved as a data file and can be

transferred into NeuroExplorer, where it can be analyzed. An example of this image is shown in

Figure 9.

<u>Figure 9. Full Map of Olfactory Bulb With Contours and Markers at 10x</u>
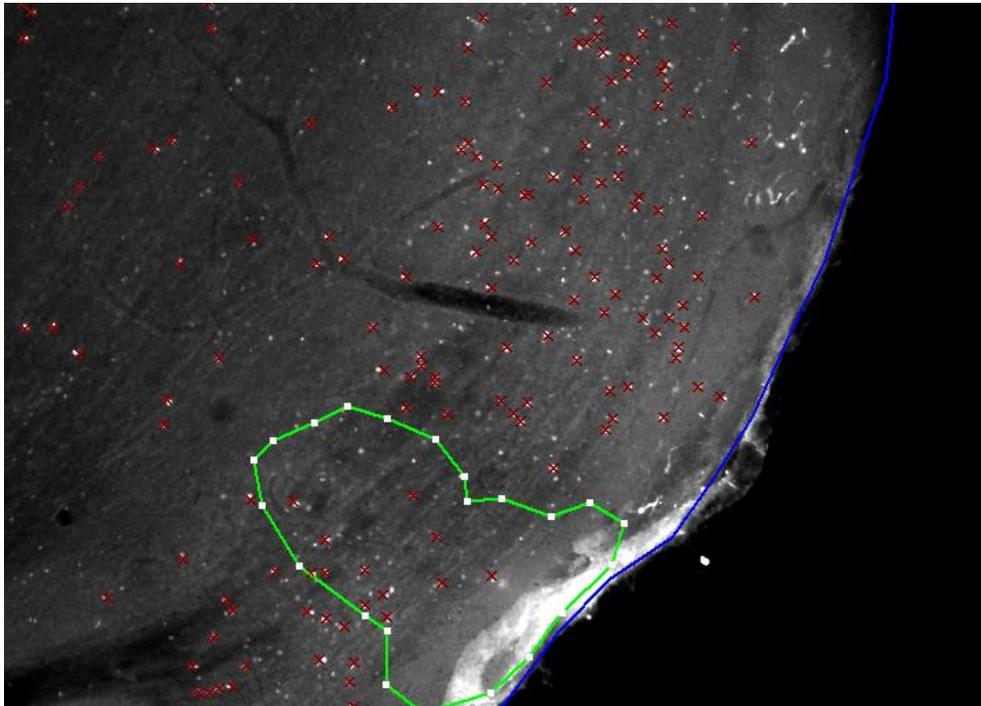

Figure 9 shows a sample of a full map of an olfactory bulb structure. This map shows both the contours for the bulb
and granule cells, as well as the markings for the new neurons.

Once the neuron maps are created for the olfactory bulb, the data file can be transferred to

NeuroExplorer, where it can be analyzed using the mathematical ability of the program. When

the maps are transferred to NeuroExplorer, analysis can be run on the markers, the contours, and

their relationships to each other. In this way, the position of the markers (new neurons) in

relation to the green contour (mature granule cells) can be seen and quantified. An example of

maps transferred into NeuroExplorer can be seen in **Figure 10**.

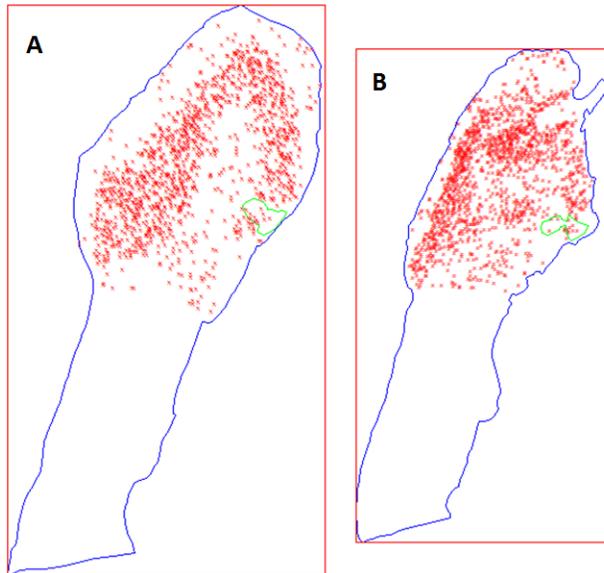## Figure 10. Example NeuroExplorer Maps for Marked Cells

Figure 10 shows two sample NeuroExplorer images produced by data files which were received from Neurolucida. As can be seen, only the bulb structure of the olfactory system is analyzed in this experiment; cells outside of the bulb are not marked. Additionally, both the red markers for new neurons and green contours for marked granule cells can be seen. Figure 10A shows a bulb which had a posterior structure injected and was the control group. Figure B is a different structure which was also injected in the posterior and is TRPA1.

The images and maps produced by NeuroExplorer alone are not enough to draw conclusions about the effects of TRPA1 on neuronal migration or even the position of new neurons in relation to the mature granule cells. However, NeuroExplorer has a mathematical function which works to quantify the positioning of markings. NeuroExplorer can instantly analyze a map such as the ones produced in Figure 10 and return data on the markers and contours in the map. For the purposes of this experiment, NeuroExplorer is used to generate data of marker distance to the green contour (granule cells) in terms of μm. This data is tabulated and shown in Tables 1-7. Tables 1-4 indicate anterior injections of the lentivirus, and Tables 5-7 are posterior injections.

- All data is given in terms of marker distance from the highlighted region in μm.

- #Marker refers to the total number of marked new neurons, while #InRegion refers to the number of markers within the highlighted contour area.

- The column St.Dev refers to the standard deviation between the marker distances for a single olfactory structure. The row StDev refers to the deviation of the averages above it.

- "Alpha BrdU/GFP" indicates what each bulb structure was immunostained for.

Table 1. Data Received for GFP2a TRPA1 BrdU alpha BRDU/GFP

Bulb	Min	Max	Mean	St.Dev	# Marker	#InRegion
1	2.1	3939.4	1438	955.5	986	44
2	0.4	4005.5	1698	928.4	1383	26
3	4.1	4057.6	1548.8	914.3	962	16
4	3	3985.1	1650.5	988.4	1180	24
5	6.6	3698.8	1659.7	900.7	1461	37
6	0.3	3464.7	1389.2	862.7	1226	36
AVGS:	2.75	3858.517	*1564.033*	925	1199.667	30.5
STDEV:	2.393951	229.9586	*127.4824*	43.69815	202.5465	10.27132

Table 1 refers to the anterior injection of the TRPA1 (experimental) vector into the olfactory bulb. The average distance of marker to granule cell area here is 1564.03 μm, while the standard deviation for this average is 127.48 μm.

Table 2. Data Received for FUGW BrdU alpha BrdU/GFP

Bulb	Min	Max	Mean	St.Dev	#Markers	#InRegion
1	9	4127.8	1087.5	802.4	944	74
2	3.4	3324.6	1158.1	773.4	802	38
3	4.3	4406.8	1130.2	817.3	1002	57
AVGS:	5.566667	3953.067	*1125.267*	797.7	916	56.33333
STDEV:	3.007214	561.8612	*35.55761*	22.3242	102.898	18.00926

Table 2 refers to the data collected from the olfactory cells which were injected with the FUGW vector (control). This injection was anterior. The average distance of marker to granule cell area here is 1125.27 μm, and the standard deviation of this average is 35.56 μm.

Table 3. Data Received for FUGW 3WS BrdU 4WS alpha BrdU/GFP

Bulb	Min	Max	Mean	St.Dev	#Markers	#InRegion
1	1.8	3097.9	1143.2	719.5	567	18
2	5.1	3641	1157.4	792.9	1148	50
3	7.8	4083.7	1235.5	834.1	838	42
AVGS:	4.9	3607.533	*1178.7*	782.1667	851	36.66667
STDEV:	3.004996	493.7514	*49.7*	58.04906	290.7181	16.65333

Table 3 refers to the data collected from the olfactory cells which were injected with the FUGW vector (control), but had a differential injection of BrdU and death than the other mice. This injection was anterior. The average distance of marker to granule cell area here is 1178.7 μm, and the standard deviation of this average is 49.7 μm.

Table 4. Tabulated Anterior Injection Results Summary

	Mean Distance	St. Deviation
GFP2 TRPA1	1564.03	127.48
FUGW	1125.27	35.56
FUGW 3WS	1178.7	49.7

Table 4 shows a simplified results table of the anterior injections of lentavirus. As can be seen, the mean distance of the TRPA1-injected olfactory bulbs is much higher than that of the FUGW bulbs. This indicates that in the TPRA1 treated cells, the new neurons were farther away from the mature granule cell cluster than they are in the control.

Table 5. Data Received for Posterior TRPA1 alpha BrdU/GFP DPA1

Bulb	Min	Max	Mean	St.Dev	# Marker	#InRegion
1	2.1	3240.8	1656.9	788	2123	16
2	5.7	3188.4	1599.1	627.5	1516	14
3	2.3	3252.9	1549.2	709.9	1248	20
4	0.9	3026.5	1345.4	730.7	1161	22
5	4.8	2926.5	1655.7	641.1	1585	5
AVGS:	3.16	3127.02	*1561.26*	699.44	1526.6	15.4
STDEV:	2.006988	143.9372	*128.6845*	66.15964	377.6563	6.618157

Table 5 refers to the data collected for the posterior injection of the TRPA1 vector into the olfactory bulb. The average distance of marker to granule cell area here is 1561.26, while the standard deviation for this average is 128.68 μm.

Table 6. Data Received for Posterior GFP FUGW alpha BrdU/GFP

Bulb	Min	Max	Mean	St.Dev	# Marker	#InRegion
1	19.5	3046.2	1697.4	685.1	1522	3
2	2.1	2808.6	1483.5	575.9	1324	12
3	9.1	2921.8	1371.5	600.6	1267	17
4	13	2930	1337.3	636	1080	16
5	3.1	2972.1	1361.3	614.5	1408	35
6	3.1	2786.5	1366.6	606.5	1585	6
AVGS:	8.316667	2910.867	*1436.267*	619.7667	1364.333	14.83333
STDEV:	6.938131	98.464	*137.7564*	37.48961	182.9783	11.30339

Table 6 refers to the data collected from the olfactory cells which were injected with the FUGW vector (control). This injection was posterior. The average distance of marker to granule cell area here is 1436.27 μm, and the standard deviation of this average is 137.76 μm.

Table 7. Tabulated Posterior Injection Results Summary

	Mean Distance	St. Deviation
TRPA1	1561.26	128.68
FUGW	1436.27	137.8

Table 4 shows a simplified results table of the posterior injections of lentavirus. Unlike the anterior injections, the results here are not as clear. Although the TRPA1 mean distance is higher, the standard deviation is large enough where it can be said that the difference is not significant.

The fact that the results in Table 7 are not as drastic as the results in Table 4 is indicative of two major outliers present in Tables 5 and 6. As can be seen, in Table 5, olfactory bulb 4 has a much lower mean distance of marker to contour than the other bulbs. In Table 6, bulb 1 has a much larger mean distance to contour. These two bulbs likely pushed the standard deviation of the averages higher, and made the two groups of data much closer in value. Without them, the average distance to contour for the TRPA1 group is 1615.23 µm (St.Dev 51.62), and the distance for the FUGW group is 1384.04 µm (St.Dev 57.13). The drastic reduction in standard deviation indicates that these two specific data results are outliers here.

In both cases, it is shown that the olfactory bulbs which had an injection of experimental vector TRPA1 had a larger mean distance between the location of new neurons and the granule cells of interest. It is believed that this is a repellent effect.

DISCUSSION

In the Results, it is shown that in both cases, for olfactory bulbs treated with TRPA1, there appears to be a repellent effect on the migration of neurons in the olfactory bulb. The average distance of new neurons to the highlighted area of granule cells is larger for the TRPA1 treated bulbs in both cases when compared to the control group. As stated, it was learned over the course of the experiment that TRP has the opposite effect of what was expected going in; it does not work to make granule cells hyperactive, but instead works to silence them. In this way, it reduces the release of neurotransmitter GABA from the granule cells. Thus, although the experiment did not work exactly as intended, it still tested the hypothesis in a different way. Instead of making the granule cells hyperactive, upon where they would release more GABA and cause recruitment of new neurons to the granule cell area, they were silenced, releasing less GABA, and showed less recruitment of neurons to the area.

It is concluded that blocking and silencing mature granule cell activity through action of the TRPA1 ion channel works to repel migration of new neurons to the area containing these granule cells. The reasoning for this is that dampening the firing of these granule cells results in less release of GABA, which results in less directed migration of new neurons to the area. Because other mature granule cells in the olfactory bulb continue to work as expected, the new neurons migrate out of the RMS towards these areas more than the experimentally altered area. In this way, although the experiment did not proceed exactly as planned, there is still evidence to support the idea that GABA works to affect the behavior of migrating cells by being released from the basal dendrites of mature granule cells. This was tested by rendering a cluster of granule cells silent, and observing a reduced migration of new neurons to the experimentally altered area.

The major portion of the lab work done involved use of the Neurolucida program to map out the olfactory bulbs, and then perform analysis on the maps provided using NeuroExplorer. This data was collected blind; although I knew the purpose of the experiment, I did not know specifically which group was considered the control (FUGW) and which group was the experimental (TRPA1) until after all the data had been collected and tabulated. Upon completion of using the Neurolucida module and presenting the data, I was given the research proposal information in order to write a detailed report. I was also told about what was learned about the true function of TRPA1 at that time. Mr. Huang, who prepared the mice and was aware of the experimental groups, did not assist in the data collection process. I believe this helped reduce any possible bias that could have occurred in the Neurolucida mapping process.

For future experiments, a greater look into the molecular mechanisms in play can be studied. Although it can be concluded that the basal dendrites and release of GABA plays a role in directed migration of neurons, the molecular mechanisms are still not fully understood. This could lead to even greater insight into the directed movement and differentiation of new neurons in the brain. Additionally, further experiments to look into this data can be performed. The same experiment can be redone with more olfactory bulbs and mice, in order to perhaps reduce the effects of outliers (seen in Tables 5-7). A similar experiment can be performed in which the original goal of this experiment, to render a cluster of cells hyperactive, is tested. Although a similar goal was achieved here, it would be interesting to view the effects of hyperactivity on cell migration in the olfactory bulb. Finally, an experiment involving genetic knockout mice could perhaps be performed, in which GABA production is knocked down and compared to the wildtype. The migration and integration of new neurons in the olfactory bulb is still not fully understood, so there are many directions in which further research can go.

The results of this experiment allow us to draw further conclusions about the role of mature neurons and granule cells in recruiting new cells in the olfactory bulb, and perhaps the brain as a whole. This MQP assisted in gaining insight into how pre-existing granule cells affect the behavior of migrating cells in the RMS through the release of GABA by mature granule cells from their basal dendrites into the RMS. In gaining a greater understanding of the mechanism which triggers the directed migration of neurons, it is believed that it will allow for greater knowledge about how new neurons integrate into existing neuronal circuits. Understanding how interneurons reach their final destination can shed light on how cell migration in the brain works, and determining the mechanisms which act here can allow for better development of strategies for brain repair.

The brain is an extremely complex organ in the human body, and many mechanisms and functions of cells in the brain are not truly understood. Attempts to repair damaged or lost neurons in the brain through methods which serve to replace cells have faced a number of roadblocks due to lack of knowledge about mechanistic development and integration of new neuronal cells into the brain. It is paramount to understanding how natural neurons integrate into brain circuits in order to begin to develop new strategies for brain repair. In severe injuries or diseases which damage neurons, there are currently no effective treatments to restore these neurons. Additionally, many neurodevelopmental disorders affect assembly of brain circuits during development. Thus, it is extremely important to understand how cells migrate through the brain, how they establish synapses with mature cells, and how they become integrated with the overall neuronal circuit. In doing this, it is hoped that this research will provide insights into brain circuit assembly, and help to design clinical strategies to achieve neuronal repair.

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