Changes in Complement Protein Expression and Its Role in Microglia-Mediated Synaptic Pruning Following RmTBIs in Adolescent Mice

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Logbook (September 2023 - February 2024)

Wooju (Cooper) Choi
Grade 11

Daily Notes

Sep | Oct | Nov | Dec | Jan | Feb

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

February 2024

Band Designer Genes Club AHS volunteer

No meetings with Tom

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1 Class 45 • Continue making sections of poster: • RQ/hypothesis • Procedures • Find or make figures/diagrams	2 • Edit draft 2 of Procedures section paper + submit to GC/Turnitin	3 • Scan logbook sections
4 • Complete C3 image analysis using parameters approved by Tom	5 Class 46 / Meeting • Run poster sections by Dr. Garcia for approval • Transfer sections into poster design of choice (leave room for graphs, discussion, conclusions)	6	7 Class 47 • No significance expected from stat analysis • Why? • Research: alternate, complement pathways, male vs. female effects	8	9 Class 48 • Finish CYSF Portal Procedures section • Research: relationship between TBI and synaptic pruning + complement	10
11 • Send Tom: • Schedule for rest of February + when can we meet next week? • My plans/dates • Completed image analysis	12 Tom returns from UK trip	13 Procedures section paper due Class 49 / Meeting	14 • Stat analysis → make graphs • Discussion: why would there be significance/no significance	15 Meeting 17 • Applications: where can my project be applied? future directions? long weekend (15-19)	16 Finish poster • Add: graphs, discussion, and conclusion section to poster • Check with Dr. Garcia if possible • Print (w/ staplist or staples)	17 Finish poster • Attach to tri-fold • Add stat analysis to Data Collection & Results section of logbook
18 • Start practicing oral presentation • Send Dr. Garcia email for time to go over presentation together	19 • Finish CYSF platform: • Discussion, conclusion, applications, etc. • Add to Discussion & Conclusion section of logbook	20 Class 50 • Practice oral presentation	21 Meeting 18 • Practice oral presentation with Tom • Ask Tom for examples of questions I may be asked + tips	22 Class 51 • Practice oral presentation	23	24 Review past background research
25	26 Class 52 • Oral presentations: Me, Jessica, Brynn, other	27	28 Class 53 • Oral presentations: Tiffany, Manisha, Elliot	29 Meeting 19 • Practice SF presentation with Tom using poster (if poster ready)		

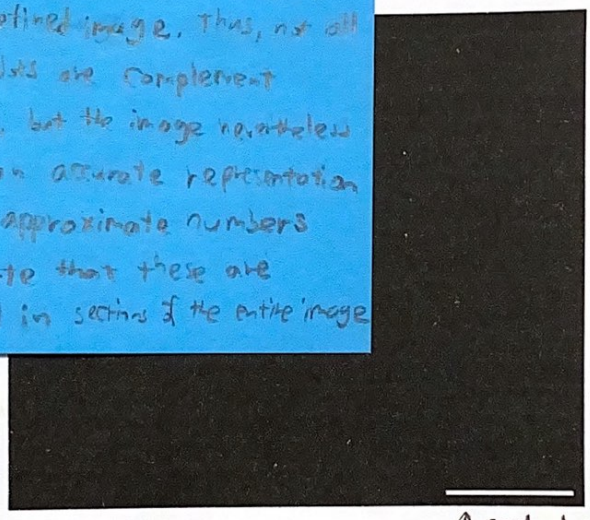
Holidays and Observances: 14: Valentine's Day, 19: Presidents' Day

Wiki Calendar

25 February

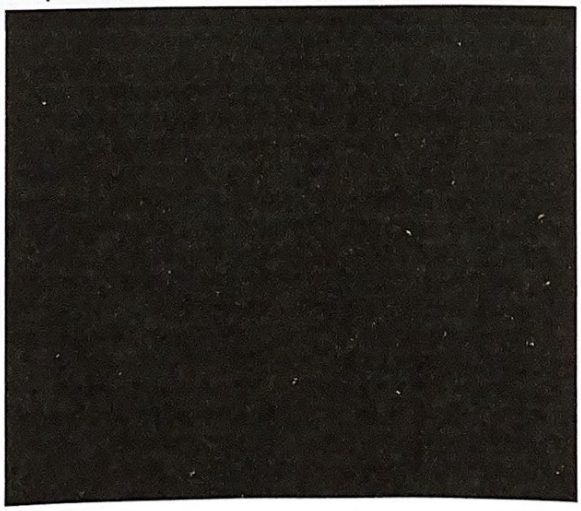
- Refine representative images + send to Tom for approval
  - visibility is not very high unless you zoom in → probably will not be an error if printed on a larger scale
  - C1q: yellow dots are particles

- Note that when background & contrast was adjusted, some background signal was included in the refined image. Thus, not all yellow dots are complement proteins, but the image nevertheless gives an accurate representation of the approximate numbers
- Also note that these are zoomed in sections of the entire image



↑ scale bar

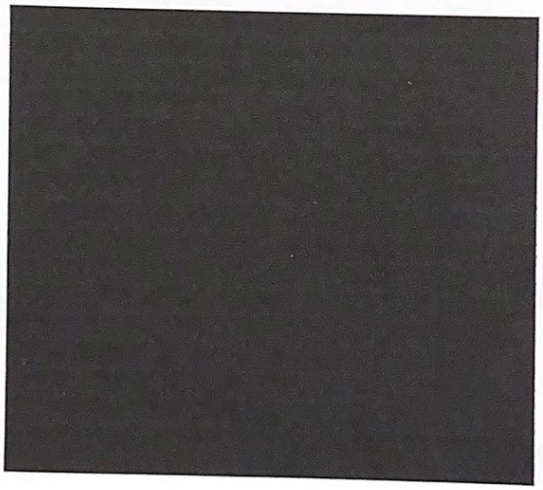
C1q RmTBI: 80-2



· C3: red dots are particles

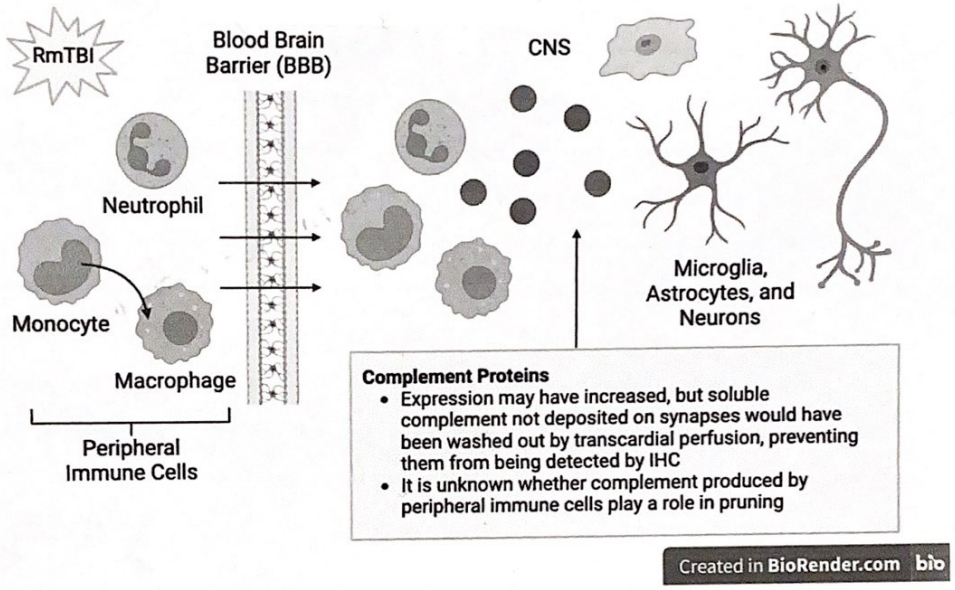
C3 Sham: 46-2

C3 RmTBI: 69-1

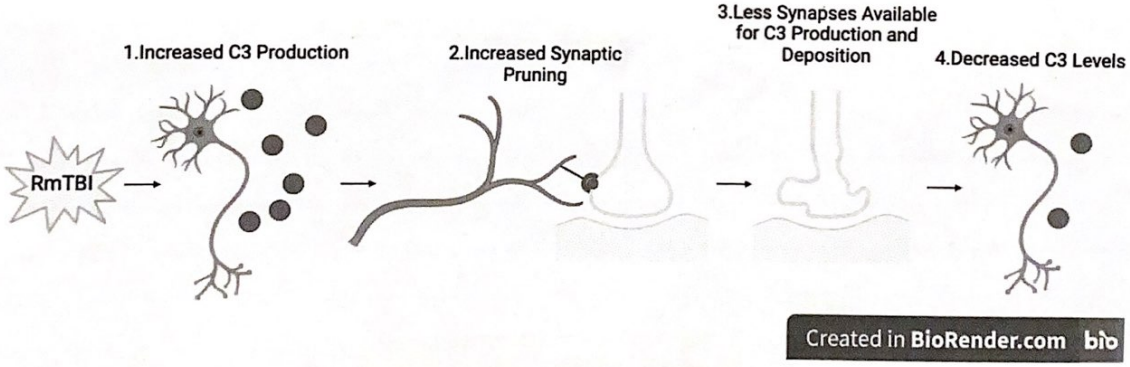


24 February

- Create/add images from Biorender to discussion/future directions section of Poster.
- Discussion – IHC did not detect soluble complement (produced by peripheral immune cells, microglia, astrocytes, and neurons:

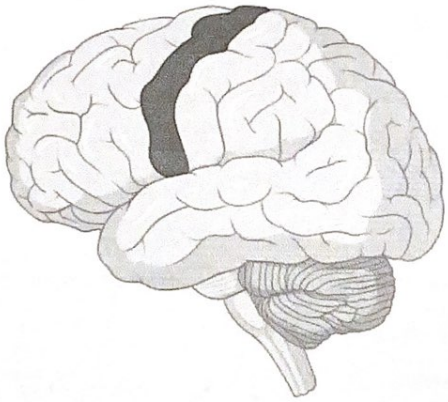


- Discussion – increased synaptic pruning may have decreased the levels of C3 detected after RmTBIs:



- Motor cortex (only used on slides for oral presentation):

Motor Cortex



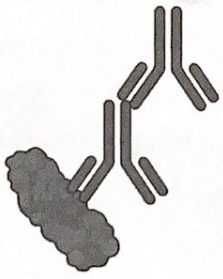
Controls motor function

Created in BioRender.com bio

- Future Directions - Using cell markers to stain neurons can be used to eliminate greater background staining during IHC

Immunohistochemistry (IHC)

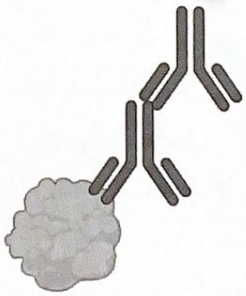
Staining



Target Complement Protein

- Deposited on synapses of neurons

Background Staining (Nonspecific Antibody Binding)



Non-Target Protein

- Bound to membranes of other molecules
- Blocking during IHC eliminates some but not all background

Created in BioRender.com bio

20 February 2024 (Meeting with Tom)

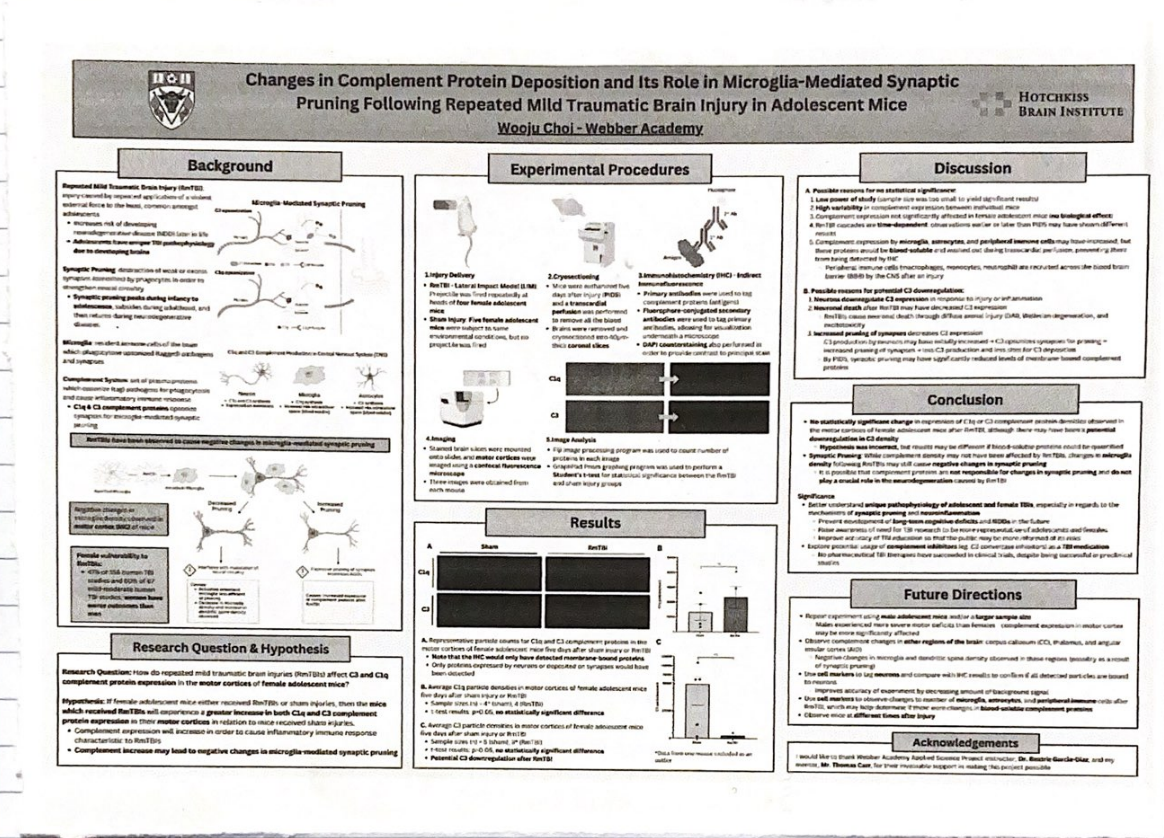
- Refining Representative Images for Poster

1. complete image analysis up to "analyze particles" ← change Image scale: 5.763 μm
2. select "Hide overlay" to hide numbers
3. crop Image to increase focus
   - Use rectangular net
   - Edit → selection → Specify
     - Scaled units (μm)
     - Width: 450
     - Height: 389.35
     - X: 332.5
       Y: 1
   - Image → crop
4. LUT: Image → lookup tables → LUT → yellow (C19) or red (C3)
5. Edit max & min of B & C to maximize visibility
6. Add scale bar (only to first image)
   - Analyze → tools → scale bar
     - Width: 100 μm
     - Height: 250 μm
     - Thickness in pixels: 20
     - Font size: 10
     - Color: white
     - Location: lower right
     - Select: horizontal, Hide text, overlay
7. Save image as PNG

- Poster Comments:
  - Shorten captions under results section
  - Add diagrams to discussion / future directions
  - Add references section + variables
- Next Meeting:
  - Maybe next week: Practice Presentation for science fair
  - Send refined representative images to Tom for checking

16 February 2024

- Finish poster: results, discussion, conclusion, and future direction sections

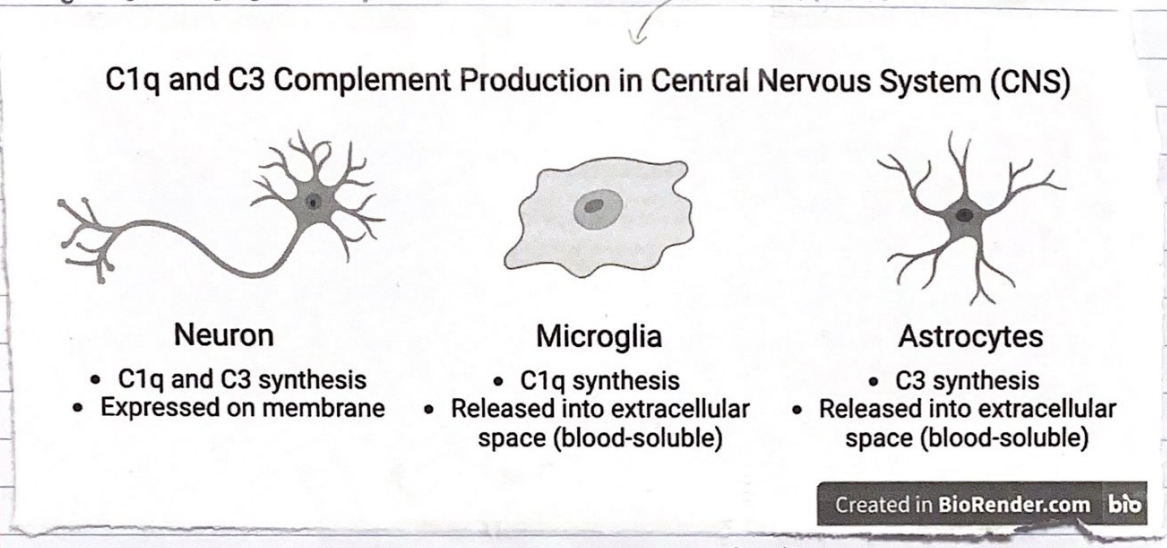


- Results:
  - C1q: $P > 0.05 \rightarrow$ no statistical significance
  - C3: $P > 0.05 \rightarrow$ no statistical significance
    - Potential C3 downregulation
- Discussion:
  - Possible reason for no statistical significance
    - Low power of study
    - High variability between individual mice
    - No biological effect
    - Time-dependent reactions
    - Blood-soluble complement not detected
  - Possible reasons for C3 downregulation
    - C3 downregulation in response to injury
    - Neuronal death after RmTBI
    - Increased pruning of synapses
- Future Directions:
  - Male adolescent mice + larger sample size
  - other regions of brain
  - Different time points
  - Cell marker staining

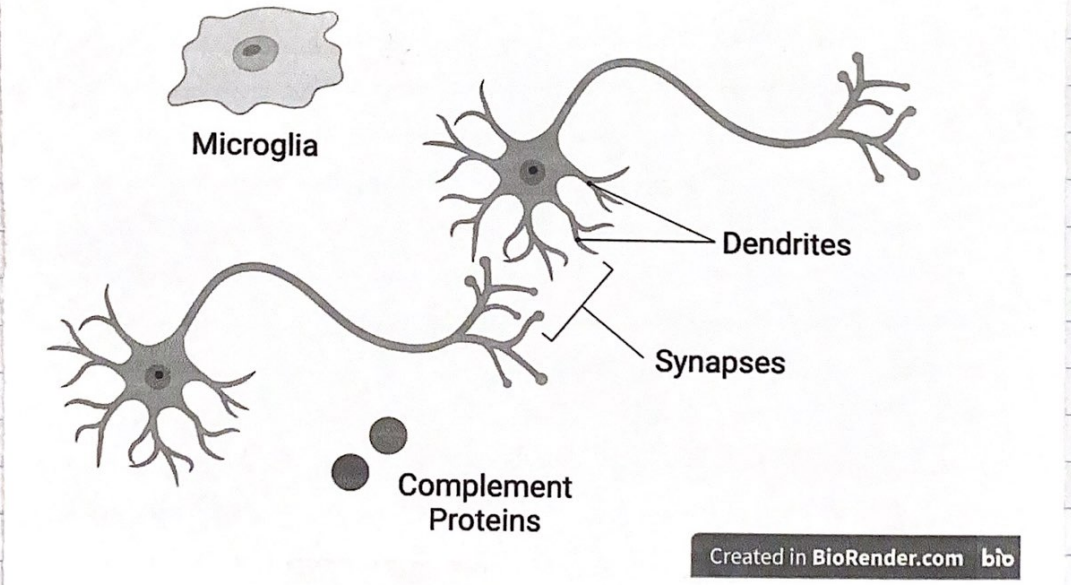
17 February 2024

- Work on final presentation - all sections
- Diagrams created for presentation:

Use with RQ + H

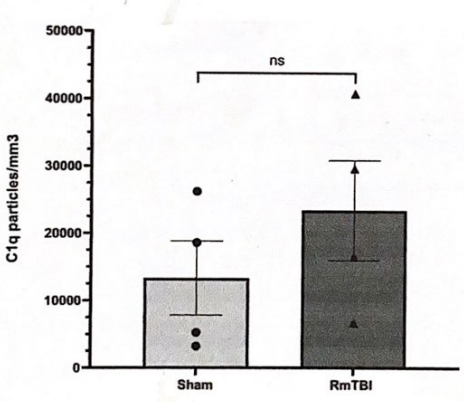


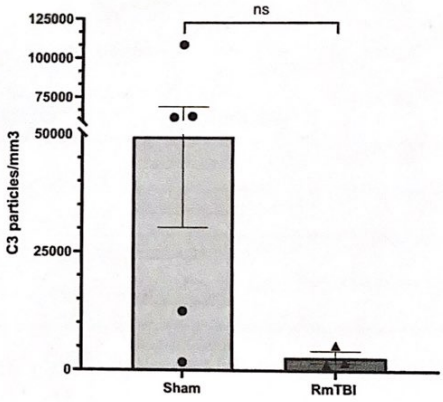
Use with Punchline



15 February 2024 (Meeting with Tom)

- Data refining
  - Sham injury group: 5 mice
  - RmTBI group: 4 mice
  - Mouse outliers:
    - C1q: #79
    - C3: #54
  - t-Test + graphing
    - No statistically significant difference between treatment groups for C1q or C3
    - Specifications:
      - t-Test: column, unpaired, 95% CI, Welch's correction
      - Graph: SEM (error bars)





- Conclusions:
  - Reasons for no significance:
    - High variability in complement expression between individual mice
    - Low power of study (n is too small)
    - Complement expression not significantly affected in females after RmTBI → males may have different results
- Analysis:
  - Potential C3 downregulation after RmTBI
    - Dendritic spine density decreased after RmTBI - C3 highly expressed on dendrites

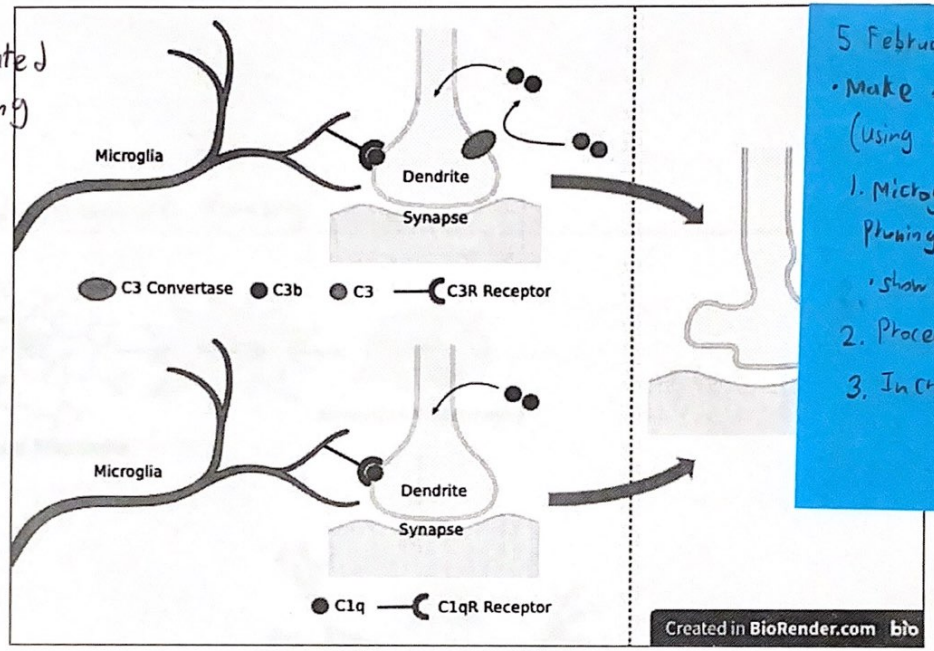
- Future Directions:
  - Different results if mice were euthanized earlier than PID5
  - Repeat experiment but with males
  - Use cellular markers to identify microglia numbers
- Applications:
  - Better understanding of TBI pathophysiology, especially in regards to inflammation
- Next meeting
  - 20 February (2PM) - go over presentation/poster

13 February 2024 (Class)

- Meeting with Tom plan - 15 February 2024
  1. Review Data Analysis
     - Review C1q/C3 parameters
     - Which mice are RmTBI/ Sham Injury? → Determine outliers for treatment Groups
  2. Stat Analysis
     - Graphpad prism → t-test (likely X Statistical Significance)
     - Send graphs to Cooper
  3. Results
     - Reasons for no statistical Significance
       - Gender Differences → more pronounced in males?
       - Animal models show better outcomes for females compared to males
       - Could decrease in microglia impair complement production?
         - C1q are mainly synthesized by microglia
  4. Application/Future Directions
     - Therapeutic use?
     - Repeat same study but with males?
  5. Sources of Error
  6. Poster
     - Flow + wording + amount of words
     - Any Tips?
  7. Other Questions:
     - Human studies show worse outcomes for females ← Can I use this in my background to frame my exigency so that I am testing female mice because I think they will have more negative reactions to TBIs?
     - The brain recruits macrophases, neutrophil, and monocytes (T cells, dendritic cells, Nk cells) across the BBB following RmTBIs ← some of these cells can synthesize complement components/receptors
       - Could I use this to support my hypothesis (microglia ↓, but other complement production sites ↑ in brain, leading to increased complement expression)
  8. Would you like to come judge for my school's SF?

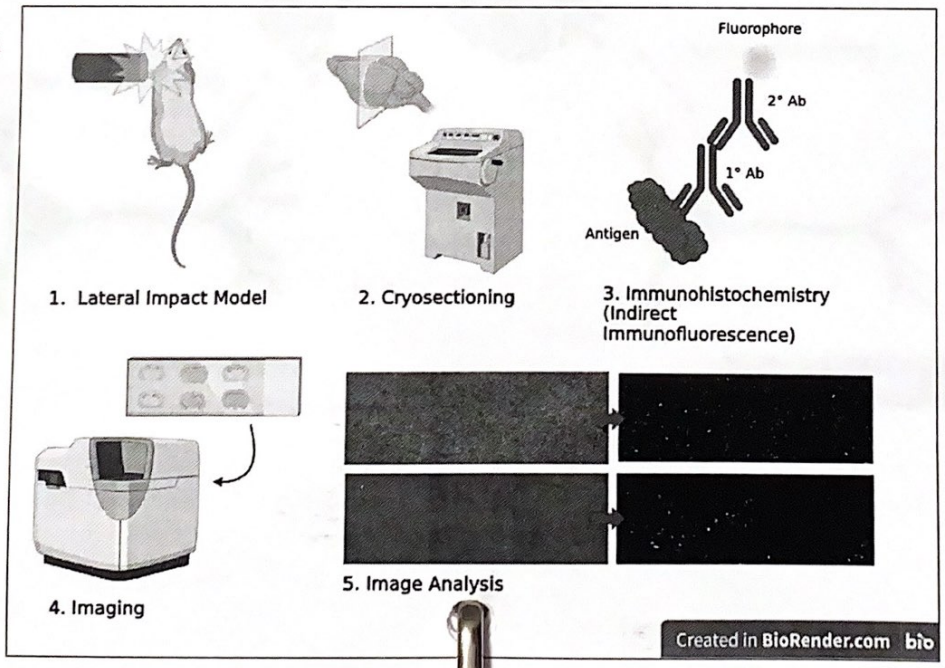
- Presentation Practice (22 Feb)
- Presentation (26 Mon)
- WASF (4 Mar)

Microglia-Mediated Synaptic Pruning



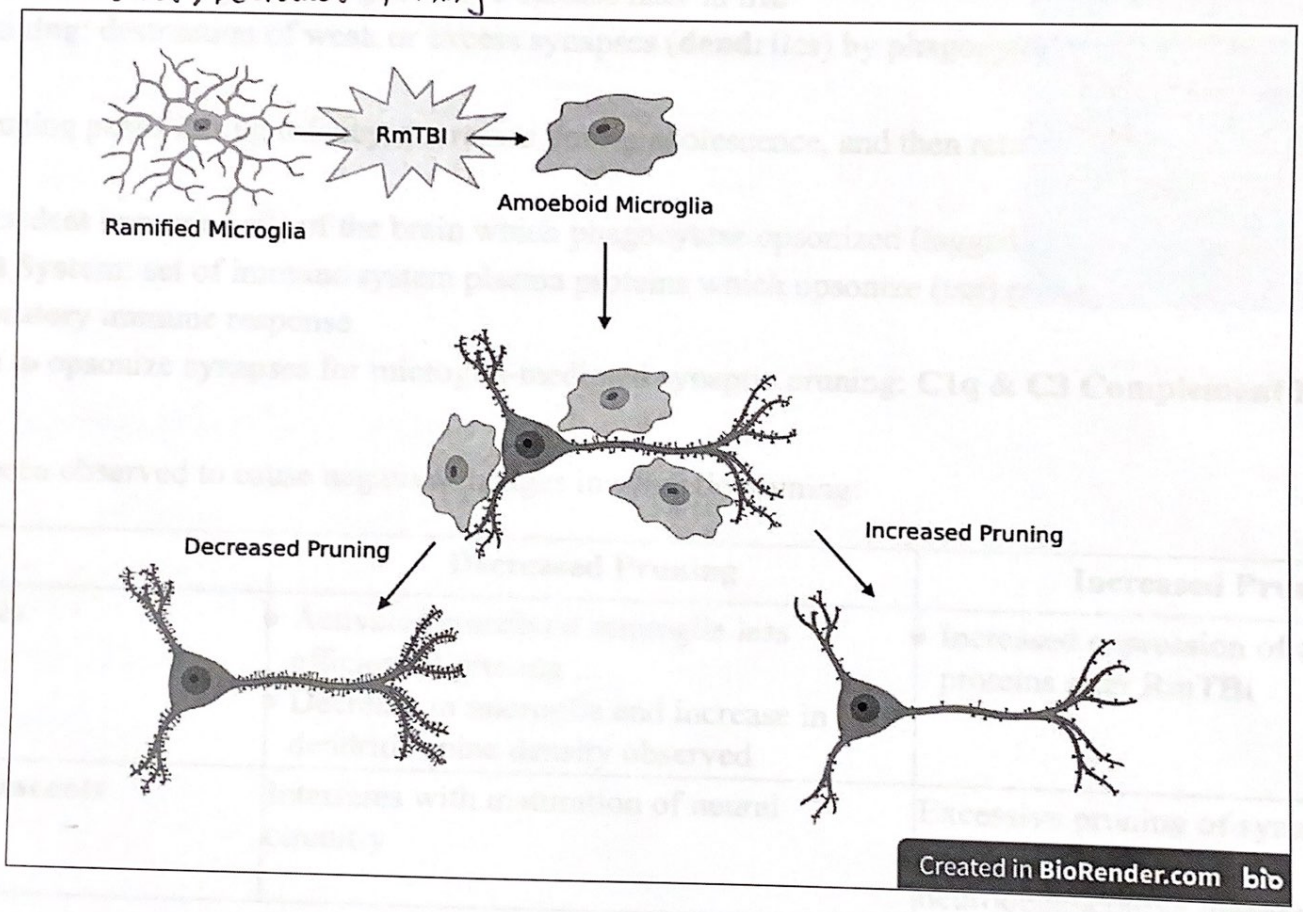
5 February 2024 (class)
- Make diagrams for poster (using BioRender)
1. Microglia-Mediated Synaptic Pruning (2020b paper)
   - show both C1q and C3 opsonization
2. Procedures Diagram
3. Increased/Decreased Pruning

Procedures



Class

Increased / Decreased Pruning



Sep | Oct | Nov | Dec | Jan

* All background research notes can be found in background research section of logbook

January 2024

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
	1 • Work on procedures section paper: IHC, imaging/image analysis	2 • Research on antibody types and classification for Abs used in experiment → create Ab info doc	3 • Work on procedures section paper: Stat analysis (finish procedures section paper)	4	5 • Make January Calendar for binder check	6
7	8 Class 39 • Send Procedures section Paper to Tom • Re-read "microglia dynamics in adolescent TBI"	9	10 Class 40 • Fill out Significant Risk form 2B • Plan for Thursday Meeting w/ Tom (write out important questions that need to be answered)	11 Meeting 16 • Go over Procedures Section Paper with Tom • Set up plan for while Tom is on UK trip • Clarify details of test cohort	12 Pick up images from Tom Send Procedures Section Paper to Dr. Garcia for analysis	13 Tom goes on UK trip
14 Midterm Break →	15	16	17	18 Send C1q image analysis samples to Tom	19	20
21	22	23	24 class 42 / Meeting • Edit Procedures Section Paper with comments from Dr. Garcia • Send edited Procedures section paper to Dr. Garcia • Send C3 image analysis to Tom	25	26 class 43 • Catch up on school work	27
28	29	30 Class 44 • Make plan for poster • Write up draft of background section of poster	31 • Finish C1q image analysis for test cohort			

No meetings with Tom →

Midterms → Tom on vacation until mid-Feb (no meetings) ↓

Holidays and Observances: 1: New Year's Day, 15: Martin Luther King Jr. Day

Wiki Calendar

24 January 2024 (Class)

- 31 Jan: Dec/Jan logbook check
- 13 Feb: Procedures section Paper (what was done + how it was done)
  - NOT why
  - Divide into subsections
- 1 Mar:
  - Mark lock at 9:00 am
  - Term 2 report cards do not include SF results

citations → Source (data sets)
Name of manufacturer:
- kits: (__, __, __)
- Equipment: (__, __, __)
- Software/apps: (__, __, __)

C1q image analysis using approved parameters from Tom
- Calculate density + IQR and determine if results outlier or not
- Results:

		C1q										
EN#	Image#	Area (mm2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound	Upper bound	Outlier?	Mouse average
46	1	0.424	33	0.01484	2223.719677						FALSE	
	2	0.424	15	0.01484	1010.781671						FALSE	
	3	0.424	94	0.01484	6334.231806	1617.250674	4278.975741	2661.72507	-2375.33693	8271.56334	FALSE	3189.577718
47	1	0.424	179	0.01484	12061.99461						FALSE	
	2	0.424	94	0.01484	6334.231806						FALSE	
	3	0.424	26	0.01484	1752.021563	4043.126685	9198.113208	5154.98652	-3689.3531	16930.593	FALSE	6716.082659
53	1	0.424	22	0.01484	1482.479784						FALSE	
	2	0.424	110	0.01484	7412.398922						FALSE	
	3	0.424	102	0.01484	6873.315364	4177.897574	7142.857143	2964.95957	-269.541779	11590.2965	FALSE	5256.06469
54	1	0.424	50	0.01484	3369.272237						FALSE	
	2	0.424	301	0.01484	20283.01887						FALSE	
	3	0.424	386	0.01484	26010.78167	11826.14555	23146.90027	11320.7547	-5154.98652	40128.0323	FALSE	16554.35759
62	1	0.424	350	0.01484	23584.90566						FALSE	
	2	0.424	592	0.01484	39892.18329						FALSE	
	3	0.424	228	0.01484	15363.8814	19474.39353	31738.54447	12264.1509	1078.167116	50134.7709	FALSE	26280.32345
69	1	0.424	767	0.01484	51684.63612						FALSE	
	2	0.424	987	0.01484	66509.43396						FALSE	
	3	0.424	67	0.01484	4514.824798	28099.73046	59097.03504	30997.3046	-18396.2264	105592.992	FALSE	40902.96496
71	1	0.424	531	0.01484	35781.67116						FALSE	
	2	0.424	274	0.01484	18463.61186						FALSE	
	3	0.424	25	0.01484	1684.636119	10074.12399	27122.64151	17048.5175	-15498.6523	52695.4178	FALSE	18643.30638
79	1	0.424	3428	0.01484	230997.3046						TRUE	
	2	0.424	468	0.01484	31536.38814						FALSE	
	3	0.424	2950	0.01484	198787.062	115161.7251	214892.1833	99730.4582	-34433.9623	364487.871	TRUE	31536.38814
80	1	0.424	92	0.01484	6199.460916						FALSE	
	2	0.424	173	0.01484	11657.68194						FALSE	
	3	0.424	1058	0.01484	71293.80054	8928.571429	41475.74124	32547.1698	-39892.1833	90296.496	TRUE	8928.571429

- wait until Tom returns for stat analysis

Send sample C3 images with parameters to Tom for approval
- Parameters (C3)
  - Brightness & Contrast: 0-25
  - Substract Background: 20X
  - Thresholding: 20-25
  - Analyze Particles: 3.50 -infinity
- Samples: Mouse number + Particle counts
  - #46: 79, 247, 2436
  - #62: 134, 213, 207
  - #80: 11, 20, 49

Jan 15/15
welldone

11 January 2024 (meeting with Tom)

- Procedures Paper Corrections:
  - Animals (2.1) & Injury Delivery (2.2)
    - N number of female mice ← only do female cohort
    - Explain P48 as Post-natal day 48
    - State:
      - Mice used for other experiments in lab prior to my experiment ✓
      - I did not perform procedures 2.2 & 2.3
    - Mouse placed on front in Gothenburg Impactor
      ↑ Prone (x supine)
  - Cryosectioning (2.3)
    - Add "Tissue Fixation" to title
    - No 5% isoflurane for euthanization
    - Transcardial Perfusion after euthanization different from that used in IHC
      - Ice-cold PBS pumped through euthanized mice circulatory systems to remove blood + 4% PFA perfused through mouse to fix tissue / avoid degeneration ✓
    - ~~frozen~~ Mounted onto circular ~~disk~~ Stand using OCT
    - Frozen at −80°C
    - 6-well plate used
    - Sectioned slices stored at −20°C
  - IHC (2.4)
    - Specify fluorescent molecules as being fluorophores
    - Blocking solution: 10% donkey/~~goat~~ serum, 1% cold fish skin gelatin, 0.25% Triton X-100
    - Explain significance of using Triton X-100 (used to permeabilize fat-rich cell membranes, allowing for easier access by 1° Abs)
    - 1° Abs ← add catalog numbers
      - rabbit anti-C1q: (Abcam, Catalog # ab 182451)
      - goat anti-C3: (MP Biomedicals, catalog # ICN 55730)
    - Immunization: target antigen/antibody + adjuvant injected into host
    - 2° Abs ← add catalog numbers
      - donkey anti-rabbit AF568: (Thermo Fisher, Catalog # A10042)
      - donkey anti-goat AF647: (TFS, Catalog # A32849)
      - Incubated 2h at room temp after 2° Ab incubation
    - washed in PBS (5x10mins) after 1° and 2° Ab incubation

- 2.5 Imaging
  - Glass slides
  - Centering during z-stacking to capture full thickness of tissue
  - Brightness & Contrast adjusted → refine signal-to-noise ratio
  - Despeckling (noise reduction)
  - Imaging parameters:
    - All: 20x magnification (0.95 NA objective), 2048 x 2048 pixel resolution, bidirectional scanning
    - DAPI: 405nm excitation laser at 2% Power + light collected through pinhole size 2AU
    - AF568: 561nm excitation laser at 1% Power + Pinhole size 1AU
    - AF647: 640nm excitation laser at 1% Power + Pinhole size 1AU
- other questions:
  - 8 male mice? - will get back to me
  - Reasons for no significance: lack of data and/or no biological effect in test cohort in test cohort
  - Tom will be away 13 Jan ~ 12 Feb
  - start conclusion + graphing when Tom returns
  - Extra help while Tom gone unnecessary / can send analyzed data to Tom before he returns for stat analysis
  - Stop by tomorrow to pick up images for female cohort
  - How many mice in female cohort?
  - Decide on imaging parameters by myself → send analyzed images to Tom for checking

10 January 2024 (class)

- Thursday - Meeting with Tom (highlighted text is a priority)
  - Questions/Tasks for Meeting
    1. Test Cohort Summary:
       - Injury: projectile only fired once?
       - 8 female or male mice?
       - Reasons for no significance?
    2. Antibodies (Rabbit α-C1q / Goat α-C3, Donkey α-rabbit / Donkey α-goat)
       - Monoclonal v.s. Polyclonal ← does it matter for experiment?
    3. Experimental Procedures/Proredure Section Paper:
       - C57BL/6 (P48) mice used?
       - Anesthetized using 5% isoflurane using 1.5s
       - Euthanized using Sodium Pentobarbital solution injection
       - Z-Stacking: at 20× magnification / 1.5μm-intervals

Ask Tom if he has time to look over it? (not a priority)

    4. Project:
       - Status on experiments; are we only doing one cohort (male or female + how many mice?)?
       - Significance:
         - Application of ~~my~~ results (therapeutics? education?)
         - How do I make an effective Conclusion?
           - Are there any topics I could research on to help me with this?
       - Help while Tom is away:
         - Is there anyone else at the lab who could help me out? - Is this necessary?
       - Posters/presentations:
         - Tips for making posters
    - Meetings for next week
      - When can I stop by to pick up images?
      - When is Tom leaving for UK trip?
    - + any papers Tom would recommend I read?
    - Do I need an alternate null hypotheses?
    - Analysis for new Cohort:
      - Do I organize results onto spreadsheet?
      - Do I decide imaging parameters?
      - Will you do the t-test + graphing? when? (I can do manually)

Sep | Oct | Nov | Dec

* All background research notes can be found in background research section of logbook

December 2023

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
					1 Class 32 • Organize all BR up to this (read through + highlight) • Plan out lit review sections (paragraph topics)	2
No Meetings With Mentor → 3 • Organize procedures onto doc: animals, injury delivery, brain removal, and cryosectioning steps	4 • Continue adding to procedures doc: IHC, mounting, imaging, and image analysis steps	5 Class 33 • Write intro section paper: RmTBI introduction + RmTBI pathophysiology	6	7 Class 34 • Write intro section paper: complement system + effect of RmTBI on synaptic pruning	8	9
10 Finish image analysis for test cohort mice brain slices	11 Class 35/Meeting • Add to hypothesis + research sections on CYSF Portal • Revise intro section format	12 Meeting 14 • Preparing gelatin-coated slides for Golgi-cox staining (last part of my experiment) • Receive new parameters for test cohort image analysis	13 Class 36 • Write Section paper: Dr. Lohman's research + RQ	14 • Finish lit review	15 Class 37 • Lit review due • Send message to CYSF ethics about 2B ethics form	16
17 Finish image analysis for test cohort mice brain slices (new parameters)	18	19 Class 38/Meeting • Complete variables and procedures section of CYSF Portal (*big task) • Rest of year plan	20 Winter Break →	21 Meeting 15 (online) • Learn data retirement (how to find outliers) + graphing + t-test	22 • Add finalized data, t-test results, and graphs of test Cohort to Experimental Procedures section of logbook	23
No meetings with mentor → 24 • Learn how to perform a t-test manually/with a graphing calculator	25 • Learn how to do a two-sample t-test • Do t-test practice problems	26 • Edit procedures doc: add images of image analysis + add data retirement and stat analysis steps	27 • Read more on Statistics in research. learn how to calculate SEM for graphing results	28 • Start procedures section paper: finish animals, Injury Delivery, and Cryosectioning sections • Add to procedures doc: ~~graphing step~~ (complete procedures doc)	29 • Research on why mouse models are commonly used in research	30 • Read Paper: Animal Models of Traumatic Brain Injury
31 • Research on antibody production + choosing 1° and 2° antibodies						

Holiday and Observances: 24: Christmas Eve, 25: Christmas Day, 31: New Year's Eve

Wiki Calendar

21 December 2023 (Meeting with Tom)

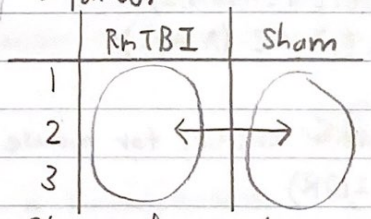
- Online Meeting: Graphing and Statistical Test
- Checking for outliers:
  - Outliers within each mouse (EN# 1, 2, 10, 11...)
    1. Calculate 1st and 3rd quartiles:
       - 1st Quartile (Q1): =QUARTILE.INC(A:A,1)
       - 3rd Quartile (Q3): =QUARTILE.INC(A:A,3)
       - eg. =QUARTILE.INC(F3:F5, 1)
         ↑ ↑ Protein densities for mouse EN# 1
    2. Calculate Interquartile Range (IQR)
       - IQR=Q3-Q1
    3. Calculate upper and lower Bounds:
       - Upper Bound: Q3+IQR*1.5
       - Lower Bound: Q1-IQR*1.5
    4. Determine if data is outlier:
       - OR(A<lower bound, A>upper bound)
       - eg. =OR(F3<J5, F3>K5) → tells if density is outlier (TRUE) or not (FALSE)
  - Outliers within groups:
    - Sort Mice into RmTBI (1) and sham (0) groups

EN#	Injury	C1q	C3
1	1	~	~
2	0	~	~
10	1	~	~
⋮			

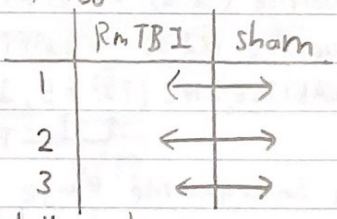
↑ ↑ Density averages go here

    - Use same process to determine if any density averages are outliers
    - Exclude outliers from total average
- Graphing
  - Use GraphPad Prism (only Tom has license → only Tom can graph)
  - Column (1 independent var) vs. Grouped (2 independent var)
  - Input average densities for C1q and C3 from each mice (separate into different sheets)

- t-Test:
  - Normality and Lognormality Test: data passes if at least one yes
  - options,
    - Choose: Unpaired v.s. paired data (doesn't really matter which is used)
      - Unpaired:



      - Paired



    - Choose: Assume both populations have same standard deviation (SD) v.s. Welch's Correction (do not assume populations have same SD)
    - Confidence Level: 95% (P-value < 0.05)
    - Test shows whether data have statistical significance
  - Graph with SD or SEM (standard error of the mean)
  - Results from Test Cohort
    - Options for t-Test: unpaired + welch's correction
    - No statistical significance for either C1q or C3 between RmTBI and Sham

12 December 2023 (Meeting with Mentor)

- Preparing Gelatin-Coated Slides (for Golgi-Cox Staining)
  - Wash slides with double distilled water (dd H_2O) and dry
  - Warm up gelatin and add dd H_2O to create desired dilution for gelatin solution
  - Filter gelatin solution
  - Load dry slides onto rack and immerse in filtered gelatin solution for ten minutes
  - Remove slides after ten minutes and dry out on tissue paper overnight
- Image analysis troubleshooting (for "test" Cohort)
  - Problem: previous parameters unsuitable for some images
  - New parameters:
    - Threshold Range:
      - C1q: 45-255
      - C3: 20-255
    - Analyze Particles:
      - Both: 3.5-infinity (Pixel units)
    - All other parameters are same
    - Exclude mouse 36 from C1q and mouse 11 from C3
- Answers to questions:
  - Antibody dilutions
    - C1q: 1:100 (1° Ab), 1:500 (2° Ab)
    - C3: 1:250 (1° Ab), 1:500 (2° Ab)
  - Same solutions for both 1° Ab and 2° Ab
  - DAPI added directly at 1:1000 dilution to slices after 2° Ab incubation
  - After IHC brain slices stored in PBS at 4°C
  - After cryosectioning brain slices stored in antifreeze at -20°C
    - Antifreeze solution: 30% ethylene glycol, 20% glycol, 1x PBS + PH 7.4
  - Reasoning ("why?") for hypothesis (Complement increase after RmTBI, which may lead to a decrease in microglia and synaptic pruning, leading to the observed cognitive deficits following a RmTBI):
    1. Complements are activated by pathogens/Complement deposition is a sign of inflammation following injury
    2. Microglia decrease have been observed after RmTBIs, likely resulting in decreased synaptic pruning (2022 paper) — there may be a correlation between this and Complement increase

- Next Meeting(s):
  - Wednesday, 13 December: move dried gelatin-coated slides
  - Next week: finish image analysis using parameters, learn data/stat analysis + graphing
  - Week of 25 December: IHC for female AND male cohorts

- Ab dilutions
  - ~~C1q Ab 1:250~~ 2°s
  - C1q Ab: 1:100 AF568 - 1:500
  - C3 Ab - 1:250 AF647 - 1:500
- Yes same solutions for both 1°s + 2°s
- Add DAPI directly to slices after they've incubated w/ 2° Ab. So the same solution as above, @ 1:1000 dilution
- PBS at 4°C

- After cryosectioning slices stored in Antifreeze at -20C
  - antifreeze is: ~~30% Ethylene Glycol, 30%~~
    - 30% EthyleneGlycol (v/v)
    - 20% Glycerol (v/v)
    - in 1x PBS
    - pH 7.4

7 December (class)

- Plan for science fair due next biweekly check
  - Dec: ~
  - Jan: ~
  - Feb: ~
  - Mar 4: science fair
- Each BIG TASK/step
  - } smaller tasks/deadlines
- Plans for now ~ end of feb
  - Presentation classes - Feb 26, Feb 28, Mar 1
  - Poster Design/Creation

11 December (Class)

- Revised Intro section Format:
  1. TBI data + need for research (keep)
  2. Define TBIs/RmTBIs (keep) ← paragraph 3
     - Shorten pathophysiology section and combine with paragraph 2
  3. Adolescent brain development (shorten) + add synaptic pruning (homeostatic role) + Microglia (Paragraph 5)
  4. the complement cascade in the CNS
  5. Effect of RmTBIs on microglia ~~+ complement~~ + synaptic pruning
  6. Effect of RmTBI on complement + relationship between complement and microglia
  7. Study by Dr. Lohman
  8. Summary
- Questions for Tom
  - Some papers state that complement activation increases synaptic pruning
  - our hypothesis: RmTBI → complement increase → microglia decrease → pruning decrease → cognitive impairment
  - Why? - evidence other than past experiments

Sep | Oct | Nov

November 2023

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

Mon	Tue	Wed	Thu	Fri	Sat	Sun
30 • Male mice brains harvested (by Tom)	31	1 Meeting 10 * Start experiment • IHC on mice brains: blocking + 1° Ab incubation	2 Class 23 • Time to catch-up on school work • 2° Ab incubation (by Tom)	3 • Mounting of brain slices (by Tom)	4	5
6 Class 24 • Read Papers on Complement System in brain • Edit RP using comments from Dr. Garcia	7	8 Class 25 • RP presentations: owen, Mariska, Natalie, Elliot • Start filling out CY2F basic info	9 Meeting 11 • Making golgi-cox solution (not part of experiment)	10 No class	11	12
13 No School	14	15 Class 26 • Finish filling out CYSF basic info/ethics form + log on to CYSF platform • Start lit review • Add meeting notes to	16 Meeting 12 • Imaging of male brain slices • Ask if more meetings/ time needed to complete experiment Cancelled	17 Class 27 / Meeting • RP presentation: Vincent • Read paper: role of microglia at complement cascade in CNS • Enter basic project info,	18	19
20	21 Class 28 • Email CYSF about Sig risk form 2B • Read paper: Microglia and complement cascade in CNS + summarize	22 logbook + • RP Presentation: Tasfia + 22, Brynn Meeting 12 • Imaging of male brain slices • ~~Ask about meetings/~~	23 Class 29 • RP presentation: Amy, Ashanki, Joel, Tiffany • Read paper: role of complement system in TBI (a review) Moved to 23 NOV	hypothesis + variables to CYSF Patel 24	25	26
27 Class 30 • Read: role of complement in Synaptic pruning + Neurodegeneration • Submit Sig risk form 2B	28 Class 31 / Meeting • Read: Synaptic pruning and key deficit in neurodevelopmental disorders • Organize November readings	29 plan during winter break Meeting 13 • Imaging of brain slices (Learn + get data from Tom)	30 • November logbook	1 Class 32 • Add new sections/info to lit review from papers read • organize BR • Start experimental procedures	2	3 • Finish lit review
4	5	6 • Ask about Dec plan	7	8 Section of logbook	9	10

29 November 2023 (Meeting with Mentor)

- Image analysis (Particle count + density); using Fiji/ImageJ program
- C19

1. Change image scale (should apply to subsequent images) ✓
   - Analyze → Set scale
     - Unit of length: mm
     - Distance in pixels: 5263 ← Scale factor: 0.19 µm/px (from microscope)
       - ↳ 5.26 Px/µm
       - ↳ 5263 Px/mm
     - Known distance: 1.00
     - Pixel aspect ratio: 1.00 ✓
2. Ctrl-shift-C or Image → adjust → Brightness & Contrast
   - Set
     - Min: 2 • Max: 25 → Ok → ~~Apply~~
3. Process → Noise → Despeckle
4. Process → Subtract Background
   - select: ✓ Sliding paraboloid, ✓ preview
   - 1.5 Px (rolling ball radius)
5. Image → Type → 8-bit (converts to grayscale) ✓
   - If needed; LUT → Yellow (add colour)
6. Image → adjust → threshold
   - B & W
   - Threshold Range: 45 ~ 255 → apply → close

only for first image:

7. Analyze → Set measurements
   - select: ✓ Area, ✓ Display Label
8. Ctrl → M → gives area of image in mm^2 ✓
   - Copy + paste to excel sheet
   - Volume = Area x (35/1000)

9. Analyze particles
   - size (mm^2): 10-infinity) • select: ✓ Pixel units
   - show: overlay Masks
   - Summarize ✓
10. Count displayed → copy to excel sheet ✓

- For next image
  - disable calibrations + messages

↳ ☐

☑

- Area + volume constant for all images
- Calculate mouse density average on sheet
- Use + save images to 8-bit folder ✓
- Titles:
  - eg. Scene 2 – Mc #1 = mouse # 1, image 2

C3 settings:
- B & C: 0~25
- Subtract Background: 20px ✓
- Threshold Range: 25~255
- Analyze Particles size (PX units): 10-infinity

0.19μm/px

1/0.19 = 5.26 px/μm

C1a
2 - 25 B+C → Despeckle
Sub Backgr. = 1.5 px
Threshold range - 45-255
Analyze particles size (pixel units)
= 3.5 - Infinity
X 36

C3
0 - 25 B+C → Despeckle
Sub background = 20 px
Threshold range - 20 - 255
Analyze particles size (pixel units)
= 3.5 - Infinity
X 11

1 November 2023 (Meeting with Mentor)

- Diluting 1% solution from 10% PBS solution
- Blocking
- 1° Ab Incubation
  1. Use pipette to transfer 1° Ab solution onto tray
  2. Use pipette to transfer 1° Ab onto tray
  3. Transfer brain slices onto tray
  4. Storage in cold room overnight
  - Maintain a negative control well on tray without 1° Ab to test for background fluorescence
- Next Meeting
  - 2 November 2023 (maybe): 2° Ab incubation

8 November 2023 (Meeting with Mentor)

- Making golgi-Cox Sdution (used for staining of dendrites/dendritic Spine density)
  - 5% Potassium dichromates (100mL double distilled water) + 5% Mercuric chloride (100mL double distilled water) + 5% Potassium Chromate (80mL double distilled water)
- Storage of solution away from light (wrapped in foil + stored in box)
- Next Meeting:
  - Immunofluorescence image analysis of mice brain slices for experiment

→ Nov Notes are great - 15/15

→ Would be good to see print outs of your image analysis for you can add to Exp. Proc or Data Collection —

→ Good tasks plan in Calendar

Sep | Oct

October 2023

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

Mon	Tue	Wed	Thu	Fri	Sat	Sun
25	26	27	28	29	30	1
Class 12 / Meeting 2 • Rewrite calendar • Read additional papers for RP (anaesthesia, stress, complement system)	3	Class 13 4 • Review RP rough draft feedback from Dr. G + edit RP • Create RP questions for Tom Meeting 5	~~Meeting 6~~ 5 Cancelled	Class 14 6 • Finish editing RP with comments from Tom	• Finish RP final draft (excluding methodology) 7	8
9 No School	Class 15 (11 Oct) 10 • Create outline for research proposal • Create Punchline	• Go over RP rough draft 11 • Factual errors • Expand on methodology • Ask about year plan	Meeting 6 12 • Go over stat analysis (methodology) • Go over plan for rest of year	Class 16 / Meeting 13 • Edit methodology • Finish RP final draft	14	15
16	• Final RP due (oct 17~19th) 17 Class 17 • Finish presentation slides • Punchline • Methodology	• Begin RP oral presentations (oct 18th-31st) 18 ~~Meeting 7~~ Cancelled	Class 18 19 • Final check over RP • Start presentation script for RP Meeting 7 • Go over RP slides with Tom	20	21	22
Class 19 23 • Edit RP slides with comments from Tom • Finish RP Presentation script	• check with Dr. G 24 TUCID/ biohazard safety?	~~Meeting 8~~ (online) 25 • Practice oral presentation • Qs: what effect does ↑ c proteins have on microglia? What can I cut from my presentation? Class 20 / Meeting	• Ask for any images to put on slides 26 Meeting 9 • Cryosectioning Practice	Class 21 27 • Send November schedule + image request to Tom • Practice RP Presentation (Practice with Dr. G at lunch)	28	29
• CYSF Permission form due to GC 30	Class 22 31 • RP Presentation • Binder check • Final RP due (if not already submitted)	• Practice oral presentation (time + cut) 1 • Qs for Dr. G. ethics form	2	3	4	5

13 October 2023 (class)

- Science Fair Info
  - WASF: March 4 → 15 to CYSF
    - March 14: Online CYSF portal closes
  - Ethics form approval → experiment start
    + Basic project info

25 October 2023 (class)

- Presentation order:
  - Oct 31
    - Jessica
    - Cooper
    - Brynn
  - Nov 6
    - Tosin & Zainab
    - Natalie
    - Owen
  - Nov 8
    - Mariska
    - Elliot
    - Amy
  - Nov 15
    - Vincent
    - Ashank & Joel
    - Tiffany
- 10 minutes (10 ~12 slides/max 15)
  - Background
    1. Title/Punchline
    2. Info
    3. Info
  - RQ + Goals — 1~2
  - Methodology — Flow Chart
  - Significance

12 October 2023 (Meeting with Mentor)

- Statistical Analysis of Image
  - Use: Fiji image processing package
  - Steps: C1q + C3 tagged fluorescent
    1. Subtract Background (caused by non-specific binding)
    2. Thresholding to convert to binary image
       - Binary Image: only two possible intensity values (0 → black, 1 → white)
    3. Analyze particles using "analyze particles" function → count for each protein per mouse
    4. Average protein count for each mouse compared between RmTBI and Sham mice
       - Use T-test (compare means of two groups for any statistically significant difference)
       - P-value threshold at <0.05 → 95% certain differences are not due to chance
    5. Calculate density of protein expression
       - Convert image dimensions from pixels to μm
       - Calculate volume of image (μm^3)
       - $\frac{\text{Count}}{\text{Volume}}$ = Density of expression

- Plan for Remainder of Year
  - Male Mice
    1. Help with Cryosectioning (week of oct. 16th) ✓
    2. IHC for C1q + C3 (week of oct. 23 – 2 meetings) ✓
    3. Mounting on slides (week of oct. 23 – 1 meeting) ✓
    4. Imaging (week of 30th ~ mid-November)
    5. Analysis (mid-November ~ early December)
  - Female Mice (harvested on Nov. 13)
    1. Cryosectioning (week of Nov. 21)
    2. IHC (week of Nov. 27)
    3. Mounting (week of Nov. 27)
    4. Imaging (week of Dec. 4)
    5. Analysis (New Year)

- Next Meeting
  - Go over presentation
    or
  - Cryosectioning Practice (Book cryostat)

Notes →

- Notes:

ALBERTA INNOVATES

Male mouse brains
- being sectioned (maybe help)
- IHC - C1q + C3 - 23rd (2 days that week) + 24th
- Mount - rest of week (25th - 27th)
- Imaging - w/c 30th → mid Nov
- Analysis - mid Nov → early Dec

Females - brains harvested on 13th Nov
- section 21st [redacted] - 24th
- IHC - C1q + C3 - 27th / 28th
- mounting 29th Nov - 1st Dec
- Imaging - 4th Dec - 18th
- Analysis - into New Year

ALBERTA INNOVATES

0 1011

Background Black — Signal White

1024 px × 1024 px

0.38 μm/px

3.42
4.68
3.48

ALBERTA INNOVATES

converted from pixels to μm

- calculate volume of each image

x μm × y μm × z μm = μm³

200 μm³

10

1500

$\frac{\text{volume}}{\text{count}}$ = density of expression

ALBERTA INNOVATES

Images processed in ImageJ, subtract background, then threshold to convert to binary image. Then the binary image is analyzed using 'Analyze particles' function, obtaining a count for each protein, per mouse.

Average particle count for each mouse was then compared between RmT8J and nhem mice, using a T-test.

T-test - stats test that compares the means of two groups for any statistically significant differences. We will set the p-value threshold at <0.05, which means we are 95% confident that any diff seen is not due to chance.

6 October 2023 (class)

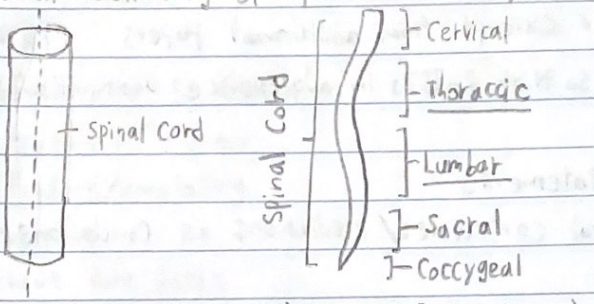
- Logbook comments
  - Show Dr. G improvements using comments
  - Include tasks + due dates in Calendar
  - Take 10~15 min every ASP class to organize logbook
    - Meeting notes, email transcripts, RP progress
    - Communicate with mentor

11 October 2023

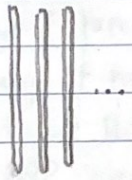
- Research Proposal
  - Turnitin + GC
    - originality
    - edits, feedback, grading
  - Deadline 31 oct
- Oral presentation (10min) → 10~12 slides
  - Big font, little text, lots of graphics
  1. Punch Line / Elevator Pitch
  2. Background Research
  3. RQ / Goals
  4. Methodology (Flow Diagram)
     - How do the results answer RQ
  - Marking
    1. Your Presentation
    2. Questions

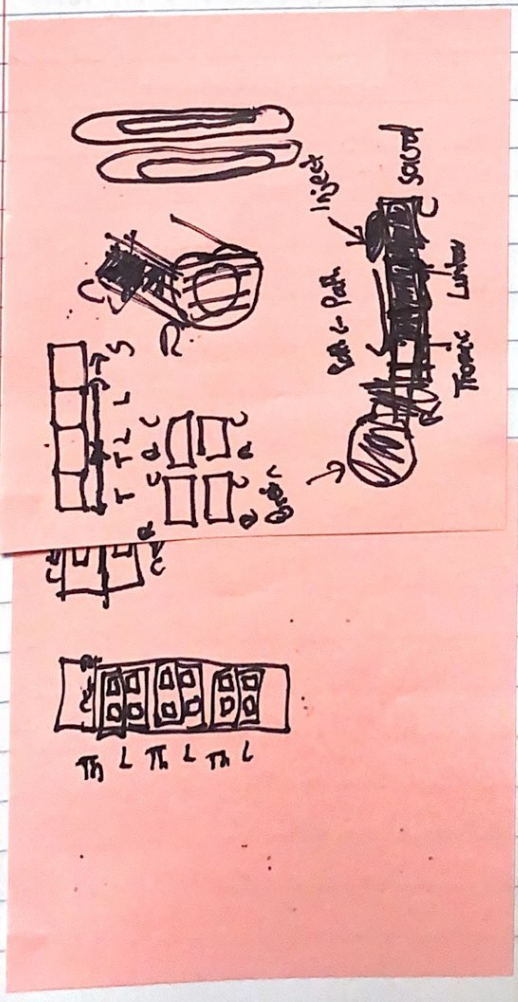
5 October 2023 (Meeting with Mentor)

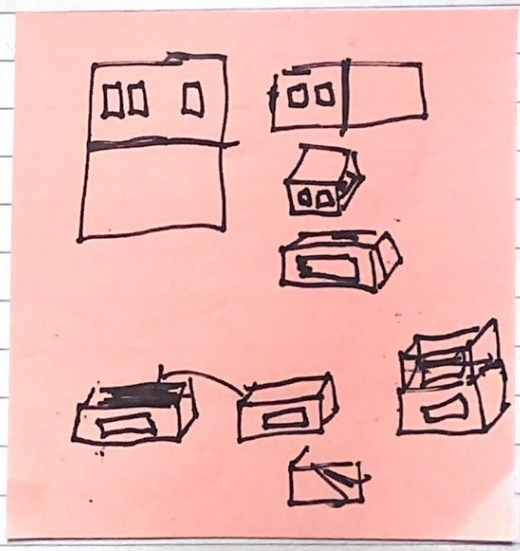
- Sagittal Sectioning of Spinal Cord Sample (Not used in my experiment)



↳ samples:







- Research Proposal Draft: General Feedback
  - Add sources + Read excerpts from additional papers
  - Reorganize order so that RmTBIs in adolescence are mentioned early in the proposal
  - Rephrase some statements
  - Avoid making general conjectures/predictions as conclusions based on relationship in data

2 October 2023 (class)

- Calendar/schedule
  - Tasks:
    - Meeting w/ Dr. Garcia
    - Meeting w/ mentor
    - Reading/annotating
    - writing
- Include due dates
- Background research/daily notes ← add enough information about daily tasks
  - Physical logbook:
    - Printout of Paper
    - Summary of Paper
    - or
      - Paste-work digitally
      - Summarize-physical
- ☆ Tasks + Due Dates
- Separate ASP calendar + regular school calendar
- Oral Presentation Outline (10~slides)
  - 1-3: Background Research
  - 4-5: Research Question/Goals
  - 6-7 • Methodology: flow chart/graphic ← variables
  - 8-10: Significance

4 October 2023 (class)

- Science Fair Info
  - CYSF.org
  - Deadlines:
    - online Portal: due March 15
    - Poster: due April 18-20
  - Webber Academy Science Fair: early March
- Research Proposals:
  - written due October 16-18
  - presentation on October 18-31

Sep

BR = Background Research
RP = Research Proposal

September 2023

Class
Meeting with Dr. Garcia
Meeting with Tom
Deadlines

Mon	Tue	Wed	Thu	Fri	Sat	Sun
28	29	30 First class	31	1 class 1 • Ask Dr. Garcia about Schedule B form/UCID	2	3
4 Finish reading brain injury paper	5	6 Class 2 • Set up Paperpile • Background research on immunohistochemistry + fluorescence imaging	7 • Finish reading Microglia dynamics/RmTBI Paper	8 Class 3/Meeting • Review Microglia/RmTBI paper • BR: glutamate excitotoxicity, synaptic pruning, complement proteins, brain anatomy Meeting 1	9	10 • Finish reading 2022 Paper
11 Meeting 2 • Go over RP sections (intro, objectives, variables, methodology)	12 Class 4 • Finish reading 2020 Paper • BR: Brain anatomy	13 ~~Meeting 3~~ Cancelled	14 Class 5 • Organize BR (all papers) • RP outline due 18 Sep • Ask for RP exemplars Meeting 3 • Learn sample mounting techniques • Ask questions about 2022 paper	15 • Gain overview of Project (BR, lab techniques) • Ask about UCID/biohazard Safety course • Ask to suggest papers	16	17
18 Class 6 • Finish RP outline • Add sections on adolescent brain development + summary of 2022 Paper • Start RP intro rough draft	19	20 Class 7 • Finish RP intro rough draft	21	22 Class 8/Meeting • Add Paperpile citations to RP rough draft • Finish RP objectives/variables/Hypothesis	23	24 • Finish RP rough draft + Send to Tom
25 Meeting 4 • Practice using cryostat for sample cryosectioning	26 Class 9 • Submit RP rough draft to Dr. Garcia for checking • Organize ASP binder (Print email communication)	27	28 Class 10 • Edit Significance section of RP • Start RP presentation	29 • Logbook check • Review logbook feedback	30	1
2	3	4	5	6	7	8

28 September (class)

- Resources (Statistics)
  - eg. 20% of Canadians are affected by disease A (...)
    - → Textbooks
    - → NHI / PubMed
      - → Disease A stats
    - → Google Scholar
    - → Health Canada.Ca

} good sources
- peer-reviewed articles
- gov. sites

✓

Sept notes are very good / Background Research is impressive!

Calendars need work - let's talk about how to make them more efficient.

avail for ASP confusing

- tasks for each class with
  - ⇒ due dates
- Plan ahead Tasks (Oct) for next month

20 September 2023 (class)

- UCID / Young persons at U of C Lab / Biohazard safety training
- General timeline for year:
  - september - Early october: finish RP
  - October - December: Experimentation / data collection
  - January - February: Data analysis
  - March: WASF
  - April: CYSF
  - May: CWSF
  - May-June: Final writing assignments

22 september 2023 (Class)

- Logbook Check: 29 September 2023
  - Digital: submit on GC
  - Notion: share permission
  - Physical:
    - Hand in 28 September (Thu.)
    - Collect 29 september (Fri. am/pm)
  - Criteria:
    1. organization; content
    2. schedule
    3. communication - Evidence
  - weekly meetings:
    - Email:
      - Report what's done
      - Ask questions/help
      - Set up meetings
      - other updates

18 September 2023 (class)

1. • Working Title: concise, informative, descriptive
   • Can be modified later
   • Abstract:
   • usually need results
   • X necessary
2. • Introduction: background knowledge
   • Make an outline
   • Broader Aspect of Field
   ↓
   Narrower Topic
   ↓
3. Ask Question (separate question and hypothesis)
   • Why do we need to study topic?
   • Lacking studies, contradictory studies, better studies
   • "Need for my study"
4. • Goals
5. • Variables + Hypothesis (only for experimental projects and certain studies)
   ↑ + Methodology (group together)
   • Am I doing a study, experiment, or innovation?
6. ↓
7. • Methodology
   • What methods do we use, why do we use it?
   • General Idea
8. • Significance: summary
   • What will it contribute to the field of study
9. : Reference
   • use paperpile

• Write more information
• No need to be too concise

• Format:
• 12pt font
• Double spaced
• Figures: legend

18 September 2023 (Meeting with Mentor)

- Cryosectioning: slicing samples using cryostat
  - Sample: spinal cords
    - Rostral ⟷ caudal
      (Front) (Back)
    - Dorsal (Top)
      ↕
      ventral (Bottom)
  - Blood washed from sample by saline diffusion
- Cryostat:
  - Frozen samples must be sectioned at low temperature in order to prevent sample from melting and avoid consequent tissue damage
  - Components:
    - Handwheel + lock lever
    - Knife/blade holder
    - Specimen clamp
    - Quick Freeze Shelf
    - Heating Glass
  - Section size: 12 μm
  - Trim size: 30 μm

- Next Meeting (21 September 2023):
  - Create/schedule Google Meet
  - Finish rough draft of introduction for research paper

18 September 2023 (class)

1. • Working Title: concise, informative, descriptive
   • Can be modified later
   • Abstract:
   • Usually need results
   • X necessary
2. • Introduction: background knowledge
   • Make an outline
   • Broader Aspect of Field
   ↓
   Narrower Topic
   ↓
3. Ask Question (separate question and hypothesis)
   • Why do we need to study topic?
   • Lacking studies, contradictory studies, better studies
   • "Need for my study"
4. • Goals
5. • Variables + Hypothesis (only for experimental projects and certain studies)
   ↑ + Methodology (group together)
   • Am I doing a study, experiment, or innovation?
6. ↓
7. • Methodology
   • What methods do we use, why do we use it?
   • General Idea
8. • Significance: summary
   • What will it contribute to the field of study
9. : Reference
   • use paperpile

• Write more information
  • No need to be too concise

• Format:
  • 12pt font
  • Double spaced
  • Figures: legend

14 September 2023 (Meeting with Mentor)

Mounting Samples onto Slides

- Samples: Coronal brain slices
- Steps:

1. Place slide with rough end facing upwards in fluid
2. Submerge slide in petti dish containing samples (excluding the rough end)
3. Use rod to float samples and observe which side they naturally orient to (the samples should be placed on the slide with that side facing upwards
4. Mount samples onto slide: push rather than pull (samples will tare); push down on sample to remove any spaces below
5. Push down on sample as you remove slide from fluid
6. Dry out slide after all samples mounted
7. Use pipette to apply fluid on onto Slide
   - Push down on plunger → insert pipette in fluid → release plunger to draw fluid
   - Apply steady pressure on plunger to release drops of fluid onto slide (DO NOT RELEASE - bubbles will form)
8. Place slide cover over slide at an angle to prevent bubbles (air becoming trapped under cover)

12 September 2023 (class)

- Schedule B Form ← signed by vice presidents
- Young Persons Accessing Uof C Labs ← Uof C/Parents - Yourselves
- Meet mentors during class time.
  1. email attend@webber
  2. CC Dr. Garcia / Email Dr. Garcia

14 September 2023 (class)

- Research Proposal:
  - Innovation:
    - no research Q
    - no variables
  - Experiment:
    - variables
    - methods
  - Study:
    - data
    - no variables
- outline:
  - Background Research
  - Research Question
  - Goals (short & long-term)
  - Methods (variables)
  - Significance

- Oct 6th (written proposal)
- Oct 11, 13, 17th (Presentation)

- Have outline for research proposal

- Statistical analysis: SPSS - Software Package

11 September (meeting with Mentor)

- Testi preparing samples with antibodies
  - Primary Antibodies/Complement proteins:
    1. Rabbit α-C1q
    2. Goat α-C3
    - In different concentrations:
      - C1q → 1:100, 1:250
      - C3 → 1:100, 1:250, 1:500
  - Secondary Antibodies:
    1. Rabbit α-C1q ← Goat α-Rb + AF568 (fluorophores)
    2. Goat α-C3 ← Donkey α-Gt + AF647
  - Procedure:
    1. wash samples 3 x 10 mins with PBS (Phosphate Buffered Saline) (brain slices)
    2. Blocking step: block non-specific binding sites on cells in samples to ensure that primary antibodies bind to POIs
       - Use:
         - PBS + 10% Goat/Donkey serum + 5% BSA (Bovine Serum Albumin)
         - +
         - Cold Fish skin Gelatin: 0.1% or 1%
         - +
         - Triton-X100: 0.1%, 0.25%, or 0.5%
           - Triton-X 100 breaks down lipids → creates holes in phospholipid (cell membrane) bilayer of cells for antibodies to enter and reach intracellular proteins
           - High concentrations of triton can destroy cell membrane + free up cell surface proteins which antibodies may accidentally bind to → different concentrations tested

    (improves signal-to-noise ratio:
    - signal: binding to PoI
    - noise: non-specific binding)

    3. Primary Antibody Incubation: 4°C, overnight
    4. Antibody Solution (BSA + Cold Fish skin Gelatin + Triton) act as carrier Proteins to deliver antibodies to where they need to be inside the cell

- Background Research:
  1. Introduction:
     a. TBI Prevalence + incidence (in US)
        - Mild TBIs/Concussions, repeated TBIs/Concussions
        - Affect on adolescent population
     ✡ b. Post-Injury Symptomatology (symptoms)
        - Loss of consciousness, vision problems, cognitive issues
     ✡ c. Post-concussion Syndrome (PCS): long-lasting/chronic symptoms after concussions
        - Repeated injuries can lead to PCS
     d. Repeated TBIs → increase risk of developing neurodegenerative diseases later in life (Chronic traumatic encephalopathy/CTE, Alzheimer's/AD, Parkinson's/PD)

Combine:
     e. Adolescent Brain Development
        - Major developmental processes (✡ Synaptic Pruning)
     f. What is Synaptic Pruning?
        - Role in brain development
        - How does it happen?
        - Role of microglia + complement proteins C1q and C3/C3R

Read →
     g. Review of Dr. Lohman's 2022 Paper
        ① - Behavioural results in mice with TBI
           - Male mice with TBI have ↓ motor behaviour + ↑ motor deficits
           - Variables: TBI v.s. Sham, Male v.s. female mice
        ② - Microglia → reference 2021 paper
           - In motor cortex of males: ↓ microglia density after TBI than after Sham
        ③ - Dendritic Spines
           - Synaptic pruning
           - In motor cortex of males: ↑ spine density after TBI than after sham
     h. I will: investigate complement protein expression
        - How expression changes in TBI
        - Relation to behaviour deficits, microglia, and spine density

2. objectives:
   a. short-Term Goals:
      - Investigate complement protein expression in motor cortex of males following TBI v.s. sham
      - optimization of immunohistochemistry techniques to detect complement proteins
   b. Long-Term Goals (Dr. Lohman's):
      - Understand how RmTBI affects complement proteins and downstream effects on behaviour, microglia, and spine density
3. variables:
   a, Independent: RmTBI v.s. sham treatment
   b, Dependent: complement protein expression
   c. Controlled:
      - RmTBI Model:
        - Speed of projectile (5m/s ± 0.2m/s)
        - Head position (lateral impact)
        - Age, sex (adolescent, male)
        - Antibody Concentration
        - Imaging parameters
   d. Confounding:
      - Anaesthesia
      - stress of mice ★
      - natural variation
4. Hypothesis: Complement protein expression increases in adolescent mice folbwing TBI
5. Methodology:
   a. RmTBI Model: lateral impact model
      - I was not involved in mouse testing ★
   b. IHC
      - Complement Expression
      - Antibodies: α-C1q, α-C3
      - 40μm brain slices

C. Confocal Imaging (Fluorescence Imaging)

- Image Complement expression in motor cortex (3 slices/mouse)

d. Experimental Design:

- RmTBI v.s. Sham
  (n=8~10) (n=8~10)
  ↑n = number of mice
- mTBIs ← 5 mTBIs delivered /24 hrs
- 5 days post injury → mice enthanized + brains sliced

: Next Meeting:

- Slide mounting + experimental techniques

- Notes:

Mouse α-TMEM
Rb α-Iba1
Rat α-CD68

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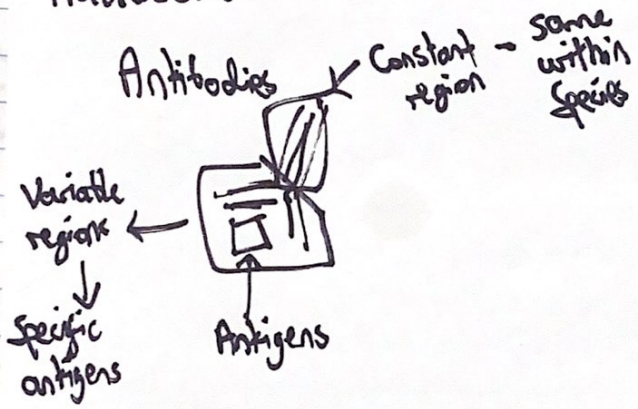
" α-mouse - AF647
" α-rabbit - AF568/594
" α-rat - AF488

ALBERTA INNOVATES

Immunohistochemistry (IHC)

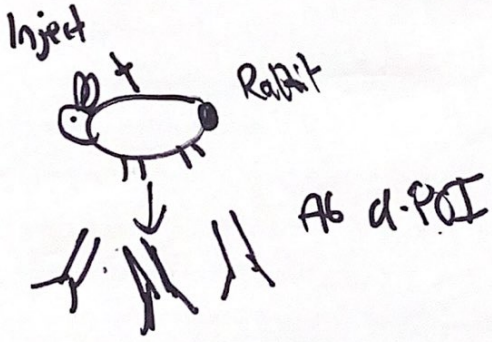
Colometric IHC -

Fluorescence IHC



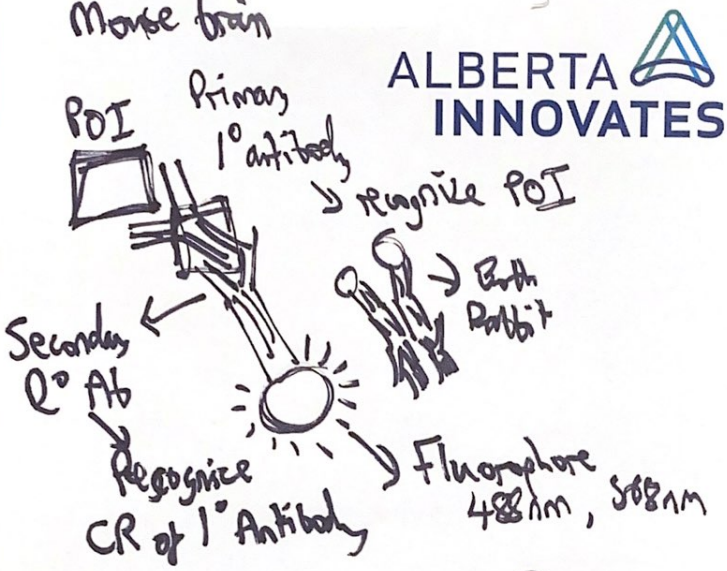
ALBERTA INNOVATES

POI + Adjuvant

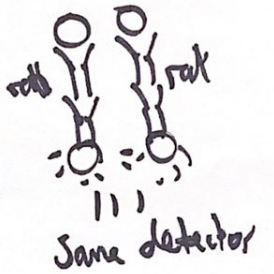


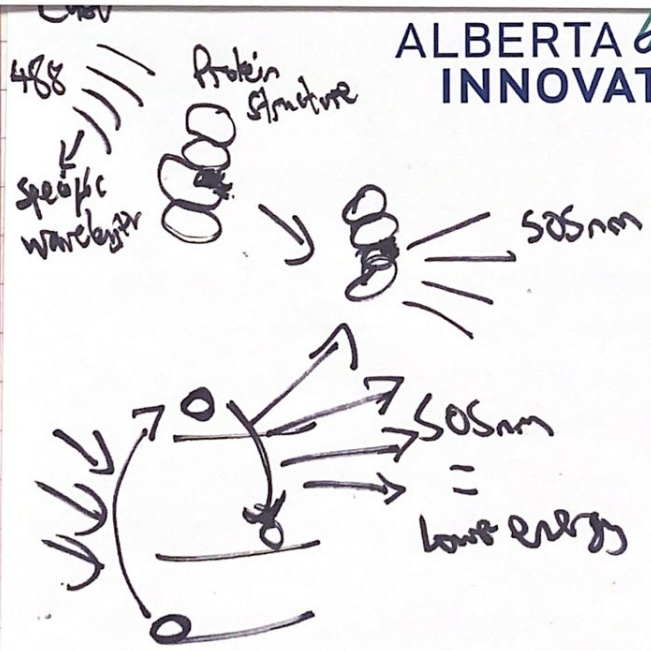
Rabbit α-Iba1 Ab

Mouse brain



Goat a-rabbit
a-rat
a-mouse
a-chicken





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Introduction

- TBI prevalence + incidence
  - Mild TBI / concussions
  - repeated TBIs / concussions
  - Adolescent population
- Post-injury symptomatology
  - loss of consciousness
  - vision problems
  - affect symptoms
  - cognitive issues
- Post-concussion syndrome (PCS)
  - Long-lasting / chronic symptoms after concussion
  - repeated injuries can lead to PCS.
- Repeated TBIs / TBIs generally ↑ risk of late life neurodegenerative diseases (CTE, AD, PD...)

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- Adolescent brain development
  - major developmental processes
  - Synaptic pruning
- What is synaptic pruning?
  - Why is it important during development?
- How does it happen?
  - microglia
  - complement proteins
    - C1q
    - C3 / C3R
- Our 2022 papers
  - Behaviour results
    - Males ↓ Motor behaviour ↑ motor deficits
      TBI vs Sham
      Male vs female
  - "2021 paper" ← Microglia - in motor cortex of males only, ↓ ramified density in TBI vs Sham

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- Dendritic spines
- 'Synaptic pruning' ↓
- Spine density ↑ in male motor cortex, TBI vs sham

- Now follow up and investigate complement protein expression - how changed in TBI, relation to behaviour deficits, microglia + spine density.

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Objectives ..

- Short term goals
  - Investigate complement protein expression in motor cortex of males following TBI vs sham:
    - Optimization of IHC to detect complement proteins.
- Long term goals
  - Understanding how RmTBI impacts complement proteins and downstream effects on behaviour, spine density.

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Variables

a) Independent variable
- RmTBI vs Sham

b) Dependent variable
- Complement protein expression

c) Controlled variable
- RmTBI model
- speed of projectile (5 m/s) +/- 0.2 m/s
- ~~accelleration~~
- head position - lateral impact
- age, sex (Adol. 8wk male)
- Antibody conc.
- Imaging parameters

d) Confounding variable
- anaesthesia
- stress of mice !!!!
- natural variability

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Hypothesis

- Related to finding in 2022 paper

" I hypothesise that complement protein expression will increase in adolescent male mice following RmTBI "

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Methodology

- RmTBI model
  - Lateral impact model
  - * mention you had no involvement in mouse work *
- IHC
  - Complement expression
  - Abs d. Cq, C3
  - 40 µm brain slices
- Confocal imaging
  - Fluorescence imaging
  - Image complement expression in motor cortex (3x slices/mouse)
- Experimental design
  - RmTBI vs Sham
    (n = 8-10) (n = 8/10)
  - mTBIs - 5%/24hrs
  - 5 days post-injury - sacrificed/euthanized ↓ brains sliced

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8 September 2023 (Meeting with Dr. Garcia)

- Narrow down research question → proposal
  - Short-term goals: what proteins,
- Variables
  - How to measure synaptic pruning?
  - What are the experimental groups
- How will the TBI be delivered?

- Try to figure out some details by yourself

- Timetable:
  - → meeting times
  - → communication
  - → set due dates (when to send proposal for feedback?)

- Have material to show mentor

7 September 2023 (Meeting with Mentor)

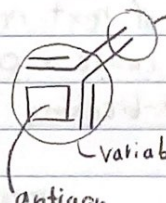
Fluorescence Microscope

- Widefield (WF): light from many focal planes (in-focus + out-of-focus planes)
  Confocal: uses pinhole to block out-of-focus light → higher resolution
- Coronal slices of mice brain (cuts parallel to Y-plane)
- DAPI Staining → Stains nucleus of all cells in sample
- observed:
  - Motor cortex: bumps on each hemisphere
  - Corpus callosum: between hemispheres
  - Neurons, astrocytes, microglia, oligodendrocytes
    - oligodendrocytes → produce myelin
    - microglia → immune cells
- Staining for:
  - TMem protein: produced by phagocytes + microglia
  - Iba1 protein: more specific to microglia
  - CD68 Protein: attached to vacuoles of lysosomes which perform phagocytosis

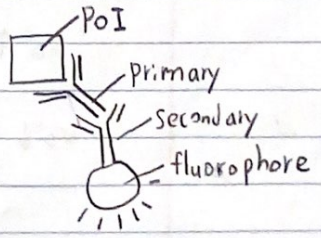
Immunohistochemistry (IHC)

1. Direct: single antibody
2. Indirect: two antibodies
   - Colometric
   - Fluorescence
     - Inject Protein of interest (PoI) + adjutant into animal
       - adjutant ↑ increases immune response (causes animal to produce antibodies targetting PoI)
     - eg. Iba1 → α-Iba1 (Iba1: protein (antigen); α-Iba1: antibody)
     - Primary antibody: recognizes PoI
     - Secondary antibody: recognizes constant region of primary antibody (allows for detection)
       - Tagged with fluorescent proteins (fluorophores)
       - often from different animals (eg. goats)

Antibodies recognize antigens (proteins)



- usually used by body to tag pathogenic antigens for destruction

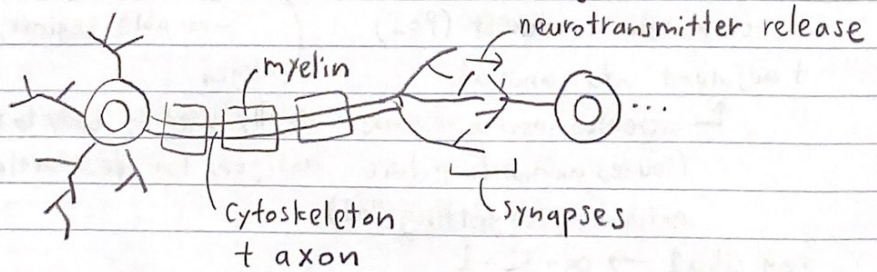


- When fluorophore is excited by light → specific wavelengths of light
  Cause shape change + light emission with higher wavelength/lower energy

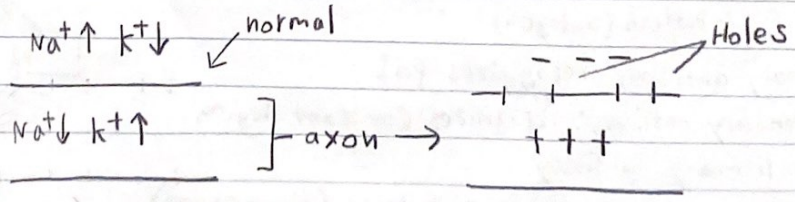
Imaging + TBIs

- White Matter Tracts (eg. Corpus callosum)
  - (Myelinated) axons are unidirectional and high density
- Grey Matter
  - Axons are polydirectional and low density
- Front ↔ back Concussion: brain moves back + forth
  - Causes a perpendicular force to the direction of axons in white matter
    → axons tear/break
  - Microglia respond → neuroinflammation
    - Repeated injuries → Chronic neuroinflammation → neurodegenerative diseases

- Can also cause holes in axon (contains cytoskeleton) instead of tearing it
  - Holes damage ion gradient → increase membrane potential → cause depolarization of axon → causes uncontrolled neurotransmitter release to synapses of next neuron
    ↑ eg. glutamate
  - Glutamate cause a calcium influx into axons → Calcium activates microtubule-breaking proteins, damaging cytoskeleton



- Depolarization:



• Further research areas:

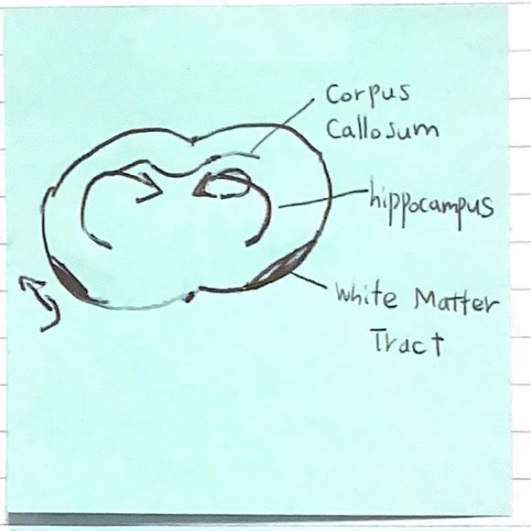
- TBI/concussion, mTBIs
  - incidence, prevalence
  - Symptoms
  - animal research
    - glutamate excitotoxicity
    - neuroinflammation
      - microglia
      - synaptic pruning
- Complement (C3, C3R, C1q)
- Adolescent brain development

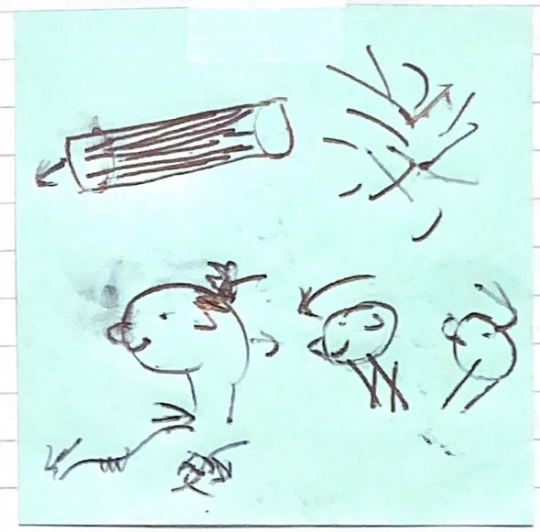
• Research topic:

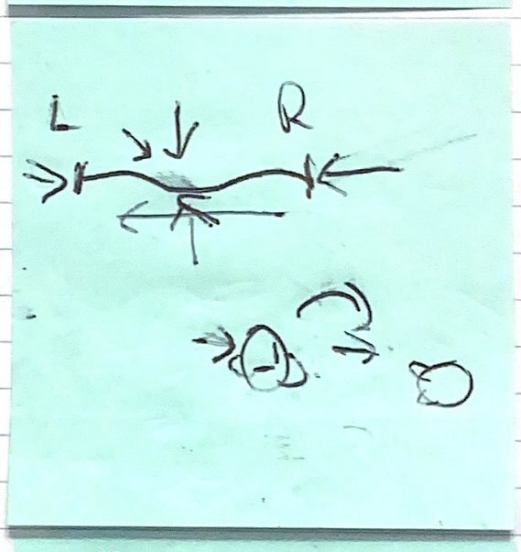
Changes in complement deposition and it's role in microglia-mediated synaptic pruning following repeated mild traumatic brain injury (rmTBI) in adolescent mice.

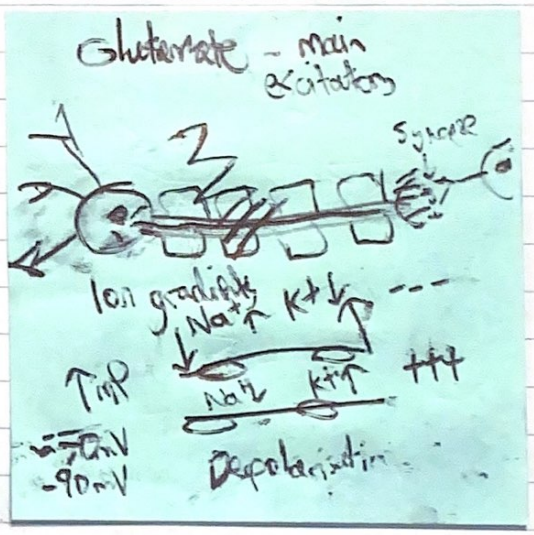
• Next meeting:
  • Staining techniques + experimentation
  • Logistics discussion
    • Research Paper: hypothesis, question, abstract, etc.

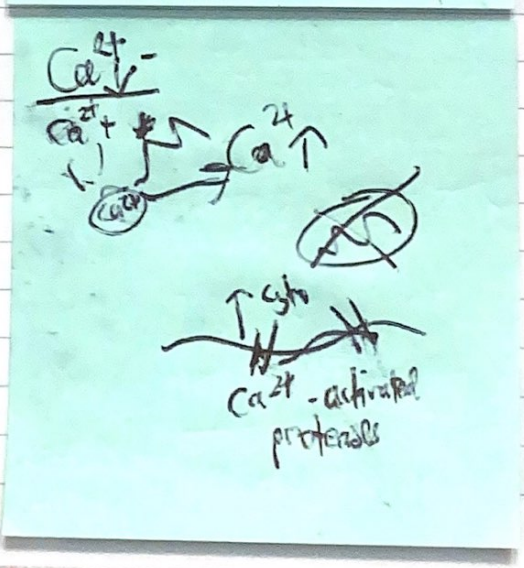
• Notes:


Corpus
Callosum
hippocampus
White Matter
Tract




L
R


Glutamate - main excitatory
Synapse
ion gradient
Na+
K+
Depolarization


Ca2+
Ca2+
Ca2+
Syn
Ca2+ - activated
proteases

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Triton
Detergent
Breaks down lipids

40 µm
15 µm
Cell surface protein
Intracellular
Transmembrane
Cell membrane
Receptors
Ion channels
- Signalling

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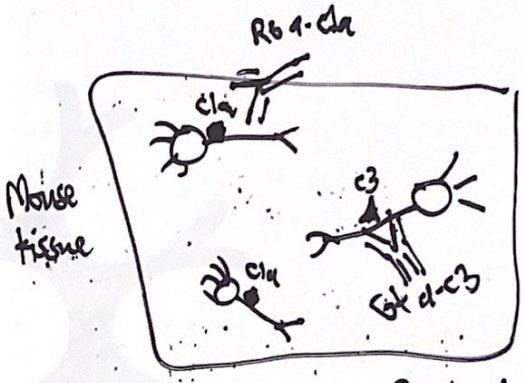
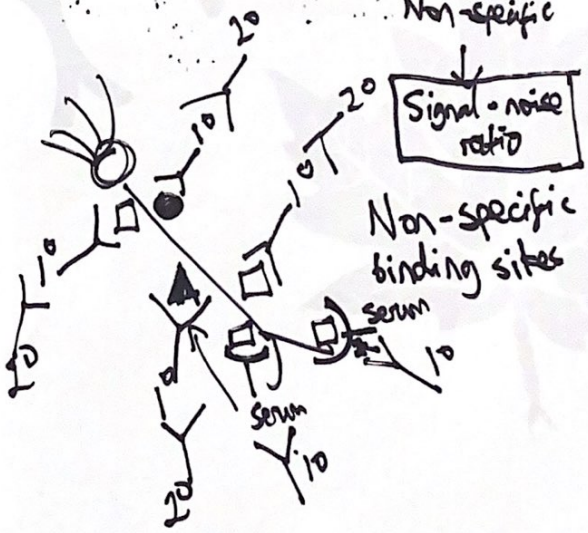


Binding to POI
Non-specific
Signal - noise ratio



Non-specific binding sites

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Test

Primary antibodies

Rabbit α-C1q } Complement protein
Goat α-C3 }

Secondary Ab

C1q 1:100 1:250 → Goat α-Rb AF568

C3 1:100 1:250 1:500 → Donkey α-Gt AF647

— — — — — —

Wash 3× 10 mins PBS
Phosphate Buffered Saline

Blocking step - PBS
10% Goat/Donkey serum
5% BSA (Bovine Serum Albumin)
Triton-X100
0.1%, 0.25%, 0.5%

Cold Fish skin gelatin
0.1%, 1%

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1° Ab incubation - 4°C, overnight
RT, 4 hrs

Ab solution
5% BSA → [Carrier Protein] - Carry Ab to whom the need to be.

FG
0.1%, 1%

Triton
0.1%, 0.25%, 0.5%

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30 October 2023 (class)

ASP Assessment/Mark Breakdown — 9 Months

- Organization/Communication (20%)
  - Monthly Mentor Evaluation (10%) ← 5 - Communication, 5 - Preparedness, 5 - Progress
  - 15-17 Biweekly Check-ins (10%)
- Logbook (20%)
  - Monthly Checks
- Oral Presentation (30%) ← Research Paper, Science Fair, Final
- Writing (30%): Scientific Paper divided over months, combine in May
  - Research Paper
  - Intro
  - Methods
  - Results/Conclusions
  - Final

Ethics Approval for Project? → (1) Ask Tom about ethics approval

What a class looks like:

- 5 min: Plan/Schedule/Communicate
- 20 min: Logbook + Reading
- 40 min: Data analysis
- 15 min: Talk with Dr. Garcia

Citations: X MLA

- AMA
- APA
- IEEE (Engineers)

Schedule B Form: Young Persons access UofC lab → (2) Ask Tom

UCID (online Training)

Meet with mentor 2~3 times a week (online or in-person)

1 September 2023 (class)

Communication - email mentor summary of meetings

- 1. ~~~ due date
  2. ~~~ wait for...
  3. ~~~

Time Management

- Schedule: ical or Google Calendar
- Task list with due dates

Logbook

1. Organization
2. Content
3. Schedule
   - September
   - October
   - [task each class]
     - Each day (90 min) must be reflected in logbook

Research Proposal (due mid-October)

NCBI PubMed: ask mentor of U of C ID to access non-free PMC articles

- Articles: review v.s. research
  - ↑ read (closest as possible to topic)
    - Read introduction (+ methodology)
- Ask mentor anything You can read
- Google unsure vocabulary
  - Use textbooks
- Use PubMed for citations
- Google Scholar

Research Paper:

- Broader aspect of field (general terminology)
  - ↓ Narrow down (+ controversies, other research, problems)
  - Research Question
- eg. Outline:
  1. Cancer statistics
  2. How Pancreatic Cancer develops

3. Role of P53 Protein Pathway
4. Studies of P53 + Cancer
5. Research Q: Role of p53 protein in Pancreatic cancer

Meetings with Mentors/Dr. Garcia
1. Schedule B Form
2. Biohazard Safety Course → How/when?
   → What's Next?
3. Research Paper/Lit Review
   - Suggest Papers to read
4. Plan Visits/Meetings

6 September 2023 (class)

Schedule/Organization/Communication (20%)

- Biweekly checks (/15) ← 10%, 6~7 1st term
- Mentor Evaluation (/15) ← 10%

Paperpile

- Cite as you write
- organize literature + references
- Use APA 7th edition

Biweekly Check / Individual Meeting Times

- September: 8, 20
- October: 2, 13, 25
- November: 6, 17, 29

Background Research

14 February 2024

Sex Differences in Traumatic Brain Injury: What we know and what we should know

- Authors: Gupte et al.
- Published: 15 November 2019
- Type: Review
- Summary:
  - Male bias in neuroscience research - have males than females recruited for clinical trials
  - Intro:
    - In General Adult population: males are 40% more likely to experience TBIs
    - In Athletics: Women suffer more TBIs than men of similar age playing same sport
    - In Military: higher in men but combat-related TBIs in women are rising
    - How are TBIs acquired? women - assault/domestic violence, men - work-related injuries, MVCs
    - Different pathophysiological responses to TBIs between men and women
    - No effective TBI therapies
      - Pre-clinical therapies fail to translate into human trials
        - 93~95% of pre-clinical studies fail to consider sex
        - Females absent in many pre-clinical studies (bc males have higher TBI incidence) → interferes with drug development
  - Analyzed 156 studies
  - Findings
    - Human Studies:
      - Worse outcomes in women than men (46%)
      - Better outcomes in women than men (26%)
      - No sex differences (18%) • Mixed results (9%)
      - When Stratified based on severity of injury:
        - Non-stratified studies: worse outcomes in women (40%)
        - Mild-moderate: worse outcomes in women (60%)
        - Moderate-severe: worse outcomes in women (34%)
      - When stratified based on number of patients:
        - 0~1000 patients: worse outcomes in women (53%)
        - 1000~10,000 patients: " (39%)
        - 10,000+: " (13%)

- Animal studies: Commonly used rats or mice
  - Females do worse (14%)
  - Females do better (44%)
  - No sex differences (14%)
  - Mixed results (28%)
  - Stratified based on intensity:
    - Mild: " (25%)
    - Moderate-severe: " (10%)
  - Stratified based on models:
    - CCI (Controlled Cortical Impact): " (0%)
    - CHI (Closed Head Injury): " (23%)
    - LFPI (Lateral Fluid percussion Injury): " (60%)
    - Other Models: " 0%
- Comparison:
  - Human studies → Women do worse
  - Animal Studies → Females do better
- Possible causes of sex Differences:
  - Injury response modulated by sex hormones
    - Women: cyclic production of estrogen and Progesterone until menopause
    - Men: declining production of testosterone over life time
    - Better outcomes for females may be due to neuroprotective effects of female sex hormones → better survival rates, BBB integrity, improved cortical blood flow, fewer sensorimotor deficits, less edema, smaller contusion volumes
    - Women in reproductive years (Post-Pubescent teenage girls) have lower mortality after TBIs than boys of similar age / Post-menopausal women have higher mortality compared with boys of similar age
    - Challenges to importance of sex hormones to TBI response
      - Neuroprotective effects of female sex hormones not observed in rats and piglets
      - College-aged women: menstrual cycle phase + oral contraceptives did not affect cognition / Postural stability after TBIs
      - Peri/post menstrual women have lower mortality than men (for 50+ years of age)
      - Progesterone fails in clinical trials as a TBI therapeutic drug

- Mitochondria
  - Mitochondria involved in: energy production, calcium homeostasis, free radical production, NT synthesis, neuron/glia apoptosis ← these processes might be performed differently in males and females
  - Under homeostatic / healthy conditions:
    - Females: higher expression of markers of neural bioenergetics (in gray + white matter)
    - Males: higher maximal respiration in cortical astrocytes
  - Under pathological / TBI conditions:
    - Under stress → males rely on carbohydrates / AA as fuel for energy production / females rely on fats
    - Differential Mitochondria response to stress:
      - Males: decreased respiration, increased autophagy, enhanced neuron death
      - Females: decreased bioenergetic markers expression
  - Effects of Mitochondrial Fusion / Fission:
    - mitochondrial Fusion: ↑ ATP production during high metabolic activity
    - Fission: facilitates transport of mitochondria to areas of ↑ E demand / removal of damaged mitochondria by autophagy
    - Males: tendency for mitochondria to undergo fission after TBI
    - Females: X studied yet

1 February 2024

Reviewing conclusions from 2020a and 2022 papers

2020a:

- TBI → secondary cascade (includes neuroinflammation)
  - activates resident microglia
  - Macrophage infiltrates BBB
  - Adaptive immune cells (T and B cells) recruited past BBB
    1. Neutrophil recruited (leukocytes)
    2. Chemokine gradient established
    3. Cause recruitment of monocytes
    4. Monocytes differentiate into macrophages: T cells, dendritic cells, NK cells
- Adolescent models
  - Markers of macrophage activation found in CSF
  - Neutrophils recruited in greater numbers compared to adults
  - No T-cell infiltration in adolescent/infant TBI
- Results:
  - Sex- and time-dependent infiltration of macrophages, reduction in microglia numbers, and infiltration of T-cells
  - Later macrophage recruitment in females compared to males
  - Fewer T cells recruited in females
  - Increased loss of microglia in males
  - Increased macrophage response in males
    - Due to: male (developing) brains have higher inflammatory mediators and reactive microglia
    - steroid hormones mediate changes in local and peripheral immune cells → males have higher number of reactive glia + stronger inflammatory responses after testosterone (steroid hormone) surge
    - males have greater tendency for more inflammatory pathways in developing brain → results in greater neuroinflammatory response after TBI
  - Loss of microglia in male mice (in MC + thalamus)
    - Contrasts many other studies: TBI induces significant microglial reactivity in animals + humans
    - Microglia may have migrated to other regions? ✗ (global reduction in microglia observed)

- Microglia may also have died or have been cleared from the brain after RmTBIs
  - Under inflammatory conditions, macrophage-derived macrophages can reduce microglia viability + modulate microglia inflammation/phagocytosis
    - In spinal cord injury
    - Infiltrating macrophages can change microglia numbers
- Effects of change in microglia:
  - Fewer mature neurons developed into/beyond adulthood → drives long-term behavioural deficits + altered neuroinflammatory responses
  - Changes in microglia + dendritic spine density seen in AD
  - After mTBI / RmTBI:
    - Spine density increase in PFC and nucleus acumbens
  - After CCI:
    - Spine density decrease in ipsi- and contralateral hemispheres

2022:

- Behavioural Changes:
  - Sexually dimorphic behavioural deficits:
    - Females have increased time-to-right (greater loss of consciousness)
      - More significant brainstem damage
    - Males have increased foot slips
      - More severe motor deficits
  - Adolescent-specific cognitive deficits (not in adults)
    - Memory deficits. (adolescents more susceptible to functional deficits)
      - May be caused by impairment of development of connections between PFC and limbic structures (eg. hippocampus, basolateral amygdala, and nucleus accumbens
    - Increased exploratory (risk-taking) behaviour
      - May be caused by development of connections between nucleus accumbens and basolateral amygdala
- Microglia and Spines
  - Male v.s. Female Brain Maturation:
    - Females: myelination occurs earlier / Males: sharper increase in myelination
    - Females: greater inter-hemispheric connectivity / Males: greater-intra-hemispheric connectivity

- Differences in gray matter maturation
  - Synaptic pruning causes gray matter reductions
- Spine density reductions pronounced in adolescents over adults (in AID)
- Synaptic loss → AD, PD, CTE, multiple sclerosis
- Developing PFC: can undergo synaptic remodeling after injury (potentially caused by microglia)
  - only seen later in adolescence (in mTBIs)
  - may be impaired in case of RmTBIs
  - May also be caused by neuronal degeneration
- Microglia
  - Neuroinflammatory cells of CNS
  - Acute period after injury: microglia have neuroprotective functions (clear cellular debris)
  - May have effect on synaptic pruning
  - Following TBI → increase in activated (pro-inflammatory) microglia → excessive phagocytose dendritic spines
    - Pruning: C3/C1q opsonize synapses ← C3R/CD11b receptors on microglia recognize tagged synapses
    - C3/C3R upregulated during adolescence + C1q upregulated after TBI/mTBI
    - RmTBIs may prime complement system → excessive pruning → early synapse loss
  - RmTBIs increase spine density in adolescent males
  - Males may be more dependent on complement-mediated (C3-C3R) synaptic pruning
  - Change in Iba1 expression OR inherent sex differences (male v.s. female) cause loss of microglia in males
    - Study by Guneykaya et al. 2018
    - Compare transcriptional profiles of male v.s. female mice

In males →
- Higher expression of Inpp5d gene (codes for SHIP-1 protein) and mTOR, both negative regulators of myeloid cell proliferation/survival
  +
  Decreased expression of Ercc1 gene, which is involved in DNA repair/recombination
- Male microglia may have limited proliferative/survival capability

ATP released by damaged neurons → ATP-mediated

In males →
- Higher expression of ionotropic P2X7R in male microglia
- P2X7R causes apoptosis through Ca^{2+}/K^{+} efflux

- Microglia numbers did not change in AID (Angular Insular Cortex)
  - But: phenotype, activation status, and phagocytic activity not observed
  - May be due to microglia priming/chronic activation following TBIs
    - these microglia poorly regulate synaptic pruning → pruning reduced

15/15 Tue

31 January 2024

Production of Complement Components by Cells of the immune system

Authors: Lubbers et al.

Type: Review

Published: 24 March 2017

- Complement production/secretion (extrahepatic - outside liver)
- Majority in liver
- Some components produced by a wide variety of cells/others only by specific cell types
- Intro
- Main complement functions: opsonization, chemotaxis, lysis + other functions in the adaptive immune system
- Facilitated by complement receptors (CR) for cleaved complement fragments
  - eg. CR1 (CD35): binds to C3b (complement receptor), competes with factor B for C3b binding + functions as a co-factor for factor I (complement inhibitor)
  - C3aR: receptor for C3a
  - Receptor for C1q uncertain

Classical Activation Pathways:

CP, LP, AP

↑ ↑ ↑

Classical Lectin Alternative

- Production
  - Mostly by hepatocytes (in liver) (C3, C4, MBL)
  - other: endothelial + epithelial cells
  - C1q, FP, factor D: only produced outside liver (immune cells)
  - Local production of complement regulates physiological processes

Due to the BBB, the CNS relies on microglia + astrocytes for immune response

- Extrahepatic production sites.
  1. Polymorphonuclear Leucocytes (PMNs): accumulate at infection sites → stimulated PMNs secrete C3 + proteases which activate C3
     - Express complement receptors: CR1, CR3, CR4, C3aR, C5aR
  2. Mast cells:
     - Responsible for inflammatory/allergic responses
     - Different subsets express C3 and C5 (can cleave C3 into C3a using local enzymes)
     - Human MC's can produce C3 and C1q
     - Complement receptors: CR1, CR4, CR2, CR3
  3. Monocytes:
     - Precursors of tissue macrophages + subset of dendritic cells
     - Produce: C1q, C3
     - Complement receptors: C3aR, C5aR1, CR1, CR3, CR4 (C3aR, C5aR1 ← C3a; CR1, CR3, CR4 ← C3b)

4. Macrophages

- Tissue-resident phagocytes: phagocytose opsonized pathogens (opsonized by C3b)
- produces: C1q, C3

human macrophages

- Complement receptors: C3aR, C5aR1, C5aR2, CR1, CR3, CR4

5. Dendritic Cells

- immature DC's → (signal) mature DC's → migrate to lymph node → activate T cells
- Produces: C1q (in immature state), C3
- Complement receptors: CR1, CR3, CR4, C3aR, C5aR1
- Differences in expression based on maturation level

6. Natural Killer Cells

- Respond to virally infected cells/tumour cells
- Not been studied whether C3 or C5 can be secreted
- complement receptors: C3aR, C5aR, C5aR2, CR3, CR4

7. B cells

- Produce antibodies (IgG and IgM antibodies can activate CP by binding C1q)
- Unclear which complement proteins are produced
- But:
  - Opsonization of antigen by C3b → stimulates B cells → lowered threshold of B cells to produce antibodies
- Complement receptors: CR1, CR4, CR2 (C3b)

8. T cells

- Subset of lymphocytes (stimulated by C3a and C5a)
- (upon activation by T-cell receptor) Produces: C3, C5
- Complement receptors: CR1, C3aR, C5aR1

- Conclusion
- Complement produced by liver hepatocytes does not reach all sites of body → local production/activation by cells is needed
- various immune cells can create local independent complement pathways

31 December 2023

More on Immunohistochemistry (2)

Choosing a Primary or Secondary Antibody

Source: Abcam

Summary:

- Polyclonal Ab: derived from different B cells ← different epitopes
- Monoclonal Ab: derived from identical B-cells produced from a parent clone ← single epitope
  - Polyclonal Ab are limited in supply, subject to variation between batches, and lack specificity
  - Monoclonal Ab have high specificity, and minimal variations between batches
- Recombinant vs. traditional Abs:
  - Recombinant Ab: Produced in vitro using synthetic genes → offers long-term secure supply
- Recombinant monoclonal or multiclonal (mixture of several monoclonal Ab → in cases where polyclonal Ab are required) Abs
  - long-term Ab supply + experimental reproducibility

Ab validation

- Some Abs have cross-reactivities with non-target proteins → use knockout validation (tests Ab specificity by testing it in a KO cell line which does not express the target protein → no signal should be produced if Ab is good)
- Choosing Primary Ab
  - Host species:
    - If indirect detection (using 2° Ab) is intended → choose a different host species as sample
    - Avoids cross-reactivity of 2° Ab (anti-immunoglobulin) with endogenous immunoglobulins in sample
      - C = antibody
      - originating from within organism
      - extent to which different antigens appear similar to the immune system → leads to background staining
  - Effect of carriers/preservatives
    - Abs usually stored in carriers/preservatives (eg. PBS, BSA, glycerol, sodium azide) → can hinder effective conjugation of labels, affect live-cell systems, and hinder with computer hardware
      - eg. BSA competes with 1° Ab to attach to label of interest → reduces conjugation efficiency
    - Choose Ab formulations without carriers or preservatives

- choosing 2° Ab
  - Detection methods:
    1. Direct: Antigen is detected by a 1° Ab conjugated to a label (no 2° Ab)
    2. Indirect: Antigen is detected by a conjugated 2° Ab that has been raised against the 1° Ab's host species and binds to the 1° Ab
       - Provides higher signal intensity (several 2° Abs can bind to a 1° Ab)
  - 2° Ab created by immunizing an animal with 1° Abs
    - Host species:
      - Must be different from 1° Ab
    - Naming:
      - eg. Donkey anti-rabbit
        - Donkey → 2° Ab host
        - rabbit → 1° Ab host / immunized against
  - Conjugates:
    - Fluorescent labels → emit light when excited by a specific wavelength
    - Enzymatic labels → produce a colored precipitate when combined with appropriate substrate
  - Avoiding Cross-Reactivity
    - Use pre-absorbed 2° Abs (used in multicolour experiments using several 1° Ab + 2° Ab)
      - preabsorption: extra-purification step which increases Ab specificity (reduces risk of reactivity between 2° Ab and endogenous immunoglobulins)
    - Use F(ab) and F(ab')2 Ab fragments instead of whole 1° and 2° Abs
      - Eliminates non-specific binding between Fc portions of Abs and Fc receptors on cells + penetrates tissues more effectively

Why does 2° Ab have to originate from a different host than the 1° Ab?
→ Abs from the same host will not recognize the other Ab and will thus not bind to it (2° Ab not reactive against 1° Ab)

Why does 1° Ab have to originate from a different host than the sample?
→ If the sample and the 1° Ab are from the same host, 2° Abs may erroneously bind to other antibodies in the tissue
→ Both reduce cross-reactivity and background staining

raised against Abs from a specific host species

31 December 2023

Polyclonal Ab: Produced by different B cells in a host animal
+ recognize multiple epitopes of a single antigen

Monoclonal Ab: Generated by identical immune cells (clones of a single parent cell)
+ recognize only a single epitope of an antigen

More on Immunohistochemistry

Introduction to Antibody production and purification

Source: ThermoFisher scientific

Summary:

- Antibodies are host proteins produced by the immune system in response to foreign molecules that enter the body
  - ↑ antigens
  - ↑ B cells
- Antibodies produced by B lymphocytes → circulate through blood
- Production mechanism for antibodies can be harnessed to detect molecules of interest in research

1. Antibody production
   - Preparation of target antigen → safe injection ("immunization") of antigens into laboratory/farm animals → high expression levels of antigen-specific antibodies in the serum → recovered from animal
     - Polyclonal Antibodies: recovered directly from serum (blood)
     - Monoclonal Antibodies: Produced by fusing antibody-secreting spleen cells from immunized mice with immortal myeloma cells to produce monoclonal hybridoma cell lines that express the specific antibody
   - special considerations:
     - Synthesize/Purify target antigen
     - Choose appropriate immunogenic carrier protein
     - Conjugate antigen and carrier protein to create immunogen
     - Immunize animals using appropriate schedules and adjuvants formulae
       - ↑ speeds up immune response
       - generates B-cells/T-cells (adaptive immune response) upon exposure to a host organism
     - Screen serum (or hybridomas) for antibody titer/isotypes
2. Ab Purification
   - Isolation of Ab from serum (Polyclonal Ab) or hybridoma cell (monoclonal Ab)
   - Methods:
     1. Crude: a subset of all the proteins in the serum (includes immunoglobulins)
     2. General: only a certain Ab class (no regard for specificity)
     3. Specific: only Abs which bind to a specific antigen
3. Ab characterization
   1. Screening: Identify which animals/hybridomas produce high levels of antigen-specific antibody

2. Titering: measure Ab concentration and functional assay titer (Ab concentration with respect to potency of Ab sample)
3. Isotyping: determine class and subclass of a monoclonal Ab
4. Ab Fragmentation
   - cleaving parts of Ab which are not necessary for binding Ab
5. Ab Labelling & Immobilization
   - Labelling: attaching molecules to the Ab to aid in detection (eg. fluorescent proteins
   - Immobilization: attaching Ab to chromatography media

30 December 2023

Animal models of traumatic brain injury

Authors: Xiong et al.

Published: February 2013

Type: Review

Summary:

- Many neuroprotective drugs developed using animal models of TBI → fail during phase II or phase III clinical trials
  - Secondary injury cascade after TBI offers window for therapeutic intervention
  - Failures may be due to pathophysiological heterogeneity amongst TBI patients, a lack of knowledge about the optimal dose, and confounding compounds given to the patient outside of the therapeutic window
  - Animal models: create a homogeneous type of injury (which may be responsible for the observed failures) but are still crucial to preclinical studies
    - New models need to be developed / existing models need to be improved
- Rodents most commonly used for TBI models
  - low cost, small size, and standardized outcome measurements
- Animal models focus on observing: biomechanical effects of TBIs + detrimental molecular cascades initiated by TBIs
- Types:
  1. Fluid percussion Injury (FPI): uses rapid injection of a fluid pulse into the epidural space to simulate a force
     - Highly Reproducible • Requires craniotomy + high mortality
     - Duplicates Concussion, Contusion, traumatic axonal injury, and hemorrhage caused in humans
  2. Controlled cortical Impact (CCI): uses an air/electromagnetically driven piston at a known distance/velocity
     - Highly reproducible • Requires craniotomy
     - Duplicates same conditions caused in humans as FPI
  3. Penetrating Ballistic-Like Brain Injury (PBBI): uses projectiles with high energy
     - Injury mechanism close to human TBI • Needs standardization
     - Duplicates same conditions caused in humans as FPI
  4. Weight-Drop Model. Drops weight directly onto exposed dura (Feeney) or onto a disk covered skull (Marmarou)

- Injury mechanism close to human TBI
- Requires craniotomy & high mortality rate
- Duplicates same conditions caused in humans as FPI

5. Blast Brain Injury: uses a detonation to generate a blast
   - Injury mechanism close to military TBI
   - Needs standardization
   - Duplicates same conditions as FPI
6. Mild TBI Models
   a. Modified Marmarou's weight-drop model
      - For lightly anesthetized mice
      - Does not require protective helmets
      - Seizures, paralysis, fractures, intracranial bleeding are rare
   b. Lateral FPI (LFPI)
      - single mild LFPIs → short-term behavioural neuropathological changes
      - Repeated mild LFPIs → Cumulative long-term behavioural impairments, neuroinflammation, neuron loss
      - Injury mechanism close to sports TBI

- Limitations of Current Animal Models:
  1. Physiological Differences
     - Differences exist between humans and non-human mammals in terms of: brain structure/function, brain geometry, craniospinal angle, gyral complexity, and white to gray matter ratio
     - Sex Differences: female sex hormones have a neuroprotective effect after TBIs / females have low comorbities + complications
     - TBI researchers do not measure other physiological variables before/after injury (eg. PCO_2, PO_2, pH, blood pressure, brain temperature)
  2. Injury Severity Assessment
     - Scoring systems for injury severity vary between laboratories and injury delivery devices (often custom-made) → makes it difficult for comparison between studies
     - Differences in timing of TBI detection/evaluation for TBIs at various severities
     - Need to find reliable biomarkers (in humans + animals) of TBI which are constant throughout all severities

- Improving Animal Models
  - Long-term v.s. short-term studies
    - Most TBI studies are short-term (hours to days after injury) → research 3 months to a year after the injury are needed
    - Therapeutic window for TBIs may extend longer than previously thought
    - Continuous treatment over several months (rather than a single early treatment post-injury) may be needed to aid in full recovery
  - Rodents can be used to model different subgroups of patients with TBI
    - TBIs produce cognitive deficits in rats similar to humans
  - TBI models with commorbities (having more than one illness at once)
    - TBI in clinical settings are heterogeneous injuries with a combination of hematomas, contusion, DAI, subarachnoid hemorrhage, hypoxia, and ischemia → need to be integrated into TBI models
      - CCI + hypoxia, hypertension
      - LFPI + hypoxia, hypertension
    - TBI models which incorporate multiple injuries (may significantly affect drug efficacy/toxicity)
      - Can also be used to identify key neurochemical mediators/mechanisms following repeated TBIs
    - TBI models which focus on the developing brain (more vulnerable to injury)
      - CCI, FPI, marmarou weight drop models used to study immature rodents and pigs
    - Greater consideration for age
      - Therapeutics which are effective in young animals may have no effect/even worsen outcome in older animals

27 December 2023

More statistics

- Standard Error of the Mean (SEM)
  - Standard error of the mean = standard deviation of the sampling distribution of the sample mean ($\sigma_{\bar{x}}$)

$$\therefore \sigma_{\bar{x}}^2 = \frac{\sigma^2}{n} \rightarrow \sigma_{\bar{x}} = \frac{\sigma}{\sqrt{n}}$$

(σ^2: variance; σ: standard deviation of original population; n: sample size)

(variance is inversely proportional to n (sample size))

- SEM indicates how different a population mean is likely to be from the sample mean
- SEM can be decreased by increasing the sample size
- Standard deviation of population (σ):

$$\sigma = \sqrt{\frac{\sum (x_i - \mu)^2}{N}}$$

(x_i = individual x values; μ = population mean; N = number of data points)

- Estimated SEM
  - Use estimated standard deviation (use s to estimate σ)

$$\sigma \approx s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

- $$SEM \approx \frac{s}{\sqrt{n}} = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n(n-1)}}$$

25 December 2023

df for two-sample t-Test = $n_1 + n_2 - 2$

t-Test Practice

Q: Electrical stimulation results in a decrease in the amount of food consumed by rats

Stimulation: (1)	No stimulation: (2)
12	8
7	7
3	4
11	14
8	6
5	7
14	12
7	5
9	5
10	8

Calculate $\bar{x}$ and S on calculator
Stat → edit → enter values for L1 or L2
Stat → calc → 1-Var Stats (1) → calculate

$\bar{x}_1 = 8.6$ $\bar{x}_2 = 7.6$

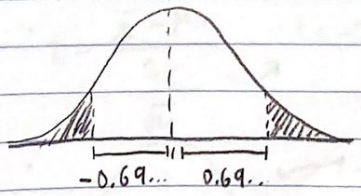
$S_1 = 3.3065...$ $S_2 = 3.1692...$

$S_1^2 = 10.9332...$ $S_2^2 = 10.0443...$

Two-sample Two-tailed:
$H_0: \mu_1 = \mu_2$ $H_a: \mu_1 \neq \mu_2$
$\alpha = 0.05$ (95% CI)

$$t = \frac{8.6 - 7.6}{\sqrt{\frac{10.93...}{10} + \frac{10.04...}{10}}}$$

$= 0.6904...$



Two-sample One-tailed:
$H_0: \mu_1 = \mu_2$ $H_a: \mu_1 > \mu_2$
$\alpha = 0.05$ (95% CI)
$t =$ "

Find P-value:

1. Use Calculator (t-Test)
   - 2-SampTTest → Pooled (X)
   - Results: $t = 0.6904...$, $p = 0.4987...$
   - $p > \alpha$ → cannot reject H_0 (no significance)
2. Use t-table
   - $t = 0.6904...$ · df: $10 - 1 = 9$ · $\alpha = 0.05$

- $0.05 \leq P < 1.00$ → inconclusive

3, use calculator (tcdf)

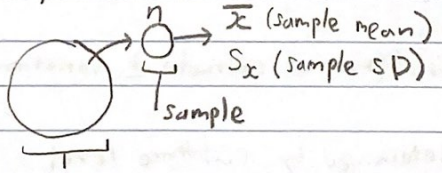
- $t = 0.6904\ldots$ $df = 9$
- $P(|t| \geq 0.6904\ldots) = 2\,\text{tcdf}(-1\times10^{99}, -0.6904, 9)$
  $= 0.5073\ldots$
- $P > \alpha$ → Cannot reject H_0 (no significance)

Sample: $\bar{x}$, s
Population: μ, σ

25 December 2023

t-Tests (continued)

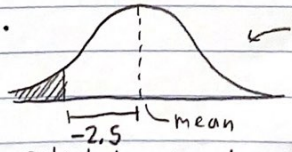
- Conditions for performing a t-Test about a mean
  1. Samples are random
  2. Samples are independent of each other
     ↳ use replacement or $n \leq 10\%$ of population
  3. Normal distribution (Sample distribution is roughly normal about the mean)
     ↳ parent population is normal or sample size (n) ≥ 30
     or distribution is normal + no outliers (from central limit theorem)
- Hypotheses for t-Tests:
  - $\bar{x}$ = sample mean
    μ = population mean
  - H_0 ← null hypothesis
    H_a ← alternative hypothesis
  - t-Test about a mean
    - H_0: $\mu = 450$
      H_a: $\mu \neq 450$
- When to use a t-Test
  - z-test ← for proportions
  - t-test ← for means
    - H_0: $\mu = \mu_0$ $H_a = \mu \neq \mu_0$



$$\rightarrow t = \frac{\bar{x} - \mu_0}{\frac{s_x}{\sqrt{n}}}$$

μ_0: assumed mean from null hypothesis
$\frac{s_x}{\sqrt{n}}$: estimated standard deviation of the sampling distribution of the sample mean

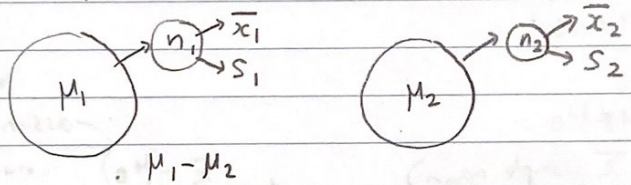
- Example 1: one-sample one-tailed t-test
  - H_0: $\mu = 5$ H_a: $\mu < 5$ $n = 25$ $\bar{x} = 4$ $s = 2$
  - $t = \frac{\bar{x} - \mu_0}{\frac{s_x}{\sqrt{n}}}$ → $t = -2.5$



  - Calculate p-value ← probability of a sample having a value -2.5 below mean
    - use TI-84: tcdf → lower bound: -1×10^{99} upper bound: -2.5 df: 25 − 1 = 24
    - tcdf $(-1 \times 10^{99}, -2.5, 24)$ =
  - use t-table: find t-value on table + use df to find p-value
  - p-value < α ← significance level → reject null hypothesis/accept alternative hypothesis

- Example: one-sample two-tailed t-test
  - H_0: $\mu = 530$ mL H_a: $\mu \neq 530$ mL
  $\bar{x} = 528$ mL $s = 4$ mL $t = -2.236$ P-value: 0.038 $\alpha = 0.05$ (95% CI)
  P-value $> \alpha$
  - Fail to reject H_0

- t-Test for a difference of two means
  - Conditions:
    1. Random
    2. Normal
       ↑ $n_1 \geq 30$ and $n_2 \geq 30$
    3. Independence
       ↳ $n_1 \leq 10\%$ of pop and $n_2 \leq 10\%$ of pop



$\mu_1 - \mu_2$

  - Construct a confidence interval (mean of estimate ± variation in estimate)
  - $(\bar{x}_1 - \bar{x}_2) \pm z \cdot \sigma_{\bar{x}_1 - \bar{x}_2}$
    - z: determined by confidence level
    - $\sigma_{\bar{x}_1 - \bar{x}_2}$: SD of the sampling distribution of the difference between the sample means
  - Find $\sigma_{\bar{x}_1 - \bar{x}_2}$:
  - $\sigma^2_{\bar{x}_1 - \bar{x}_2} = \sigma^2_{\bar{x}_1} + \sigma^2_{\bar{x}_2}$
    $= \frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}$ ← unknown

estimate → $\approx \frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}$

$\sigma_{\bar{x}_1 - \bar{x}_2} \approx \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$

- $(\bar{x}_1 - \bar{x}_2) \pm t \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$
  ↑ works better than z when estimating $\sigma_{\bar{x}_1 - \bar{x}_2}$

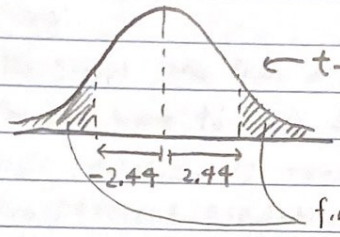
Conservative degrees of freedom
- $df = (\text{Smaller of } n_1 \text{ or } n_2) - 1$

α (significance level) ← probability that event could have occurred by chance (no statistical significance)

- Hypotheses:
  - H_0: $\mu_{difference} = 0$
  - H_a: $\mu_{difference} \neq 0$ or > 0 or < 0
- Example: two-sample two-tailed t-test
  - $\alpha = 0.05$ $H_0: \mu_A = \mu_B$ $H_a: \mu_A \neq \mu_B$ $\bar{x}_A = 1.3m$ $\bar{x}_B = 1.6m$
    $s_A = 0.5m$ $s_B = 0.3m$ $n_A = 22$ $n_B = 24$

$$t = \frac{\bar{x}_A - \bar{x}_B}{\sqrt{\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}}} \rightarrow t = \pm 2.44$$

$df = 22 - 1 = 21$



find probability of sample having a value 2.44 above or below mean:

Probability ~~(p-value)~~ $\rightarrow P(|t| \geq 2.44) \approx 2\,tcdf(-1 \times 10^{99}, -2.44, 21)$

$\approx 0.024 = $ P-value

P-value $< \alpha \rightarrow$ reject H_0 / accept H_a

$0.024 < 0.05$

24 December 2023

Student's t-Test

Intro to t-Tests

Distribution symmetric about mean ("bell curve")

- t-Test: statistical test used to compare means of two groups for statistically significant difference
  - only for two groups (more than two groups → use ANOVAs)
  - Parametric Test → assumes that:
    - Data is independent
    - Data has normal distribution
    - Data has similar amount of variance within each group
- Types:
  - options:
    1. Do groups come from a single population or two different populations?
    2. Do you want to test difference in a specific direction?

→ 1. Single population (eg. measure before/after treatment) → paired t-Test
2. Two different populations (e.g. two different species) → two-sample/Independent Test

One group compared against a standard value (eg. compare acidity of a liquid to pH 7/neutral) → one-sample t-Test

→ 2. want to know if populations are different from each other → two-tailed t-Test
want to know if one population mean is greater/less than that of the other → one-tailed t-Test

- Formula:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)}}$$

ratio of difference in group means

Standard error of both groups

SEM (Difference of population mean from sample mean)

- t = t-value $\bar{x}_1, \bar{x}_2$ = means of each group
  s^2 = sample variance (spread between numbers)
  s = standard deviation (spread of numbers from mean)
  n_1, n_2 = number of observations in each group
- Large t-value → difference between group means is greater than pooled standard error of the mean (significant difference)
- Compare t-value to critical value → calculate p-value
  use degrees of freedom
  + 95% confidence interval

Example:

Q: older and younger adults' scores on life satisfaction test shown below → is there a statistical difference between two groups?

Older Adults: (1)	Younger Adults: (2)
45	34
38	22
52	15
48	27
25	37
39	41
51	24
46	19
55	26
46	36

Null hypothesis: no difference in test scores

Alt hypothesis: difference in test scores

Use: Two-sample two-tailed t-Test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

$\bar{x}_1 = 44.5$ $\quad n_1 = n_2 = 10$

$\bar{x}_2 = 28.1$ $\quad S_1 = 8.6826...$ (SD) $\quad S_2 = 8.5433...$

$S_1^2 = 75.3888...$ (var) $\quad S_2^2 = 72.9888...$

$df = 20 - 2 = 18$ $\quad$ Confidence interval = 0.95 $\quad$ Critical value = 2.1013

↳ p-value = 0.05

$$t = \frac{44.5 + 28.1}{\sqrt{\frac{75.3}{10} + \frac{72.9}{10}}}$$

$= 4.257...$

t-Test > critical value → there is significant difference between test scores

$\bar{x}_1 > \bar{x}_2$ → older adults have higher life satisfaction

Degrees of Freedom: maximum number of logically independent values

- df = Number of items within data sample − 1

- Mechanism triggering complement activation is unclear
  - synaptosis (apoptosis of synapse without neuron death)
    - Exposure to phosphatidyl serine / other signals
    - A lack of CD47 at synapses
    - Hypoenergetics
  - overexpression of Crry (complement inhibitor in mice) → reduced synaptic pruning
- Neuroinflammation in brain + Therapeutic Potential
  - C3a/C5a → inflammation/activation of cells with C3aR/C5aR1 receptors → chemotaxis + cytokine production
  - Many diseases involve CNS-produced complement (some involve peripherally-produced complement)
  - AD
    - Complement system activated by fibrillar Aβ + hyperphosphorylated tau
    - C1q, C3 and C4 co-localize with amyloid plaques
    - complement plays a role in excessive synaptic loss (in both pre-plaque and abundant-plaque stages)
    - Complement activation → C5a generation → binds to C5aR1 → neuroinflammation
      - C5aR1 inhibition → ↓ memory loss, prevent neuron loss, ↓ amyloid pathology, ↓ glial reactivity, ↓ synaptic/cognitive deficits
  - TBI
    - Increased levels of C1q, C3b, C3d, and MAC in brain/CSF
      - Decreased levels of regulatory proteins CR1, CD59, C4BP in plasma astrocyte-derived exosomes
    - C3 is commonly targeted in therapeutic approaches
      - eg. chimeric composed of CR1g to target C3b and CD59 to suppress MAC-mediated injury
      - others: C6 inhibition → block MAC synthesis / C5 inhibition → C5b blocked → block MAC synthesis
    - Inhibiting alternative pathway using CR2-fH → improvement in cognition
      - Alternative pathway plays major role in TBI

23 November 2023

The role of the complement system in traumatic brain injuries: a review

Authors: Hammad et al.

Published: 22 January 2018

Type: Review

Summary:

- secondary cascade of TBIs cause irreversible damage
  - Complement system plays a major role in inflammatory reaction of secondary cascade
    - Can have deleterious effects + play role in neurogenesis/plasticity after injury
  - Role of specific complement pathways in TBI + role of complement in post-TBI repair + therapeutic potential of targeting complement
- Inflammatory Response to TBIs
  - M1 microglia → pro-inflammatory mediator
  - M2 microglia → anti-inflammatory mediator
    - M1 microglia: cell debris/foreign antigen clearance but can also damage healthy cells + exacerbate inflammation
    - M2 microglia: cellular survival/tissue repair + promote neurite growth
  - Can be activated by complement proteins
    - C1q causes shift to M1 microglia
    - M1 microglia → release inflammatory cytokines → astrocyte activation → glial scars → block axonal regeneration/growth
  - BBB compromised by initial injury → leukocyte infiltration across BBB
- other TBI-related injuries
  - Na^+-K^+ pump disrupted → depolarization of neuron → glutamate release → excitotoxic neuron death (by rise in calcium levels → activate enzymes + free radical generation)
- Complement System
  - Part of innate immune system (protect body from antigens)
  - Made of 30 proteins + zymogens (inactive enzyme precursors)
  - Complement cascade activation pathways: classical, lectin, alternative
    - classical: C1q binds to IgG/IgM antibodies → C1r and C1s bind to C1q (forms C1qrs complex) → C1s cleaves C4 to C4a/C4b + C2 to C2a/C2b → C2b + C4b = C4bC2b (acts as C3 convertase) → C3 convertase cleaves C3 to C3a/C3b (C3a = anaphylatoxin + causes acute inflammatory response to injury)

- Lectin: generates C3 convertase (C4bC2b)
- Alternative: generates C3 convertase (C3bBb)
- C3 convertases form C5 convertases → cleaves C5 to C5a/C5b
  - C5b → MAC synthesis → lyse non-nucleated cells
  - C5a → (Pro-inflammatory molecule) chemokines, cytokines, ROS synthesis
- Also regulates T cell / B cell activation
- Activation regulation:
  1. Decay accelerating factor (DAF): breaks down C3/C5 convertases → interferes with MAC formation
  2. Serum factor I / membrane cofactor protein (MCP): cleaves C3b to iC3b → inactivates C3 convertase
  3. Factor H: breaks down C3b
- In Healthy CNS (homeostatic brain)
  - Clears cellular debris / apoptosing cells, clears Aβ plaques (opsonization for microglia), protects from infection/inflammation
  - mice with absent C1q / C3 → more susceptible to infections
  - Infection/inflammation → upregulation of complement mRNA expression
  - synaptic pruning: C1q/C3 (synthesized by astrocytes) opsonize synapses
- In CNS Injury (Particularly in Secondary injury)

Complement expression now in healthy CNS

- TBI → BBB breakdown → influx of proteins (including complement) + complement-activating immune cells
  - C1q, C3b, C3d, + MAC levels elevated after TBI (in penumbral regions)
  - C1q, C3, fB, MAC levels increased after TBI (in CSF)

Clinical tests

- Applicability of mice TBI models on humans
  - Models: intracerebral hemorrhage (ICH), cryoinjury, Controlled cortical impact (CCI), standardized weight drop
  - Weight-drop model: mimics mechanical injury successfully but are not reproducible/reliable
  - CCI: more variables can be controlled → more reproducible + applicable on humans
- C3 convertase suppressed or C3 null mice: reduced neutrophil infiltration + reduced leukocyte infiltration, microglia activation, edema build up (after a TBI)
- Cry protein overexpression → reduced tissue loss + improved neurological outcomes

C5a receptor
C3a, C5a
to investigate role of anaphylatoxins in injury
- Interfering with C5a function / introducing C5aR: reduces damage after TBI, reduction in leukocyte infiltration + edemas
- Interfering with MAC formation: reduces neuronal death, microglia activation, and neurological deficits

- Role of different complement pathways in injury
  - Alternative pathway inhibited: reduced neuronal loss, upregulation of anti-apoptotic proteins/downregulation of pro-apoptotic proteins, reduced inflammatory response, upregulation of neuroprotection genes
  - Proteins involved in classical/lectin pathways inhibited: decreased brain tissue damage, reduced motor deficits, reduced contusion size
- Complement system in CNS repair:
  - Neurogenesis
    - occurs in subgranular zone (SGZ) of dentate gyrus (DG) of hippocampus + subventricular zone (SVZ)
    - C3a/C3aR play a role in neurogenesis
    - C3 null mice: reduced neurogenesis post-TBI (compared to null mice) in SVZ
  - Neuroprotection
    - C1q-exposed neurons: greater survivability + more neurites
    - C5: protects neurons against death by excitotoxicity
    - C5a: reduces apoptotic cell death
    - C3a:
    - MAC (at lower concentrations): inhibits oligodendrocyte apoptosis → reduces demyelination after TBI + oligodendrocyte proliferation → enhanced axonal myelination
    - C5: reduced wallerian degeneration + reduced gliosis
      ↑ result of remyelination
- Clinical Applications
  - TBI treatment can target Complement System for downregulation (insert therapeutics in complement pathway)
  - Future research:
    - Determine which pharmacological agents are most appropriate
    - Which pathways they must be administered at
    - When after initial injury to administer them.

21 November 2023

From development to dysfunction: Microglia and the complement cascade in CNS homeostasis

Authors: Zabel & Kirsch

Published: 16 Feb 2013

Type: Review

Summary:

- Neurogenesis/Synaptic regulation: neurons, microglia, + complement proteins involved
  - Recedes during adolescence but returns in old age/after injury or disease (eg. Alzheimer's)
- Even with an intact BBB, neurons, astrocytes, and microglia (innate immune system) are active (neuroimmune system)
  - Microglia regulate synapses growth
  - Mechanisms of microglia-mediated synaptic pruning may cause disease/pathological alteration of synapses if left unchecked

1. Microglia
   - Function (normally): phagocytosis of foreign pathogens
   - other roles:
     - phagocytosis of damaged/nonfunctioning synapses to allow tissue regeneration after an injury (eg. inflammatory stimulus)
       - During mice brain development: microglia increase + distribute themselves uniformly in white/grey matter (3 Plate white matter more in human brains)

2. Synapses/circuit Development
   - During nervous system development: excessive number of neuronal connections produced by accident (phagocytosed by ramified microglia)
     - Amoeboid microglia enter embryonic brain and become ramified (specialize) → phagocytose weak synapses
   - Decreased synaptic pruning → pruning of inactive synapses + strongest survive

3. Microglia recognition of synapses
   - Neuron-to-microglia signalling: ↙ controls recruitment of microglia
     - mediated by fractalkine (FKN) / CX3CL1 (chemokines)
       - Microglia contain CX3CL1 receptor: CX3CR1
   - Complement in microglia-mediated synaptic remodeling
     - MHC I and complement play role in synaptic Pruning
     - C1q and C3
       - Astrocyte mediated activation of C1q → activation of C3b → C3b

C1q: initiates complement activation
C3: enhances process ↑

deposits on neurites (tagged for elimination)

- No C1q or C3 → increased synaptic connectivity + epileptiform activity
- C1q opsonization mediated by complement receptor 1 (CR1) or C1q Receptor (C1qRP)
- C3b likely plays a greater role
- Alternate C1q-mediated synaptic pruning pathway: C1q binds to neuronal Pentraxins → destabilize dendritic spine receptors → decrease in communication between pre- and post-synapses → synaptic weakening
- Microglia have phagocytic receptors
- Complement Receptor 3 (CR3/CD11b) plays a major role in synaptic pruning (CR3 → C3b)
- Microglia and complement-mediated synaptic remodeling:

A. Microglia recognize active synapses through neurotransmitter-mediated signaling and avoid them + active synapses experience high calcium influxes → repress transcription of complement factors (eg. C1q)

B. C1q or FKN expressed by neurites set for destruction → microglia recognize them through receptors (CR3 and CX3CR1) → phagocytosis
- C1q expression cleaves C3 into C3b → deposits on target synapse (tags for phagocytosis)

C. Microglia also respond to targeted synapses by expressing pro-inflammatory molecules → cause neurons to express complement factors or FKN

- Neurodegeneration Pathways are similar to those involved in neurogenesis + synaptic pruning
- Microglia start re-expressing complement receptors at start of neurodegeneration
- Microglia also bind/clear amyloid in AD brains
- C1q in AD
- Localized in neuritic plaques (amyloid β → causes AD) + astrocytes/microglia + neurons
  ↳ suggests neuronal synthesis of complement increases in AD brain
- Lack of C1q → increase in C3 levels
- Microglia have more robust activation + increase intracellular calcium + release proinflammatory mediators when stimulated by C1q
- Does not increase Aβ uptake/ decreases Aβ uptake by C1q → causes Aβ accumulation

- Aged/diseased AD neurons increase C1q expression → C1q deposits on neurons → possibly cause microglial migration + phagocytosis of neurons/dendrites → cognitive decline

- C3b in AD
  - C3: joins all three complement pathways
    - cleaved into C3b (acts as an opsonin) + C3a
      - C3b further cleaved into other fragments
      - C3b deposited on AD-affected neurons (allow phagocytosis by microglia)
  - C3 activation may NOT be harmful
    - C3 activation suppression → increase in Aβ deposition
      → neuron loss
      → alter microglia phenotype (↑ anti-inflammatory cytokines)
    - C3 may be responsible for Aβ opsonization + removal.
  - C3 activation may be harmful
    - C3b primes microglia → respond vigorously upon second stimulus
    - C3b accumulation → bind to microglial CR3 → second stimulus causes myelin phagocytosis + proinflammatory cytokine release → MAC → cellular damage
    - AD patients show increased CR3 ↔ C3b + Aβ binding
      - C3b-bound microglia → ramified phenotype

AD/Alzheimer's Disease; caused by beta-amyloid plaques and neurofibrillary tangles (created from tau proteins)

15 November 2023

Complement and Microglia Mediate Early Synapse Loss in Alzheimer Mouse Models

Authors: Hong et al.

Published: 31 March 2016

Type: Research

Summary:

- Microglial complement in AD → mediate synapse loss in early AD, + neuroinflammation in late AD
  - C1q increases (C1q initiates classical complement cascade pathway) ← receptor
  - Inhibition of C1q, C3, or CR3 (on microglia) reduces number of phagocytotic microglia + reduces early synapse loss
    - C1q needed for toxic effects of soluble β-amyloid (Aβ) oligomers on synapses + hippocampal long-term potentiation (LTP)
    - Microglia need CR3 receptors to prune synapses when exposed to soluble Aβ oligomers
- Development period: C1q + C3 mediate synaptic pruning by microglia
  - AD brain: this pruning pathway is activated
- Structured illuminated microscopy (SIM) on transgenic AD mice:
  - Significant synapse loss in hippocampus at 3~4 months old (before plaque formation)
  - C1q increase in hippocampus + frontal cortex (~1 month)
  - C1q increase is dependent on soluble Aβ levels (oligomeric Aβ/oAβ induces C1q deposition) ← oAβ
- oligomeric Aβ increase C1q and microglial phagocytic activity
- Complement is necessary for synapse loss and dysfunction in AD mice
- Microglia engulf synapses via CR3 upon oligomeric Aβ challenge
- conclusions:
  - → Region specific increase in microglia, C1q, and C3 in pre-plaque AD brains
  - → Microglia prune synapses when challenged by soluble oAβ (deletion of CR3 blocks this process)
  - → Inhibiting C1q, C3, and CR3 activity decreases synaptic loss/dysfunction

Changes in Complement Deposition and Its Role in Microglia-Mediated Synaptic Pruning Following Repeated Mild Traumatic Brain Injury in Adolescent Mice

INTRODUCTION:

A Traumatic Brain Injury, or TBI, is a type of brain injury caused by the application of a violent external force to the head. TBIs are extremely prevalent, often being caused by falls, contact sports, motor vehicle collisions, physical abuse, and armed combat (Mayo Clinic Staff, 2021). In the United States, there are as many as 2.8 million cases of TBI annually, and TBIs are responsible for approximately 30% of all injury-related fatalities (Faul et al., 2010; Taylor et al., 2017). Similarly, 500 out of every 100,000 Canadians suffer from a TBI annually (Langlois, 2004). This trend is repeated worldwide, where there are about 42 million cases of TBI every year (Gardner & Yaffe, 2015). The variety of potential causes and the high rates of incidence associated with TBIs identify it as an internationally significant healthcare concern which is deserving of extensive research.

The majority of TBIs are mild traumatic brain injuries (mTBIs), better known as concussions. Studies conducted by the Centers for Disease Control and Prevention have confirmed that around 75% of all TBIs are mTBIs, making them a critical area of interest in brain injury research (Gerberding & Binder, 2003). Symptoms of mTBIs include headaches, dizziness, blurry vision, ringing in ears, difficulty sleeping, loss of consciousness, nausea, and a variety of cognitive issues (*What Are Common Symptoms of Traumatic Brain Injury (TBI)?*, 2020). Symptoms usually resolve within three months but certain patients have been known to experience post-concussive syndrome (PCS), where symptoms persist beyond the regular period (Permenter et al., 2022). The pathophysiology of mTBIs consist of a primary injury cascade followed by a secondary injury cascade. Immediately upon injury, a primary injury cascade

causes diffuse axonal injury (DAI). The outer gray matter and inner gray matter of the brain have ~~a~~ different density and ~~orientation~~; consequently, when a force is applied, either matter moves at different speeds and causes the axons to tear, killing the neurons. Neuron death continues throughout the secondary injury cascade; axonal stretching induced by the ~~primary~~ ~~cascade~~ causes mechanoporation, or the creation of holes in the axon, allowing an influx of ions which triggers depolarization. This causes the pre-synaptic neuron to release an excessive amount of neurotransmitters, namely glutamate, to the post-synaptic neuron, which subsequently initiates an influx of calcium ions into the neuron. Calcium ions also activate microtubule-breaking proteins (microtubules comprise the axonal cytoskeleton), cause mitochondrial dysfunction, and release cytotoxic molecules, killing the neuron (Eyolfson, Khan, et al., 2020).

An overlooked fact regarding mTBIs is that adolescents and young adults aged 15 to 24 are amongst the most vulnerable populations. Adolescence is characterized by a period of peak neural reorganization, ~~which has the potential~~ making it a vulnerable period to injury to cause neurological disorders. Previous studies observing neuroanatomical changes during adolescence concluded that there was a decrease in gray matter and increase in white matter during ages 10 to 18. The gray matter loss was attributed to synaptic pruning, the destruction of excess synapses and neuropil (dendrites, dendritic spines, axon terminals) as the brain matures; the white matter gain was thought to be caused by axonal myelination, the encasing of axons by conductive myelin sheaths to increase the speed of signal transfer. There was also a reduction in electrical activity in ages 10 to 20, which was suspected to be caused by synaptic pruning in the cortices (Whitford et al., 2007). In particular, synaptic pruning is a critical component of neural maturation during infancy through adolescence. The destruction of excess or weak synapses allows for the strengthening of the remaining synapses. ~~Synapses are tagged by complement proteins C3 (converted to C3b using~~

C3 convertase) and C1q; this allows microglia, which contain C3 and C1q receptors, to bind to the synapses and phagocytose them. However, mTBIs have been shown to either decrease or increase synaptic pruning in adolescents. Pruning may decrease due to an increase in activation of amoeboid microglia which are less efficient at pruning. Inversely, pruning may increase as the expression of C3 complementary protein increases with injury (Eyolfson, Khan, et al., 2020).

Microglia are amongst the three main glial cells (the others being astrocytes and oligodendrocytes) whose function is to support neurons. Since immune cells are normally prevented from entering the brain by the blood-brain barrier (BBB), microglia serve as the resident immune cells of the brain instead. When microglia detect an infection, they trigger an inflammation and phagocytose the pathogens; they are also responsible for destroying malfunctioning proteins (Ozturk & Wu, 2022). Beyond participating in the brain's immune response, microglia have a wide variety of functions throughout the various stages of life. In the prenatal brain, microglia play a role in neurogenesis (neuron generation), phagocytosis of neuronal progenitors (neuronal precursors), axonal growth, and neuronal fasciculation (formation of neuron bundles). In postnatal stages to early adulthood, the main function of microglia shifts to synaptic pruning in order to support neural maturation. The neuroinflammation triggered by microglia after injuries is a hallmark of mTBIs. Upon experiencing a mTBI, microglia can either cause neural repair or exacerbate damage. When a brain experiences a mTBI, microglia respond by phagocytosing any cellular debris/cytotoxic molecules which are produced and initiating neural repair. Damaged neurons release damage-associated molecular patterns (DAMPs), which activate microglia from their ramified surveillance state to their activated amoeboid state. Microglia have a highly plastic nature and amoeboid microglia may either adopt pro-inflammatory (M1-like) phenotypes or anti-inflammatory (M2-like phenotypes). The

~~DAMPs and~~ amoeboid microglia ~~also weaken the BBB, making the brain vulnerable to foreign~~ immune cells, neutrophils, macrophages, T cells, and dendritic cells, cross the BBB and contribute to neuroinflammation (Eyolfson, Khan, et al., 2020).

mTBIs leave the brain vulnerable for uncertain periods of time, during which additional impacts can lead to repeated mild traumatic brain injuries (RmTBIs). RmTBIs exacerbate neural damage and are responsible for causing chronic neuroinflammation. They are especially a major concern amongst adolescents, with a study concluding that 19.5% of surveyed adolescents experienced at least one mTBI and 5.5% experienced RmTBIs (Veliz et al., 2017). Sustaining RmTBIs during adolescence has also been linked to increased risk of developing neurodegenerative disease later in life, the most common being chronic traumatic encephalopathy (CTE), Alzheimer's disease, and Parkinson's disease.

Previous research has identified RmTBIs amongst adolescents to be a common and potentially life-changing injury. However, there is currently a dearth of research concerning the pathophysiology of RmTBIs and how they affect adolescent populations. ~~A 2022 study by Eyolfson and colleagues investigated the sex- and age-dependent effects of RmTBIs on mice.~~ A similar study by Eyolfson and colleagues in 2020 investigated sex-dependent effects of RmTBIs on adolescent mice. Analyzing changes in the cortex, hippocampus, thalamus, and corpus callosum, they observed that RmTBIs caused sexually dimorphic behavioural deficits, induced ~~sexually dimorphic/time-dependent neuroinflammation, and only~~ reduced microglia density amongst male mice. ~~Males were discovered to have worse behavioural deficits than females; they also experienced microglia loss~~ (in cortex, thalamus, and corpus callosum) ~~and earlier immune cell infiltration/recruitment through the BBB, leading to amplified neuroinflammatory responses. In particular, the loss of microglia in the adolescent brain was predicted to decrease~~

* Delete here — Start after 2020 work was described

the number of mature neurons and increase the risk of developing neurological disorders later in life (Eyolfson, Carl, et al., 2020). Using these findings, the 2022 study conducted by Eyolfson and colleagues delivered either RmTBIs or sham injuries to adult or adolescent and female or male mice. Five injuries were delivered every 24 hours and a series of cognitive tests were performed after the injuries. Mice were subsequently euthanized and microglia/dendritic spine density were calculated in the motor cortex/agranular insular cortex. Eyolfson and colleagues concluded that RmTBIs induced sex- and age-dependent behavioural deficits and changes in microglia/dendritic spine density. Male mice experienced increased motor deficits while female mice experienced increased loss of consciousness. In corroboration with the 2020 study, male adolescent mice experienced a decrease in microglia density. Male adolescent mice also experienced a decrease in dendritic spine density in the motor cortex. These results suggest that a loss of microglia impairs synaptic pruning and leaves the brain vulnerable to neurodegenerative diseases later in life (Eyolfson et al., 2022). The studies conducted by Eyolfson and colleagues confirms the threat posed to cognitive function and neural development by RmTBIs amongst RmTBIs; they further identify adolescent males as being more vulnerable to RmTBIs than adolescent females.

Despite the advances in our understanding of adolescent RmTBIs made by Eyolfson and colleagues, the effect of RmTBIs on microglia function during adolescence remains unclear. In particular, the role of complement proteins in RmTBIs remains relatively unexplored and is deserving of additional research. As mentioned, complement proteins C3 and C1q tag excess synapses for pruning by microglia. RmTBIs are thought to increase the expression of complement proteins, causing excessive synaptic pruning and leading to the emergence of neurodegenerative disease later in life. The complement cascade is a critical component of the

immune system, but can also exacerbate neuroinflammation or cause neurodegenerative disease. Once the cascade is activated by the presence of pathogens, complement proteins are cleaved and deploy a variety of immune responses. For example, C3b proteins (cleaved C3 proteins) perform surface opsonization, where they tag pathogens for phagocytosis; C5b proteins (cleaved C5 proteins) initiate the assembly of C5b-9 complexes, or membrane attack complexes (MACs), which are capable of causing pathogen lysis. Other cleaved proteins, such as C3a (cleaved C3 proteins) and C5a (cleaved C5 proteins), can recruit pro-inflammatory cells and cause neuroinflammation. Thus, a failure of the strict regulatory mechanisms controlling complement cascades could lead to neuroinflammation and neural damage (Alexander et al., 2008). In order to better understand the effects of RmTBIs on complement protein expression, this study will investigate the changes in complement deposition and its role in microglia-mediated synaptic pruning following RmTBIs in adolescent mice. ~~In order to expand upon the work completed by Evolfson and colleagues,~~ this study will also focus on identifying the relationship between complement protein expression and behavioural deficits, microglia density, and spine density.

QUESTION:

How do repeated mild traumatic brain injuries (RmTBIs) affect complement protein expression in the motor cortex of male adolescent mice?

OBJECTIVES:

The main short-term objective of this study is to investigate changes in complement protein (C3 and C1q) expression in the motor cortex of male adolescent mice following RmTBIs and sham injuries. Due to the critical role complement proteins C3 and C1q play in

microglia-mediated synaptic pruning during neural maturation amongst adolescents, the study hopes to understand what function complement proteins serve in neural damage following RmTBIs. This study also hopes to make advances in the optimization of immunohistochemistry techniques to detect complement proteins. Immunohistochemistry (IHC) is a staining technique which utilizes the binding that occurs between specific antigens and antibodies to detect antigens in certain tissue (Magaki et al., 2019). Immunohistochemistry usually follows an indirect method which involves the injection of primary antibodies into a tissue; these primary antibodies bind to an antigen, or protein of interest. In order to detect the primary antibodies using a light microscope, secondary antibodies tagged with fluorescent proteins are subsequently injected into the tissue. This study will attempt to determine the effectiveness of immunohistochemistry in staining complement proteins within a tissue.

The long-term objectives of this study are to provide additional insight into the mechanisms through which RmTBIs induce downstream effects on behaviour, microglia density, and spine density. These factors have been determined by Eyolfson and colleagues to undergo negative sex-specific and/or age-specific changes in RmTBI-affected brains; this study seeks to further corroborate these conclusions and provide potentially valuable information regarding the role of complement protein in RmTBIs.

VARIABLES:

The independent variable of this experiment is whether male adolescent mice received RmTBI or sham treatment.

The dependent variable of this experiment is the level of complement protein expression in the motor cortex of adolescent mice.

The controlled variables/constants of this experiment are the speed of the projectile used in the RmTBI model (5m/s ± 0.2m/s), the head position in the RmTBI model (lateral impact model), the frequency of injury in the RmTBI model (5 injuries/24 hours), age/sex of the mice (adolescent/male), antibody concentration, and imaging parameters. The consistencies maintained in the RmTBI model minimize any variation in the intensity of injury. In particular, controlling the frequency of injury ensures that the mice are at specific periods of post-injury vulnerability when they receive successive injuries. Since research ~~conducted by Eyolfson and colleagues~~ already identified male adolescent mice as highly susceptible to neural damage caused by RmTBIs, sex and age will not be independent variables in this experiment and can be kept constant. Differences in primary or secondary antibody concentration can result in different levels of staining during immunohistochemistry. Antibodies are usually titrated to determine the optimal antibody concentration for staining. [Imaging of samples will be performed using confocal imaging on a ZEISS Celldiscoverer 7 microscope under constant parameters.]

The main confounding variables of this experiment are the anaesthesia of mice prior to injury, the stress level of mice, and natural variation between mice.

HYPOTHESIS:

If male adolescent mice either received repeated mild traumatic brain injuries (RmTBI) or sham injuries, then the mice which received RmTBIs will experience an increase in complement protein expression in their motor cortex in relation to the mice which received sham injuries.

[RmTBIs have been shown to cause neurological deficits by triggering ~~excessive~~ decreased microglia-mediated synaptic pruning; complement proteins allow microglia to identify synapses for pruning and an increase in their expression is therefore associated with RmTBIs.]

METHODOLOGY:

The RmTBI group and sham injury group will each be assigned eight to ten male adolescent mice. A lateral impact model (LIM) will be used to deliver RmTBIs and sham injuries. The author of this study will not be participating in any form of testing involving live mice, but the general RmTBI injury delivery procedure can be summarized as follows: mice will be anesthetized, after which a projectile will be fired at a speed of 5m/s ± 0.2m/s at their heads; a "helmet" covering the head of the mice will distribute the force laterally across the brain. The projectile will be fired five times over 24 hour intervals to simulate repeated injuries. Mice in the sham injury group will also be anesthetized but a projectile will not be fired against their heads.

Five days after the injury, the mice will be euthanized. Their brains will be removed and frozen at 4°C before being cut into 40μm-thick coronal slices (cut parallel to the y-axis) by cryosectioning; three coronal slices will be obtained from each mouse. Primary and secondary antibodies will then be inserted in order to stain the target complement proteins using immunohistochemistry. In order to perform a IHC analysis, the samples will be washed three times over 10 minute intervals using phosphate buffered saline (PBS). Then, blocking will be performed to block non-specific binding sites on proteins; this increases the "signal-to-noise" ratio by preventing the primary antibodies used in IHC from binding to non-target proteins (the "noise") and thus allowing them to bind in greater numbers to the protein of interest ("the signal"). An antibody solution will be created through a mixture of PBS, goat/donkey serum, 5% bovine serum albumin (BSA), cold fish skin gelatin, and triton X-100. Primary antibodies will be incubated at 4°C overnight in order to equilibrate the temperature between them and the sample. The antibody solution will act as carrier proteins, transferring the primary antibodies to their

target proteins; in particular, triton X-100 creates holes in the phospholipid membrane of cells and delivers antibodies to intracellular proteins. Rabbit α-C1q and goat α-C3 primary antibodies, which target complement proteins C1q and C3, respectively, will be used; the primary antibodies will bind to antigens (the complement proteins) at their variable regions. Following primary antibody labelling, samples will be washed using PBS again and labelled using secondary antibodies. The secondary antibodies will bind to the constant region of the primary antibodies and be tagged with fluorophores (fluorescent proteins); the fluorophores undergo shape change and emit light when excited by specific wavelengths of light, allowing for detection by microscopes. The sample will then be counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in order to illuminate the ~~labelled complement proteins~~. Afterwards, the samples will be mounted onto slides.

Fluorescence microscopy will be performed on the samples using a ZEISS Celldiscoverer 7 confocal microscope. Z-stacking will be used to image and measure the complement protein expression in the motor cortex.

SIGNIFICANCE:

Adolescent health is an incredibly important issue given the variety of critical ~~physical~~ and physiological changes that occur during this period of growth. ~~In particular~~, the brain undergoes significant neuroanatomical and neurophysiological changes during adolescence which have longlasting implications in the future. However, given statistical data and the high level of physical activity that usually occurs during this period, adolescents are especially vulnerable to traumatic brain injuries (TBIs), especially repeated traumatic brain injuries (RmTBIs). Past research has determined that adolescent males affected by RmTBIs experience

neurological deficits and are at high risk of developing neurodegenerative diseases later in life. Nevertheless, the research into the pathophysiology of RmTBIs amongst adolescents is relatively scarce. Microglia-mediated synaptic pruning is a hallmark of adolescent maturation in the brain which is suspected to play a major role in the development of neurological deficits post-injury. This process makes heavy use of complement proteins as tags for target synapses. By studying the changes in complement protein expression, this study attempts to better understand the pathophysiology of RmTBIs in adolescence. The results of this study will be invaluable in developing safety procedures to protect adolescents from the negative effects of RmTBIs.

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conjugated: tagged with a fluorescent molecule

- How to select a Secondary Antibody:
  - Using a secondary Ab > conjugated primary Ab (↑ conjugated)
    - Advantages:
      - Can choose conjugate no matter primary Ab
      - Enhance detection by localizing more conjugate at antigen
      - Conjugated Ab are more specialized/rare/costly
  - Consider:
    1. Host/Target species
       - Host: animal in which secondary Ab was generated
       - Host of secondary Ab should always be different from host of primary Ab
    2. Cross Reactivity/Specificity
       - Secondary Ab may bind to multiple targets (cross reactivity)
       - Secondary Ab must bind to correct target (specificity)
    3. Detection/purification method
       - Conjugated to correct fluorophore
    4. Additional Requirements for storage (placed in a certain solution)

Primary: Rb α-c19 + Gt α-C3

Secondary:

- Fluorophore (Yellow + far red emission): AF488, AF568, AF594, AF647 (Green, Orange, Red, Far-red; Alexa Fluor)
  - Yellow (550-599 nm): AF568
  - Far Red (680-729 nm): AF647
- Rb α-c19 ← Gt or Do α-Rb AF647
- Gt α-C3 ← Do or Rb α-Gt AF568

conjugated: tagged with a fluorescent molecule

- How to select a Secondary Antibody:
  - Using a secondary Ab > conjugated primary Ab > conjugate
    - Advantages: ↑ conjugated
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- Gt α-C3 ← Do or Rb α-Gt AF568

4 october 2023

Use of Confocal Microscopy in Comparative Studies of Vertebrate Morphology
Authors: Collazo et al.
Published: 29 April 2005
Type: Research

- Confocal Fluorescence Microscopy: collects light from only one plane of focus
  - Z-stack: collection of multiple focal planes to create 3D image
  - Steps: (along the z-axis)
    1. Labeling/mounting specimen
    2. Optimizing Image on Confocal
    3. Collecting/ analyzing confocal image data

1. Labeling Specimens: label specimen with fluorophores
2. Mounting specimens
3. optimizing image on Confocal
   - Varies based on microscope brand
   - Adjust z-section thickness: adjust pinhole size
   - Adjust image brightness:
     - Adjust laser power (low as possible → minimize photobleaching)
     - Adjust sensitivity of photo multiplier tube/PMT (detects emitted light from sample)
4. Collect Image data
   - Microscopes collect images at repeated intervals → z-series stacks
5. Analyze confocal data
   - often z-series stacks are projected down to one plane of focus so that all planes of focus are visible

Widefield v.s. Confocal fluorescence Microscopy

- Fluorescence Microscopy
  - fluorescent molecule excited by photon (at specific wavelengths/energy) (e^- excited) → when e^- returns to ground state (emission of energy less than the original photon)
    - ↑ energy, ↓ wavelength
  - Fluorescent microscopes use wavelengths to image fluorescent molecules (fluorophores)
  - Important to avoid photobleaching (exciting e^- too many times weakens emitted photons ← too weak to be detected)
- widefield:
  - fluorophores introduced to tag certain structures
  - Light emitted onto sample (excitation filter only allows specific wavelengths to pass through)
  - Sample emits energy → wavelength detected by microscope → imaging on computer
- Confocal:
  - Same procedure but:
    - Energy emitted by sample focused through a pinhole (eliminate background fluoresence)
    - Small portion of sample imaged at a time but at a higher resolution
  - More susceptible to photobleaching

- widefield v.s. Confocal Microscopy
- Light microscopy types

1. widefield:
   - Single light source illuminates sample → 2D image of whole sample
   - Light passes through out-of-focus structures in sample → blurred image
   - Still sufficient resolution

2. Confocal:
   - Laser beam scans sample at a set depth (laser beam focused through a pin hole → only in-focus light passes through)
   - Sharper 3D image
   - only captures images on in-focus plane

4 October 2023

The Development of Brain White Matter Microstructure
Authors: Catherine Lebel, Sean Deoni
Published: 3 January 2018
Type: Research

- Significant brain remodeling from infancy to early adulthood
- Using MRI techniques to observe microstructural changes in brain white matter during maturation
  - Infancy (ages 1~3): increased myelination + axonal packing (↑density)
  - Continued white matter maturation throughout childhood/adolescence (albeit at slower pace)
  - Regional variation in development:
    - Earlier maturation in central regions compared to peripheral regions + posterior corpus callosum compared to anterior CC
    - Sensory/Motor regions mature the earliest
    - Emotional/cognitive regions in frontal/temporal areas mature the latest

Immunohistochemistry (IHC)

1. Tissue collection + Perfusion
   - Preserve tissue to prevent breakdown
   - Perfusion: tissue rinsed free of blood (often using saline) in order to remove blood-derived antigens which may interfere with target antigens
2. Tissue Fixation
   - preservation of tissue in life-like state
   - most common fixative formaldehyde (formalin)
   - Embedded in paraffin wax for sectioning → called formalin-fixed paraffin-embedded (FFPE) tissue
3. Tissue Embedding
   - FFPE

     or
   - Sensitive samples (X chemical) fixation) are frozen and then cryosectioned
4. Sectioning / Mounting
   - Sectioned into slices as thin as 4 ~ 5 μm
   - sections dried overnight at room temperature
5. De-Paraffinization + Antigen Retrieval
   - paraffin removal (paraffin obscures target antigens); often uses xylene
   - Formaldehyde create methylene bridges that can mask antigen (epitope: antibody binding site on antigen) → Antigen retrieval: boil de-paraffinized sections in various buffers
6. Blocking Non-specific Sites
   - Antibodies can bind nonspecifically (weakly) to sites on non-antigen proteins → cause background staining
   - Samples incubated with a blocking buffer
7. Immunodetection:
   - Primary/secondary Ab diluted into a buffer (stabilize Abs, promote diffusion into sample, discourage non-specific binding)
   - Sample must be rinsed between steps to remove weakly-bound Ab (to non-specific sites) + unbound Ab
8. Counterstaining (Provide contrast to primary stain)
   - Often cell-structure specific (eg. DAPI → nucleus)

3 october 2023

Axon degeneration: Molecular mechanisms of a self-destruction Pathway

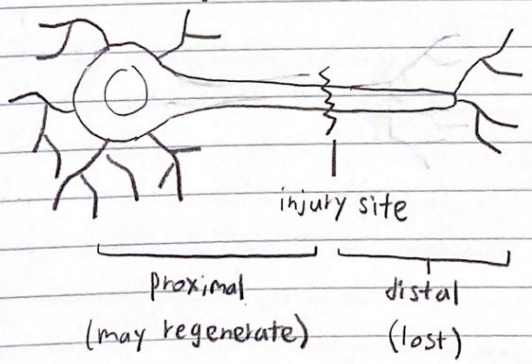
Authors: Wang et al.

Published: 9 January 2012

Type: Review

- Wallerian Axon Degeneration
  - Associated with many neurodegenerative conditions and traumatic injuries
    - Expression of Wallerian degeneration slow transgene/WldS → slows nerve degeneration
  - Proposed model
    - Damage to nerve leads to impaired expression of local axonal survival factors → increase in calcium levels inside → calcium-dependent cytoskeleton breakdown → breakdown of BBB → infiltration of reactive glial cells to remove axonal/myelin debris
  - Steps:

1. Axonal segments proximal (left) and distal (right) to injury site undergo acute axon degeneration (AAD)
   - Influx of Ca^{2+} into axons → activation of Ca^{2+}-dependent protease Caplain inside axons (triggers apoptosis)
2. Distal axon segment remains stable
3. Distal axon segment rapidly degenerates (cytoskeletal breakdown)
4. Increased glial influx
   - Clear axonal debris
   - Promote regeneration by proximal axon segments



1 October 2023

Lasting effects of general anesthetics on the brain in the young and elderly: "mixed picture" of neurotoxicity, neuroprotection, and cognitive impairment

Author: Wu et al.

Published: 11 March 2019

Type: Review

- False: general anesthetics (GAs) is reversible and the CNS can be reverted to its original state following removal of anesthetic
  - Long lasting/undesirable effects
- Anesthetics received during surgery associated with cognitive impairment in young/elderly populations
- GAs use receptor proteins to regulate neuronal activities / exert amnesic, analgesic, sedative, and immobilizing effects
  - Receptors: GABA receptor, NMDA receptor, etc.
  - Abundance of receptors may cause long-term cognitive dysfunction
- Anesthesia-induced developmental neurotoxicity
  - Important mechanism: neuroapoptosis → impairments in neuronal communication + faulty formation of neuronal circuitries

30 September 2023

Neurobiological and Systemic Effects of Chronic Stress

Author: Bruce S. McEwen

Published: 10 April 2017

Type: Review

- Brain performs primary response to stress
- Promotes adaptation (allostasis) → imbalance of neural circuitry affecting cognition, decision-making, anxiety, and mood
- Contributes to pathophysiology (allostatic load/overload) if dysregulated
- Highly plastic in response to stress
- Adaptations may be beneficial in short term (acute stress), but requires therapeutic intervention if it becomes chronic
- Sex differences in brain response to stress

- Stress: Good/Tolerable/Toxic

- Allostasis: achieving stability by activating physiological systems
  - physiological responses can promote pathophysiology if overused/unbalanced (results in allostatic load/overload)

- Effect of stress on brain:
  - Healthy brain → plastic (neural circuitry adapts to new situation + changes in gene expression)
  - Persistence of stress on unhealthy brain (excessive activation of excitatory amino acids) → irreversible damage → eventually leads to Alzheimer's
  - Adrenal steroid Receptors in Hippocampus:
    - Hippocampus (involved in episodic/spatial memory + mood regulation):
      - Stress + glucocorticoids cause dendritic shrinkage/spine loss
    - Amygdala (Basolateral Amygdalae/BLA)
      - Acute stress → increased spine density
      - Chronic stress → loss of spines/shrinkage of dendrites
    - Prefrontal Cortex (PFC)/medial Prefrontal Cortex (mPFC):
      - Chronic stress → debranching of neurons/shrinkage of dendrites
    - Orbitofrontal Cortex
      - chronic stress → expand dendrites
  - Key Role of Excitatory Amino Acids:
    - Excitatory Amino Acids (eg. glutamate): play key role in structural/functional changes in brain + excess causes damage/inflammation

- BLA, orbitofrontal cortex: dendrites expand

- Hippocampus:
  - chronic stress → shrinkage of dendrites in neurons
  - Acute stress → increased glutamate levels
- mPFC:
  - stress-induced NMDA-dependent dendritic remodeling

N-methyl-D-asparate

glutamate activity

- Excess glutamatergic activity after injuries → Permanent neuronal loss (exacerbated by glucorticoids)
  - Hippocampus: depressive-like behaviour, shrinkage of dendrites, suppression of neurogenesis
  - Related to aging / dementia
- Sex Differences
  - Male and females do not share the same pattern of neural remodelling
  - Hippocampus: dendrite remodeling does not occur in females after chronic stress, different cognitive consequences
  - mPFC: no dendritic remodeling in females
  - BLA: expansion of dendrites in females

30 September 2023

Commentary on Mild Traumatic Brain Injury Research Needs in General Population

Author: Emi Isaki

Published: July 2021

Type: Forum

- Statistics:
  - 2M Patients in US
  - 80%-90% are mTBIs
  - Many go unreported → actual number is higher
  - High-risk Populations: Soldiers, athletes
- Research has only focused on high-risk populations
  - But has improved: prevention measures, diagnosis, therapeutic treatments, reintegration of patients into society
  - Military: effect of overlay of mTBI from blast injuries and posttraumatic stress disorder from battle
  - Athletes: risk of RmTBIs and CTE (chronic traumatic encephalopathy)
- More research about mTBIs in general population needed
  - Lack of rigid guidelines for follow-up care amongst general mTBI patients
  - Education on mTBI risk
  - Need for standardized treatment procedures/consensus amongst professionals

24 September 2023

The Complement System in the Central Nervous system:
From Neurodevelopment to Neurodegeneration
Authors: Chen et al.
Published: February 2022
Type: Review

- Role of complement system in immunity: opsonization, cell lysis, inflammation
- Role of complement proteins in CNS (extends beyond immunity) is relatively unknown
  - Research therapeutic application of anti-complement strategies for neurodegenerative disease treatment
- function of Complement System in CNS:
  - phagocytose pathogens, aberrant proteins, cellular debris
- Complement proteins produced by neurons and glial cells (local production ← faster response)
- Complement system regulation malfunctions → neurodegenerative disease
- Complement system activation pathways:
  1. Classical: C1q binds to antigen-antibody complex → C1r + C1s activated → cleave C4+C2 into C4b + C2a
  2. Lectin: mannose binding lectin (MBL) binds to mannose → activates MBL-associated serine proteases (MASPs) → cleave C4 + C2 into C4b + C2a
     - C4b + C2a → forms C3 convertase → cleaves C3 into C3b/C3a
  3. Alternative: Spontaneous hydrolysis of C3 → forms C3 convertase (Produces C3b to amplify complement cascade)
     - 80-90% of complement cascades
- C3a → downstream activation (amplification of complement cascades)
- C3b → Phagocytosis
  → create C5 convertase (cleave C5 → C5a / C5b)
  → C5b → used to assemble MACs → cell lysis
- C3a/C5a → anaphylatoxins (induces inflammation)
- complement protein production in CNS:
  - C3: astrocytes
  - C1q: microglia
  - Microglia also synthesize complement protein receptors (CR3/CR4, C3aR/C5aR)
    - CR3/CR4 interact with C3b/C4b → phagocytosis (involved in neurodegenerative diseases)

- C3aR/C5aR: anaphylatoxin receptors (inflammatory response), tau/amyloid pathology in Alzheimer's, synaptic loss, cognitive dysfunction
- Microglia can also synthesize C3

- complement system and Neurodegenerative Diseases
  - Alzheimer's Disease (AD): most common
    - Caused by: β-amyloid plaques, tau aggregation, neuroinflammation, synaptic loss (result of microglia-mediated synaptic pruning)
    - C1q:
      - Active in frontal cortex + hippocampus
      - Increases when Aβ plaques and tau are present → C1q causes neuropathological changes in AD through microglia-dependent synaptic pruning
    - C3:
      - Increased levels in brain/CSF of AD patients
        - From astrocytes (can be produced by neurons)
      - when Aβ plaques and tau are present → CR3 recognizes C3 (+ its cleaved forms) → initiates microglial phagocytosis → synaptic loss (only produced by microglia)
      - C3 interacts with C3aR → disruptions in neuronal/dendritic morphology, aberrations in synaptic plasticity (neuronal C3aR)
        → alters expression of immune networks, mediates neuroinflammation, modulates amyloid/tau pathology (microglial C3aR)
      - C3 activation accelerates Aβ clearance
    + Multiple sclerosis
      Amyotrophic Lateral Sclerosis
      Parkinson's Disease
      Huntington's Disease
      Perioperative Neurocognitive Disorders
- Therapies Targeting Complement System
  - Anti-complement agents: inhibit convertase assembly/cleavage, MAC Formation
    - Agents targeting C5 most successful
  - Target: C1q inhibition
    - ANX005 (anti-C1q antibody): binds with C1q to inhibit classical pathway
  - Target: C3 inhibition
    - Intravenous Immunoglobulin (IVIg) → Not yet approved for safety/clinical trials
    - Compastatin

Research Proposal – Introduction outline

a. TBI Statistics

- US:
  - Up to 2.8M cases of TBI, causing ~30% of all injury-related deaths ✓
  - ~75% of TBIs are mTBIs
  - Total healthcare costs: ~22 B
  - Adolescents (15-24) among most vulnerable groups
- Canada:
  - 500 out of 100,000 suffer TBIs annually
    - 19.5% → concussion (mTBI)
    - 5.5% → RmTBI
- World wide:
  - 42 B cases of TBI annually · 80% are mTBIs

b. Post-Injury Symptomatology:

- Symptoms of mild TBI: Headaches, dizziness, blurred vision, ringing in ears, sleep problems, loss of consciousness, cognitive issues (memory, concentration, thinking), nausea ✓

c. Post-Concussive Syndrome (PCS): symptoms persisting for 3+ months

- only occurs in some patients (TBI symptoms usually resolve with 10-14 weeks)

+ mTBIs: also known as concussions

- Caused by a bump/blow/jolt to the head or a hit to the body causing the brain to move back and forth
- Falls, Sports (football, hockey, soccer), MVCs, Combat, physical abuse, repeated Concussions
- Higher prevalence amongst males
- Brain is vulnerable after mTBIs ✓
- Pathophysiology
  - Primary Injury Cascades: DAI
  - Secondary Injury Cascades:
    - Reduced cerebral blood flow (↓ glucose, oxygen in brain)
    - Mechanoporation → depolarization of neurons → uncontrolled NT release (glutamate) to post-synaptic neuron → calcium influx → neuronal death

d. RmTBIs increase risk of developing neurodegenerative diseases later in life (eg. CTE, AD, PD)

e. Adolescent Brain Development
- Gray matter loss ← synaptic pruning
- White matter increase ← axon myelination
- Decrease in electrical activity ← synaptic pruning

f. Synaptic Pruning: removal of excess synapses
- Steps:
  1. Excess/weak synapses tagged by complement proteins C3 → C3b & C1q
  2. Microglia (containing C3 & C1q receptors) bind to complement proteins and phagocytose synapses → strengthen remaining synapses
- Following RmTBI:
  - Pruning ↑: ramified microglia ↑ (more efficient at synaptic pruning)
  - Pruning ↓: C3 expression ↑

g. 2022 paper: mice with RmTBI v.s. sham (M v.s. F)
1. Behavioural Deficits:
   - Male mice: ↑ motor deficits
   (- Female mice: ↑ loss of consciousness)
2. Microglia (2021 paper)
   - Male Adolescent mice: ↓ microglia density in thalamus / cortex
3. Dendritic Spines
   - Male Adolescent Mice: ↑ spine density in motor cortex

h. I will investigate complement protein expression
- How expression changes in TBI
- Relation to behaviour deficits (1), microglia (2), and spine density (3)

22 September 2023

An Introduction to the Performance Immunohistochemistry
Authors: Magaki et. al.
Published: 1 January 2020
Type: Review

Immunohistochemistry (IHC): uses binding occurring between antigen (molecule triggering immune response) and antibody (molecule produced to bind to antigen and destroy it) to detect antigens in certain tissue
↑ aka immunoglobulin
- Common staining tool in anatomic pathology
- Used to determine cell type/organ of origin
- often used with formalin-fixed paraffin-embedded (FFPE) tissue
- steps:
  1. Antigen Retrieval (AR): Pretreatment of tissue to retrieve antigens and make them more accessible to antibody binding
     ↑ masked by fixation
     - Methods: break protein cross-links caused by fixation through chemical/physical means
  2. Primary Antibody
     - Titrated (determine optimal antibody concentration for staining)
     - Polyclonal Ab: made using multiple different immune cells, targets multiple epitopes (attachment points on an antigen)
     - Monoclonal Ab: made using identical immune cells, target single epitope
  3. Antibody visualization under Light Microscope:
     - Two Methods:
       1. Label primary Ab: rarely used due to lack of signal amplification
          ↑ needs higher Ab concentration
       2. Label Secondary Ab
          - Tagged with fluorescent molecules/enzymes
  4. Decreasing Background Staining
     - Background staining may be due to: nonspecific antibody binding + endogenous peroxidase activity (reacts with chromogens to produce stain)
     - Methods:
       1. Preincubation of Ab with normal serum from same species as secondary Ab
       2. Blocking
          ↑ substance that can be turned into a colored compound through a reaction
     - Controls:
       - + Control: tissue with antigen known to stain with certain antibody

- - Control. Sample tissue undergoes identical staining conditions (excluding primary Ab)
- Eliminates possibility of nonspecific Ab binding with secondary Ab

18 september 2023

The complement cascade: Yin-Yang in neuroinflammation - neuro-protection and degeneration

Authors: John Alexander et. al.

published: 24 october 2008

Type: Review

- Complement Cascade: necessary for health but can exacerbate inflammation/ degenerative disease
  - Part of the 'immune system': recognition, trafficking, elimination of Pathogens
  - Activation: classical, alternative, or lectin pathways
    - cleavage of complement proteins
      - C3 → C3b (Surface opsonization: C3b deposits on pathogens and signals phagocytosis)
      - C5 → C5b (assembly of C5b-9/membrane attack complex → lyses pathogens)
    - C3a/C5a released (induce inflammation)
  - Strictly regulated to prevent self-injury

results

- Also participates in:
  - Neurogenesis (synthesis of nervous tissue)
  - Liver regeneration
  - B-cell Proliferation
  - Synaptic plasticity
- Complement Proteins primarily synthesized by hepatocytes (liver cells)
  - In CNS, synthesized by: neurons, microglia, astrocytes, oligodendrocytes

18 September 2023

Brain Maturation in Adolescence: Concurrent Changes in Neuroanatomy and Neurophysiology

Authors: Whitford et al.

Published: March 2007 ✓

Type: Research

- Investigate effect of age on neuroanatomy/neurophysiology (aged 10~30)
  - use MRI + EEG/Electroencephalography (measures electrical activity in brains)
  - Data divided by gray/white matter
- Introduction:
  - Dramatic structural changes in brain occurs during Perinatal + addlescence
    - Adolescent brain development: Peak neural organization + early onset of major mental illnesses ✓
  - previous studies:

MRI studies:

- Gray matter loss in ages 10-18 (Peripubescent period)
  - occurs most at association cortices (cerebral surface)
  - Causes: synaptic pruning (X neuron death)
    - Elimination of excess synapses + neuropil (dendrites, dendritic spines, axon terminals)
- White matter increase in ages 10-18
  - occurs most at frontal lobe + hippocampus
  - Caused by axonal myelination → development of language/memory skills

- EEG power decreased in ages 10-20
  - Caused by a reduction in the number of cortical synapses ✓

Glial cells form myelin sheaths around axons → insulation, increases speed of signal transfer

14 September 2023

Repetitive Mild Traumatic Brain Injuries in Mice during Adolescence cause Sexually Dimorphic Behavioural Deficits and Neuroinflammatory Dynamics

Authors: Eyolfson et al.
Published: 15 December 2020
Type: Research

- Introduction:
  - mTBI: blow/jolt to head
    - cause different velocities in white/gray matter → DAI
  - RmTBIs → secondary cascades → neuroinflammation + neuronal dysfunction → damaged neurons release DAMPs → activate microglia → activate pro-inflammatory (damage) / anti-inflammatory (repair) + increase BBB permeability (allow peripheral immune cells to enter brain)
- Methods: RmTBI v.s. sham
  - RmTBIs delivered by: Gothenburg Impactor Device, 5 x RmTBIs
  - MRIs: cortex, hippocampus, thalamus, and corpus callosum observed
  - Behavioural Testing
    1. Beam Walk Assay: motor coordination; number of foot slips recorded
    2. Light-dark Box; anxiety; total time spent in light / dark recorded
    3. Open Field: locomotor/exploratory behaviour; total distance travelled / time spent in middle of field recorded
    4. Three-chamber Assay; social behaviour; total time spent in zones recorded
    5. Novel object Recognition: cognitive function; total exploration time with each object recorded
    6. Forced Swim: depression; time spent immobile recorded
  - Transcardial Perfusion Fixation: mice euthanized
  - Immunohistochemistry + cell counting
  - Flow cytometric Immunophenotyping
  - Statistical Analysis
    - One-way ANOVAs: injury (RmTBI or sham)
    - Two-way ANOVAs: sex + injury
- Results:
  1. Lateral Impact module (LIM)-induced RmTBIs cause common behavioural deficits in adolescent male mice
     - Adolescent mice: ↑ T2R, ↑ foot slips,
     - Increase in brain volume (ctx, hipp, cc)

T2R measured immediately after RmTBI/Sham injury: time required for mouse to recover from supine to prone position (indicates loss of consciousness)

2. Adolescent RmTBIs cause sexually dimorphic behavioural deficits
- T2R: no significant difference between sexes (injury effects)
- BWA: males have higher deficits (injury effects)
- OFA: females travelled farther (no injury effects)
- LD, NoR, FS: no injury/sex effects
- Compare shams vs. RmTBI:
  - 0: RmTBI mice performed as well or better than shams
  - 1+: RmTBI mice performed worse than shams
  - Majority of RmTBI mice: 3-4 (average)

3. RmTBIs drive sexually dimorphic, time-dependent neuroinflammation
- used flow cytometry to quantify microglia and infiltrating immune cell numbers
- Microglia decrease in males (time-dependent)
- Macrophage/T cell increase in males (time-dependent)
  - 1h (M), 6h (F)
  - 1d (M), 3d (F)

4. RmTBIs reduced microglia density only in adolescent male mice
- CC: decrease in microglia density for males
- Thal; crtx: decrease for males
- Hipp: no change for either

- Discussion:
- Purposes:
  1. characterize LIM in mice
  2. Define changes that occur in adolescent brains after injury
  3. Differences in male/female response to RmTBIs

1. White Matter changes
- Confirm white matter tract damage attributable to DAI after RmTBIs
- Increased volume may be a result of:
  a. edemas
  b. reduced clearance of waste products due to impairment caused by RmTBIs

2. Sexually Dimorphic Impairments
- Adolescent mice: ↑ loss of consciousness, ↑ motor disfunction, ↑ cognitive deficits (compared to shams)
  - Worse in males than females
  - No significant effects on anxiety, depression, social behaviour

3. Time/Sex-Dependent Neuroinflammation
- Neuroinflammation: key secondary cascade
  - Caused by: activation of microglia, infiltration of macrophages, recruitment of adaptive immune cells (T/B cells)
- Adolescent mice: sex/time-dependent microphage infiltration, ↓ microglia, T cell infiltration
- Females: later recruitment of macrophages, fewer T cells recruited
- Males: increased microglia loss
  - Greater propensity towards neuroinflammatory responses
  - Progressive microglia loss in adolescents
    - Brain-wide + specific reductions in Crtx & Thal
    - Causes: microglia migrated to other regions (x) or microglia are dying from RmTBIs
    - Consequences.
      - Fewer mature neurons (microglia responsible for synaptic pruning) in adulthood
      - Neurological disorders later in life

12 September 2023

Repeated mild traumatic brain injuries in mice cause age- and sex-specific alterations in dendritic spine density

Authors: Eric Eyolfson, Thomas Carr, Erik Fraunberger, Asher Khan, Isabel Clark, Richelle Mychasiuk, Alexander W. Lohman

Type: Research

- Abstract:
  - RmTBI: influenced by age and sex at injury
  - Study: Effect of closed-head lateral RmTBIs on adolescent/adult and male/female mice
    - → Neurobehavioural deficits
    - → Microglia response
    - → changes in dendritic spine density
- Introduction:
  - Pathophysiology of mTBI
    - → Most (80%) recover
    - → 20% develop PCS
    - → High risk of neurodegenerative diseases
  - Adolescents: critical time for development yet limited studies
    - Synaptic pruning (destroying of excessive synaptic connections) occurs
      - Alterations in synaptic pruning → brain damage
    - Previous Papers:
      - RmTBI → behavioural deficits
      - Adolescent male mice: ↓ Iba1+ microglia density in motor cortex
      - Effects of ↓ microglia in synaptic pruning
  - Area of focus:
    - Motor cortex + agranular insular cortex in mice
      ↑ equivalent of orbitfrontal insular cortex in humans
- RmTBI v.s. Sham group: 5 injuries/24h, time-to-right (loss of consciousness) measured
  - 1 day after injury: beam walk (number of foot slips while walking along beam)
  - 2 days after injury: open field (movement of mice in box tracked)
  - 3 days after injury: novel object recognition (time spent discovering a novel object v.s. familar novel)
  - 4. Immunohistochemistry (mice euthanized, brain slices analyzed + cell density calculated
  - 5. Golgi-Cox staining (average dendritic spine density calculated per hemisphere)

• 6. Statistical analysis: three-way ANOVAs

• Results:

1. RmTBIs induced sex/age-dependent behavioural deficits

• Age-dependent:

a. Adolescents: ↑ time-to-right, ↑ time spent in centre of field

b. Adults: ↑ foot slips, ↑ distance travelled in field ✓

• Sex-dependent:

a. F: ↑ time-to-right

b. M: ↑ foot slips

2. RmTBIs induced sex/age-dependent changes in microglia and dendritic spine density (in AID/MC)

• Microglia density:

a. ↑ microglia density in adolescents (AID/MC)

b. ↓ Iba1+ microglia expression in males (MC)

• Dendritic spine density:

• ↑ spine density in adolescents (MC-males / AID-females) for RmTBI group

• ↑ spine density in males (MC-adolescents / AID-adults)

• Discussion:

1. Behavioural Changes ✓

• F: increased loss of consciousness

• M: severe motor deficits (adults)

2. Microglia and Spines

• Adolescence: myelination / myelinogenesis, synaptic pruning occurs (synapses (dendrites))

• RmTBIs cause spine density loss (pronounced in adolescents)

→ decrease in synaptic pruning → neurodegenerative diseases later in life

• Secondary Injury Cascades:

→ neuroinflammation can cause deficits / hinder recovery

• Microglia: resident CNS inflammatory cells; neuroprotective functions after injury + assist in brain maturation (through synaptic pruning)

• Complement proteins C1q and C3 tag synapses for pruning

• C1q/C3 - microglia receptor interactions → pruning

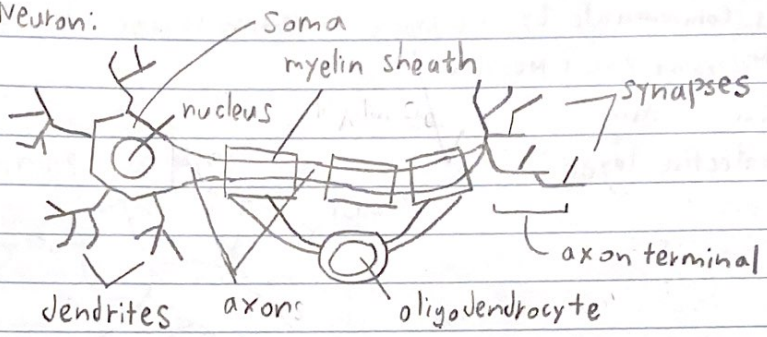
• TBIs increase expression of C1q/C3 → excessive pruning → spine density loss

8 september 2023

Brain Anatomy and How it Works

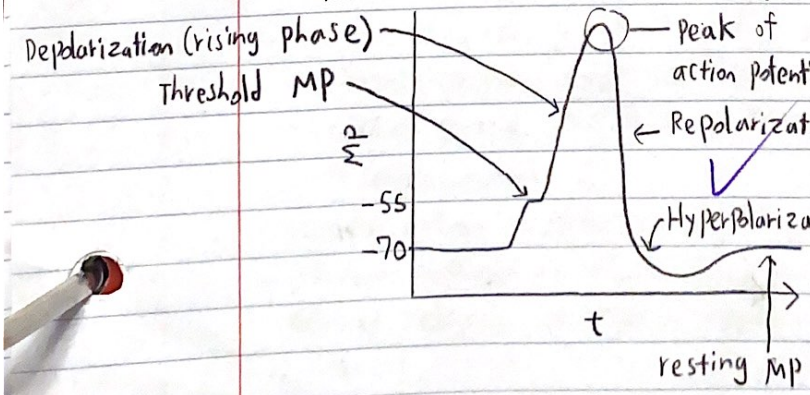
Source: Johns Hopkins Medicine

- Gray matter (outer portion): made of neuron somas
- White matter (inner portion): made of neuron axons
- Neuron:



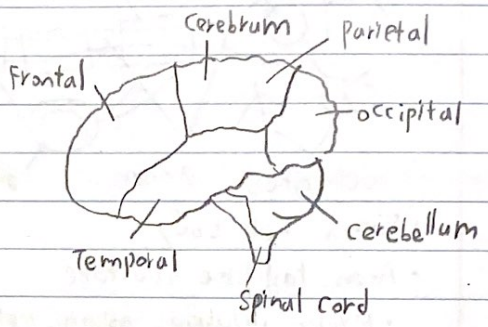
- Soma: cell body
- Axon: tail-like structure
  - Myelin: insulates axon, helps conduct electrical signal
- Dendrites: receive signals
- Send signals using action potentials
  - Caused by change in membrane potential across a membrane
  - Membrane potential: difference in charge between the inside and outside of a neuron ↑ caused by ions
    - At rest:
      - → Inside: ↓ ions
      - → outside: ↑ Na^+, ↑ Cl^-, ↑ K^+ + ↑ anions
      - Resting MP: -70 mV
      - Maintained through Na^+/K^+ pump: 3 Na^+ out, 2 K^+ in
    - Action potential: reversal in membrane potential
      1. Neurotransmitters bind to receptors on dendrites → depolarization (↓ MP)

Depolarization: less negative charge inside cell than outside



2. Threshold MP reached → Na^+ channels open → Na^+ in → depolarization of neuron
3. Peak: Na^+ channels close, K^+ channels open → K^+ out → repolarization
4. Hyperpolarization: overshoots resting MP
5. Returns to resting MP

- Cerebrum: front of brain
  - Cerebral Cortex: outer gray matter of cerebrum
  - Divided by the Sulcus/interhemispheric fissure into two hemispheres
    - Hemispheres communicate by the corpus callosum (bridge-like structure)
- Brainstem: Midbrain + Pons + Medulla
- Cerebellum: back of brain
- Meninges: protective layer
  - Dura Mater
  - Arachnoid
  - Pia Mater
- Lobes: Part of the Cerebrum
  1. Frontal Lobe
  2. Parietal Lobe
  3. Occipital Lobe
  4. Temporal Lobe
- Other:
  - Pituitary Gland
  - Hypothalamus
  - Amygdala
  - Hippocampus
  - pineal Gland
  - Ventricles



5 September 2023

Microglia dynamics in adolescent traumatic brain injury

Authors: Eric Eyolfson, Asher Khan, Richelle Mychasiuk, Alexander W. Lohman

Published: 29 October 2020

Type: Review

- Abstract:
  - Repetitive, Mild Traumatic Brain Injuries (RmTBIs) in adolescents (neuroinflammation causes damages neurological function)
  - Microglia (immune cells in the brain) regulate the number of neurons and synapses formation/elimination
  - Microglia may activate neuroinflammatory phenotypes upon injury
  - what role does microglia play in RmTBIs?
- Background:
  - Research focus on secondary mTBIs instead of primary mTBIs
    - Primary mTBIs occur immediately upon injury
    - mTBI diagnosis is late
- TBIs:
  - Males experience more TBIs than females
  - Individuals who experienced mTBIs are at high risk for RmTBIs
  - Brain is in state of vulnerability for an uncertain time window after mTBIs
  - Pathophysiology:
    - Primary Injury cascades: Diffuse Axonal Injury (DAI)
      - Result of different speeds of white and grey matter in brain upon injury
      - DAI: microtuble damage → calcium influx → axonal swelling → axon breakage
    - Secondary Injury cascades:
      - Reduction of cerebral blood flow (hypoperfusion) → low oxygen/glucose in brain
      - Axonal stretching → mechanoporation (membrane damage) → depolarization (Na^+, Ca^{2+} ions in)
      - Lack of glucose → failure of ATP-dependent ion transporters → depolarization
      - Depolarization of pre-synaptic neurons
      - Calcium influx → mitochondrial dysfunction, reactive oxygen species release → neuron death

- Neuroinflammation
  - Microglia:
    - First-responders to injury
    - Highly plastic: can change from M1-like (pro-inflammatory) to M2-like (anti-inflammatory) phenotypes rapidly
      - Able to cause injury repair or exacerbate damage
    - After a TBI: ramified microglia → amoeboid microglia
      - DAMPs (molecules indicating injury) released by damaged cells → activate microglia → microglia differentiate amongst M1-like and M2-like phenotypes → synthesize cytokines/chemokines
      - DAMPs can also weaken the blood brain barrier → peripheral immune cells enter the brain and cause inflammation
  - Microglia + CNS Development
    - Microglia Function in Prenatal Brain:
      - Neurogenesis regulation
      - Balancing neuron survival and death
      - Phagocytose (digest) neuronal progenitors (precursors)
      - Regulate axonal growth
      - Promote neuron fasciculation (involuntary muscle movement)
    - Microglia Function in Posthatal Brain:
      - Regulate synapses number in neurons by synaptic pruning (destroying excessive synapses)
        - Recognizes excessive synapses by protein tagging (C3 proteins)
        - RmTBI: Synaptic pruning can increase or decrease
          - Produces amoeboid microglia (less efficient at pruning) → decrease
          - Causes increased expression of C3 tagging proteins → increase
      - Microglia transition from ramified surveillance to activated phenotype ↑ primed microglia (susceptible to inflammation with subsequent injuries) states and increase in density with aging
      - In individuals affected by RmTBI
        - RmTBIs during adolescence may cause neurological issues in adulthood through primed microglia

Background Research 3 September 2023

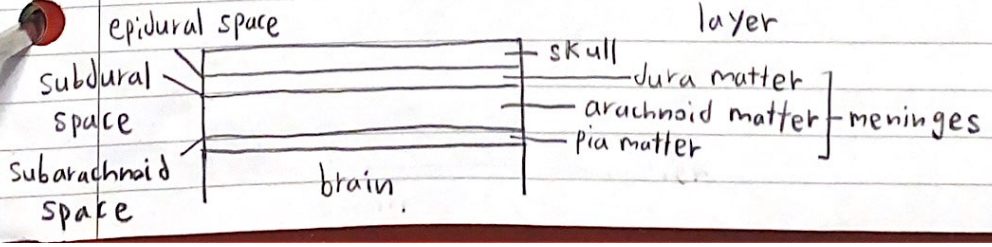
Pediatric Traumatic Brain Injury: Characteristic Features, Diagnosis, and Management

Authors: Takashi Araki, Hiroyuki Yokota, Akio Morita

Published: 20 January 2017 Type: Review

- Abstract:
  - TBIs are the leading cause of death/disability in children
  - Pediatric TBI is associated with age-specific anatomical/physiological characteristics
- Epidemiology
  - Injury characteristics according to age: (ongoing bleeding, clotted bleeding)
    1. Newborns → delivery head injury, hemorrhage, hematoma
       - Low birth weight & hypoxemia increase risk of hemorrhage
    2. Infants → accidental head injury, abusive head trauma (AHT)
    3. Toddlers → accidental head injury
       - Causes: motor vehicle accidents, pedestrian injuries
    4. Adolescents → bicycle/motorcycle accidents, sports injuries
  - Structural characteristics in pediatric population
    1. Skin:
       - Poor cushioning, susceptible to tearing, high water retention, microvascular breakdown causes hematomas, blood accumulation under skull, hematomas cannot be calcified
    2. Cranium (skull):
       - High craniofacial ratios (loose cranial structures), continuity of skull well-maintained (no fragments)
    3. Brain/Nerve Fibers: (myelination protects brain from TBI)
       - Undeveloped myelin sheaths on neurons, high water concentration, more flexible fibers, vulnerable to contusions
    4. Neck/Cervical Spine:
       - Poor head support/undeveloped neck muscle, vertebrae vulnerable to dislocation
- Primary TBI: mechanical injury produced by trauma
  1. Skull Fracture
  2. Intracranial Injury
     a. Acute Epidural Hematoma (AEDH): between skull and meninges (dura matter)

     b. Acute Subdural Hematoma (ASDH): between dura matter and arachnoid layer



- AHT
- Subdural Fluid Accumulation

3. Traumatic Subarachnoid Hemorrhage (tSAH)
4. Intraventricular Hemorrhage (IVH)
   - ventricles: spaces in brain
5. Cerebral Contusions (bleeding inside brain due to ruptured capillaries)
6. Diffuse Axonal Injury (DAI; tearing of axons/nerve fibers)
7. Intracerebral Hemorrhage (ICH)

- Secondary TBI: Physiologic response to initial trauma

1. Diffuse cerebral Swelling (DCS)

Experimental Procedures

Animals

C57BL/6 Mice:

- Source: Charles River Laboratories
- Gender: Female
- Age: Post-natal Day 48 (P48)
- Number of Mice: 10
- RmTBI and Sham Injury groups each assigned 5 Mice

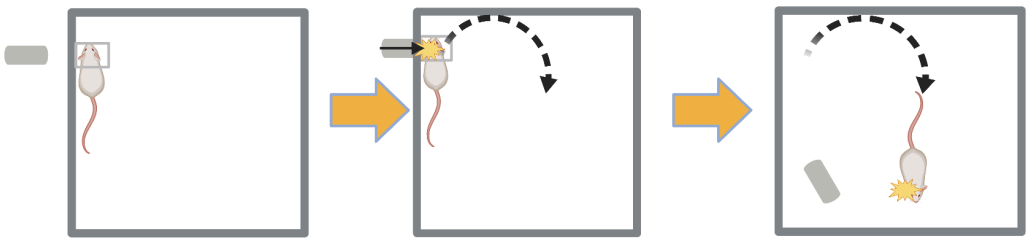
RmTBI and Sham Injury Delivery

Lateral Impact Model:

1. Mouse anesthetized for 15 seconds using 5% isoflurane
2. Mouse placed in prone position inside Gothenburg Impactor Device
    a. Left side of head beside a sheet of metal (used to simulate a "helmet")
3. 50g cylindrical projectile fired at head of mouse five times at 24 hour intervals
    a. Speed of projectile: 5m/s ± 0.2m/s
    b. "Helmet" distributes force onto lateral surface of head
    c. Force causes mouse to accelerate away from initial position, turn 180°, and then rapidly decelerate (see diagram below)

Sham Injury Delivery: steps 1-3 repeated, but without firing a projectile at the mouse

- Intended to minimize effects of confounding variables (neurological effects of anesthesia, stress caused by environmental factors)

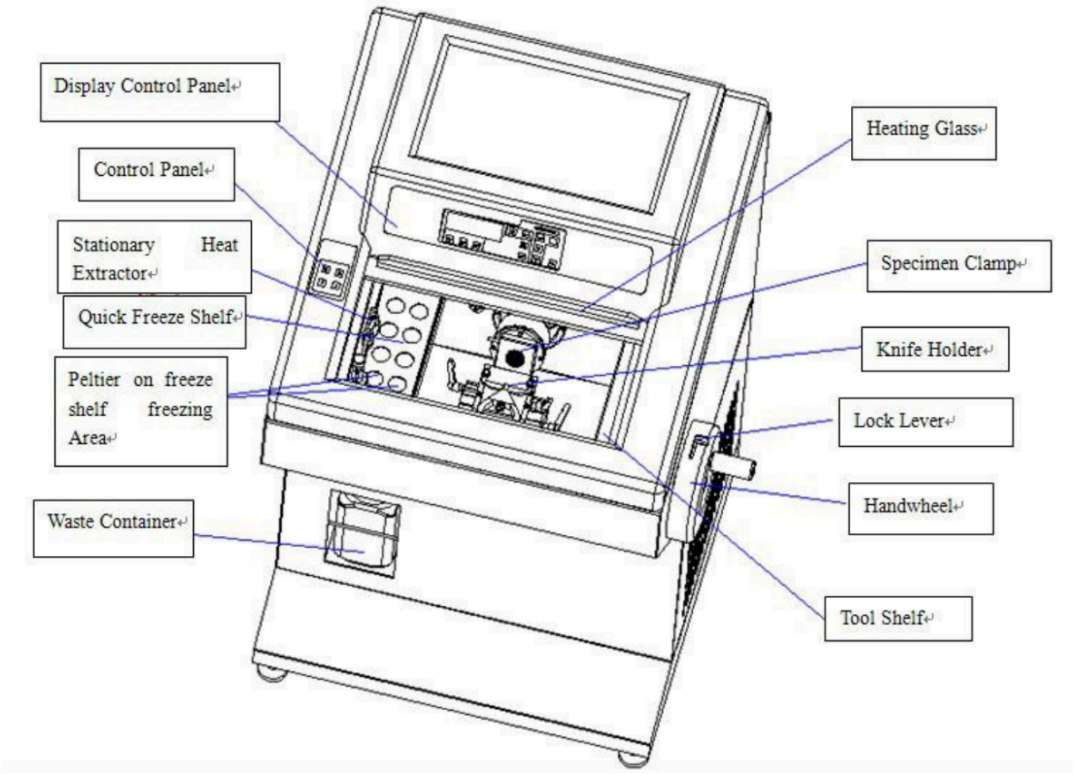


Tissue Fixation

1. On Post-injury day 5 (PID 5) mice anesthetized using 5% isoflurane
2. Mice euthanized using an intraperitoneal injection of sodium pentobarbital solution (0.05mL of 240 mg/mL solution)
3. Transcardial Perfusion: ice-cold phosphate-buffered saline (PBS) pumped through mice circulatory systems to remove all the blood
4. 4% paraformaldehyde perfused through mice to fix the tissue and avoid degeneration in the future
5. Brains stored in 4% paraformaldehyde (PFA) solution for 24 hours
6. Brains transferred to 30% sucrose solution at 4℃ for storage

Cryosectioning

- Leica HM550 cryostat/microtome used (see below)

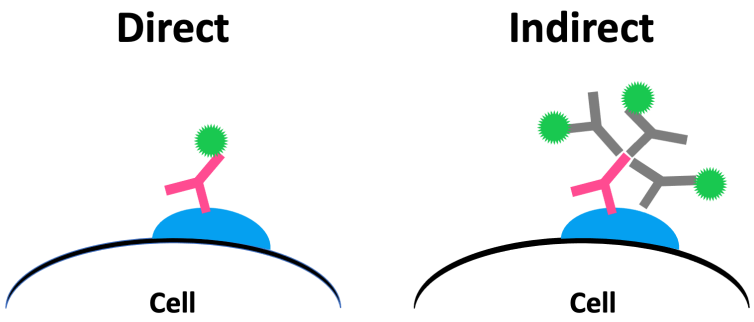


1. Brain freezed using optimal cutting temperature (OCT) compound onto circular stand compatible with cryostat
    a. OCT freezes at -20℃
2. Stand placed onto specimen clamp on cryostat and angle of clamp adjusted to line up with blade as needed
3. Slice thickness set to 40µm on control panel
4. Handle rotated clockwise to cut brain into 40µm-thick coronal (front-to-back) slices
5. Each brain slice transferred to six-well plate filled with PBS
    a. Serial sectioning used to gain an accurate representation of the brain (each consecutive brain slice transferred to a different well, and then repeated when all six wells filled, starting again from first well)
6. Brain slices transferred to antifreeze solution
    a. Antifreeze Solution: 30% ethylene glycol, 20% glycerol, 1x PBS + pH 7.4
7. Brain slices in antifreeze stored at 20℃

Immunohistochemistry

- Immunohistochemistry (IHC): staining technique which uses specific binding between antigens and antibodies to visualize target antigens
- Indirect Immunofluorescence used:

- Primary antibody tags target antigen
- Secondary antibody conjugated to a fluorophore (fluorescent molecule) tags primary antibody
- Fluorophore visible underneath confocal fluorescence microscope



Blocking:

- Block non-specific binding sites (epitopes) on antigens where antibodies may erroneously bind to and increase background fluorescence
- Increase signal-to-noise ratio
  - "Signal"=target antigens (C1q and C3)
  - "Noise"=background fluorescence (non-target antigens)

1. Blocking solution created using PBS, 10% donkey serum, 5% bovine serum albumin (BSA), cold fish skin gelatin, and 0.25% triton X-100
   a. Triton X-100 permeabilizes membranes of cells, allowing primary antibodies to access the interior of the cell more easily
2. Brain slices incubated in blocking solution

Primary Antibody Incubation:

- Primary Antibodies:
  - C1q: rabbit α-C1q (Abcam, catalog # ab182451)
  - C3: goat α-C3 (MP Biomedicals, catalog # ICN55730)
    - Primary antibodies must have different host from sample (mouse) - if primary antibody and sample have the same host, the secondary antibody (raised against a primary antibody produced by that host) may erroneously bind to other binding sites in the sample, increasing background fluorescence
- Primary Antibody Solution (buffer) - same solution used for both C1q and C3: PBS, 5% bovine serum albumin (BSA), cold fish skin gelatin, triton X-100
  - Primary antibody diluted in buffer to:
    - C1q: 1:100 concentration
    - C3: 1:250 concentration

    - Dilution in buffer stabilizes antibody, promotes its uniform diffusion across the tissue, and discourages nonspecific binding
1. Primary antibody solution moved to well plate using pipette
2. Primary antibodies moved to well plate using pipette
    a. One well maintained as a negative control (primary antibodies not introduced) in order to observe background fluorescence
3. Brain slices transferred to well plate and incubated in primary antibody solution at 4℃ overnight
4. Brain slices washed five times at 10 minute intervals using phosphate-buffered saline (PBS) and 4% paraformaldehyde
    a. Washing rinses out primary antibodies weakly bound to nonspecific sites and unbound primary antibodies

Secondary Antibody Incubation + DAPI Counterstaining

- Secondary Antibodies:
    - Rabbit α-C1q: donkey α-rabbit (Thermo Fisher, catalog # A10042)
    - Goat α-C3: donkey α-goat (Thermo Fisher, catalog # A32849)
    - Secondary antibodies must have different host from primary antibodies - if the secondary and primary antibodies had the same host, they would not recognize each other and thus not bind to each other
- Fluorophores:
    - Donkey α-rabbit: AF568
    - Donkey α-goat: AF 647
- Secondary Antibody Solution (buffer) - same for both C1q and C3: PBS, 5% bovine serum albumin (BSA), cold fish skin gelatin, triton X-100
    - Secondary antibodies diluted in buffer to:
        - C1q: 1:500 concentration
        - C3: 1:500 concentration
- Counterstaining: Provide contrast to primary stain
    - Stain: 4′,6-diamidino-2-phenylindole (DAPI)
        - Stains cell nuclei to provide contrast for principal stain (stain performed on complement proteins using the antibodies)

1. Secondary antibody solution moved to well plate using pipette
2. Secondary antibodies moved to well plate using pipette
3. Brain slices transferred to well plate
4. DAPI counterstain added to secondary antibody solution at dilution of 1:1000
5. Brain slices incubated in secondary antibody solution at 4℃ overnight
6. Brain slices washed five times at 10 minute intervals using phosphate-buffered saline (PBS) and 4% paraformaldehyde
7. Washed brain slices stored in PBS at 4℃ until mounting

Mounting:

- Each slide contains six brain slices (two rows of three slices) obtained from serial sections

1. Brain slices mounted onto side of slide with rough end
2. Slide submerged (excluding white end) in PBS (contains brain slices)
3. Side which brain slices naturally orient to observed (this side will be mounted facing up)
4. Samples mounted onto slide
    a. Push rather than pull on brain slices
    b. Push down on brain slices to remove bubbles
5. Slides dried out after all samples mounted
6. Pipette used to apply fluid onto slide
    a. Pushed down then released to draw fluid
    b. Steady pressure applied to release droplets of fluid onto slide
7. Slide cover placed on at an angle to prevent bubbles from being trapped

Imaging (Confocal Fluorescence Microscopy)

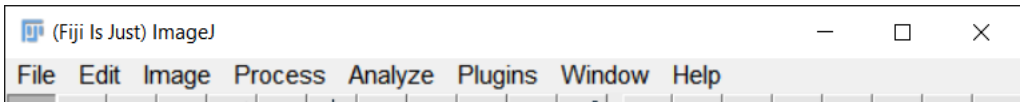
- ZEISS Celldiscoverer 7 confocal fluorescence microscope used
    - Confocal fluorescence microscopy creates higher resolution images (especially in the z-axis) by restricting light emitted from the sample to a pinhole, only allowing in-focus light from the focal plane to be captured and making it less likely for scattered light from above or below the focal plane to be captured
        - This is in contrast to widefield microscopy, the other type of light microscopy, where both out-of-focus and in-focus light is captured, resulting in more blurry images
    - Three slides can be imaged at the same time
    - Exposure of samples to light must be minimized in order to avoid photobleaching (repeated excitation of electrons in fluorophore weakens light emitted by fluorophore)

1. Set of three slides placed inside microscope
2. Imaging region (motor cortex) delineated in each brain slice using a tiling function
    a. Each region about three blocks in size
3. Images for DAPI, AF568 (C1q), and AF647 (C3) selected
    a. Imaging Parameters:
        i. General: 20X magnification (0.95 numerical aperture objective, or NA objective), 2048 X 2048 pixel resolution, and bidirectional scanning
        ii. DAPI: excited using a 405nm excitation laser at 2% power, and light collected through a pinhole at a size of 2 AU (airy units)
        iii. AF568: excited using a 561nm excitation laser at 1% power, and light collected through a pinhole at a size of 1AU
        iv. AF647: excited using a 640nm excitation laser at 1% power, and light collected through a pinhole at a size of 1 AU

4. Confocal z-stacks (collection of 2D images from multiple focal planes to create a 3D image) obtained at 1.5µm intervals
    a. Z-stacks centred in order to capture full thickness of tissue
    b. Each imaging cycle (three slides) took around 50 minutes to complete
- Three images (DAPI, AF568, and AF647) produced for each brain slice
- Images underwent maximum intensity projection to collapse the 3D-stack into a single 2D image and were exported as TIFF files labelled with corresponding mouse and brain slice number (eg. brain slice number 1 from mouse number 1 → scene-1-#1-MC)
    - Treatment type excluded from label to avoid bias during image analysis
    - Each mouse had three brain slices/images

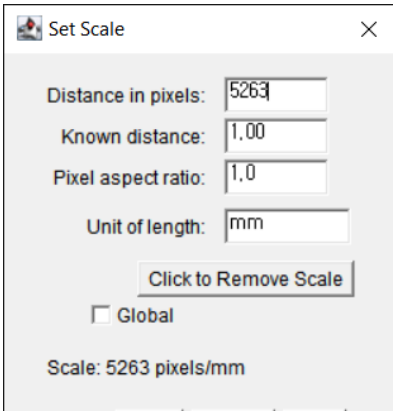
Image Analysis

- Fiji image processing package used to obtain complement protein count:

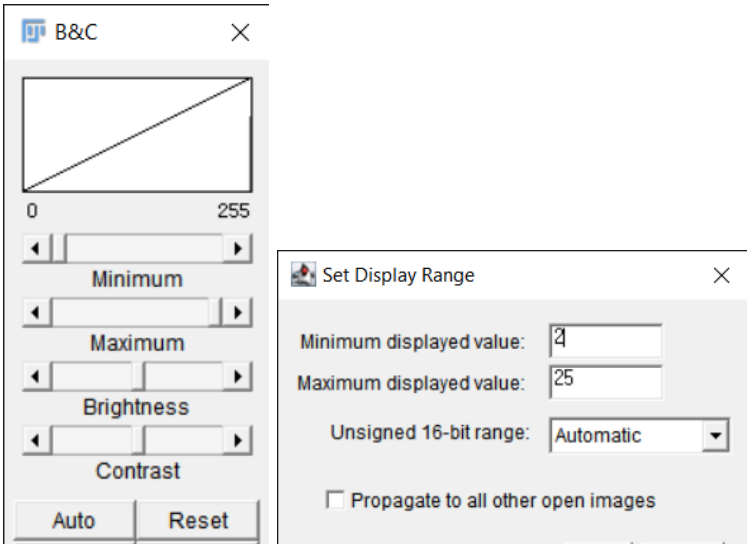


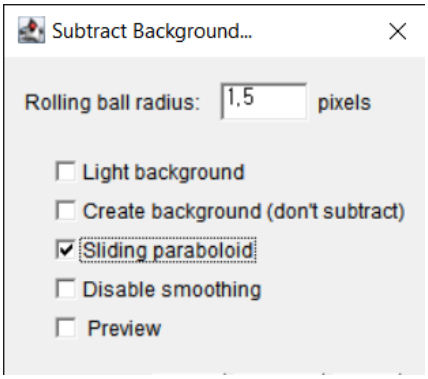
- Imaging parameters must be kept consistent between all images for each protein
    - Parameter values may vary between cohorts

1. Images converted from RGB TIFFs to 8-bit TIFFs
2. Image scale changed (analyze→set scale)
    a. Unit of length: mm
    b. Distance in pixels: 5263
    c. Known distance: 1.00
    d. Pixel aspect ratio: 1.00

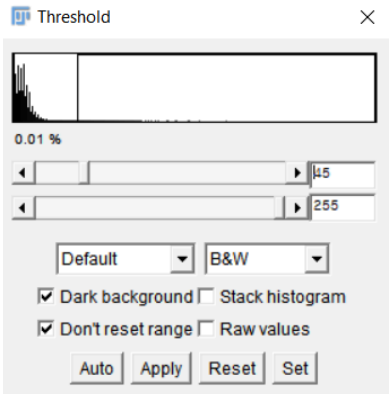


3. Brightness & contrast of image adjusted (ctrl-shift-C or image→adjust→brightness & contrast)
    a. "Auto"
    b. "Set" (min-max):
        i. C1q: 2-25
        ii. C3: 0-25

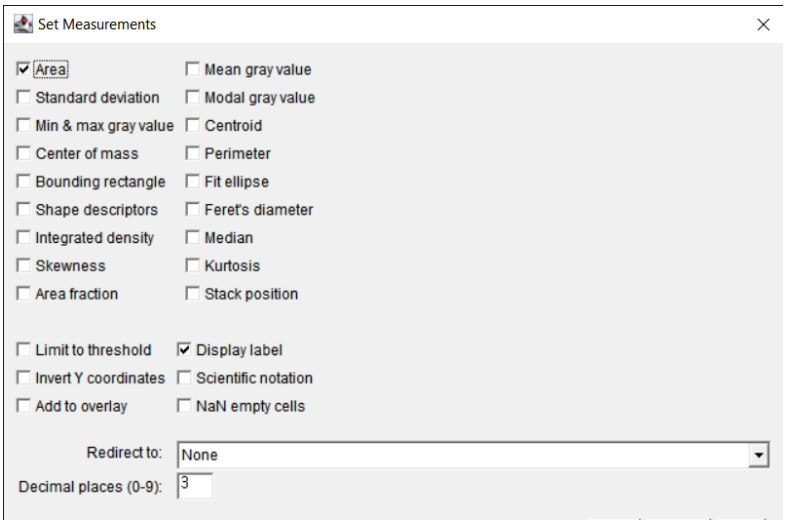
- 

4. Image despeckled (process→noise→despeckle)
5. Background subtracting performed on image (process→subtract background)
    a. Select: sliding paraboloid, preview
    b. Rolling ball radius
        i. C1q: 1.5px
        ii. C3: 20px
- 

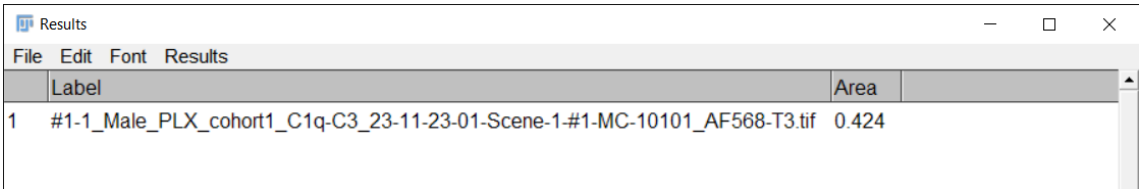
6. Image converted to 8-bit/grayscale (image→type→8-bit)
7. Thresholding performed on image (image→adjust→threshold)
    a. Select: B&W (black&white)
    b. Threshold Range:
        i. C1q: 45-225
        ii. C3: 20-255



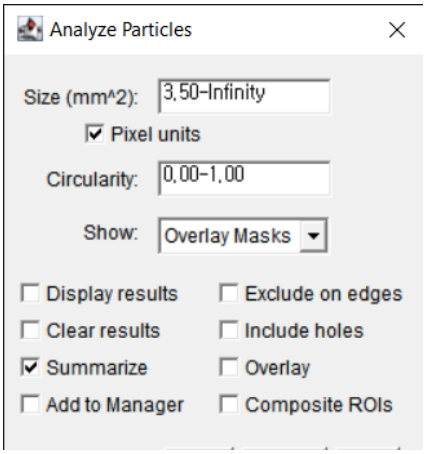
8. *Measurements for image analysis set (analyze→set measurements)
    a. Select: area, display label



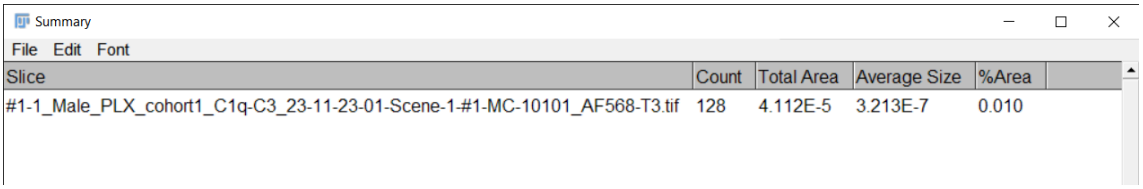
9. *Area of image calculated (ctrl-M)
    a. Area given in mm^2
    - Example of results:



10. Number of proteins in image/"particle count" calculated (analyze → analyze particles)
    a. Size:
        i. C1q: 3.5-infinity
        ii. C3: 3.5-infinity
    b. Select: pixel units
    c. Show: overlay masks
    d. Select: summarize

- 

- Example of results:



*Only needs to be performed once

Data Organization/Refinement

Volume, Density, and Average Calculations for Each Image:

- Image area (mm^2) and particles count data organized into spreadsheet beside corresponding mouse and brain slice number
    - Image area constant for all images
    - C1q and C3 data separated into different tables

1. Image volume (mm^3) calculated for each brain slice
    a. Formula: volume = area*(35/1000)
        i. Depth of image (from microscope): 35µm = 35/1000mm
2. Particles density (particles/mm^3) calculated for each brain slice
    a. Formula: volume = particles count/volume
3. Particle density average (particles/mm^3) calculated for each mouse
    a. Each mouse has three brain slices/images
    b. Formula: particle density average = ∑particle density/3
4. Steps 1-3 repeated for other protein

- Example of data table (C3):

		C3				
EN#	Image#	Area (um2)	Particles count	Volume (mm3)	Density (particles/mm3)	Mouse average
1	1	0.424	303	0.01484	20417.78976	
	2	0.424	325	0.01484	21900.26954	
	3	0.424	401	0.01484	27021.56334	23113.20755
2	1	0.424	25	0.01484	1684.636119	
	2	0.424	36	0.01484	2425.876011	
	3	0.424	21	0.01484	1415.09434	1841.868823

Checking for Outliers Within Each Mouse

1. First and third quartiles of particle densities calculated for each mouse
    a. Formula:
        i. 1st quartile (Q1) = QUARTILE.INC(particle density for brain slice 1:particle density for brain slice 3,1)
        ii. 3rd quartile (Q3) = QUARTILE.INC(particle density for brain slice 1:particle density for brain slice 3,3)
2. Interquartile range of particle densities calculated for each mouse
    a. Formula: interquartile range (IQR) = Q3-Q1
3. Upper and lower bounds of particle densities calculated for each mouse
    a. Formula:
        i. Upper bound = Q3+IQR*1.5
        ii. Lower bound = Q1-IQR*1.5
4. Determined whether particle densities of any of the brain slices were outliers
    a. Formula = OR(particle density<lower bound,particle density>upper bound)
        i. Eg. OR(F3<J5,F3>K5)
            1. "$": absolute cell reference - prevents cell from changing when formula is copied and pasted
    b. Cell stated either "TRUE" or "FALSE"
        i. "TRUE": particle density was outside the range and was an outlier
        ii. "FALSE": particle density was within the range and was not an outlier
    c. If a particle density is an outlier, it was excluded from the average for the corresponding mouse
5. Repeated steps 1-4 for other protein

- Example of data table after checking for outliers within each mouse (C3):

		C3										
EN#	Image#	Area (um2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound	Upper bound	Outlier?	Mouse average
1	1	0.424	303	0.01484	20417.78976						FALSE	
	2	0.424	325	0.01484	21900.26954						FALSE	
	3	0.424	401	0.01484	27021.56334	21159.02965	24460.91644	3301.886792	16206.19946	29413.74663	FALSE	23113.20755
2	1	0.424	25	0.01484	1684.636119						FALSE	
	2	0.424	36	0.01484	2425.876011						FALSE	
	3	0.424	21	0.01484	1415.09434	1549.865229	2055.256065	505.3908356	791.7789757	2813.342318	FALSE	1841.868823

Checking for Outliers Within Each Protein Group

1. Mice sorted into RmTBI and sham injury groups and corresponding particle density averages recorded into a spreadsheet
    a. The RmTBI group was denoted by "1" and the sham injury group was denoted by "0"
2. First and third quartiles of average particle densities calculated for each injury group (RmTBI or sham)
3. Interquartile range of average particle densities calculated for each injury group
4. Upper and lower bounds of average particle densities calculated for each injury group
5. Determined whether any of the average particle densities were outliers
    a. Outliers excluded from the group average
6. Steps 1-5 repeated for other protein

- Example of data table after sorting mice based on treatment and checking for outliers within each injury group (C3):

EN#	Injury	C3	Q1	Q3	IQR	LB	UB	Outlier?
2	0	841.86882						FALSE
11	0							FALSE
20	0	92969.45						FALSE
35	0	47371.96	24606.91824	70170.70979	45563.79155	-43738.76909	138516.3971	FALSE
1	1	3113.2075						FALSE
10	1	190.47615						FALSE
25	1	84973.04						FALSE
36	1	34905.66	17632.52471	47422.50674	29789.98203	-27052.44834	92107.47978	FALSE

*Grey cell denotes that staining for that particular mouse failed and thus its data was not used

Statistical Analysis

t-Test and Graphing (Manual):

- Two-sample two-tailed t-test used:
    - Two-sample (independent) t-test: used when two different populations are present
    - Two-tailed t-test: used when experiment only wants to determine if means of the two selected populations are different from each other (as opposed to if experiment wants to determine if the mean of one population is greater/less than that of the other population - an one-tailed t-test use in this case)

- Formula for two-sample t-test:

Two-Sample T-Test

$$t = \frac{(\bar{X}_1 - \bar{X}_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

$\bar{x}_1$ = observed mean of 1st sample
$\bar{x}_2$ = observed mean of 2nd sample
s_1 = standard deviation of 1st sample
s_2 = standard deviation of 2nd sample
n_1 = sample size of 1st sample
n_2 = sample size of 2nd sample

  - In this experiment:
    - Population 1: RmTBI group
    - Population 2: sham injury group
    - 95% confidence interval used - 95% probability that a value will fall within the range produced by the confidence interval (calculated using t-value)
    - Level of significance (α) - probability of obtaining a difference due to chance: 0.05
      - α = 1-0.95
    - Degrees of freedom (df)
      - Formula: df (for a two-sample t-test) = n1+n2-2
  - Conditions for a t-test:
    - Samples are randomly selected
    - Samples are independent of each other
    - Sample distribution is roughly normal about the mean

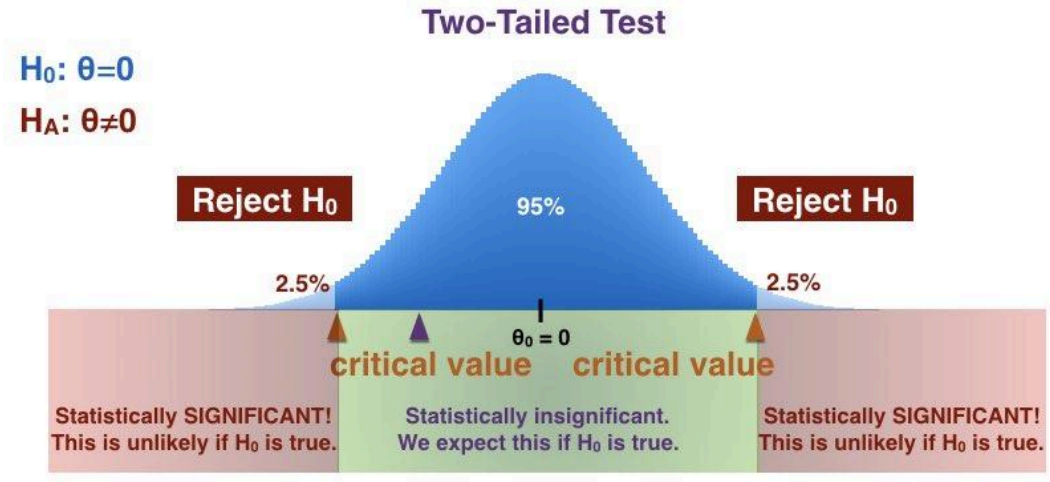
1. Formulate null hypothesis (H0) and alternative hypothesis (Ha)

$$H_0 : \mu_1 = \mu_2$$
$$H_A : \mu_1 \neq \mu_2$$

   a.
      i. μ1: mean of population 1 (RmTBI group)
      ii. μ2: mean of population 2 (sham injury group)
2. Calculate t-value using collected data
   a. t-value can be negative
3. Calculate p-value (t-value=t)
   a. p-value: the probability of sample (mouse) having a value (average particles density) t units below or above the mean

i. P(|average particle density|>t) = p-value
ii. Formula:
    1. If t<0: p-value = 2*tcdf(-1*10^99,t,df)
    2. If t>0: p-value = 2*tcdf(t,1*10^99,df)
iii. tcdf (Student's t cumulative distribution function): can be found in graphing calculator
    1. tcdf(lower bound,upper bound,degrees of freedom)
    2. As tcdf only calculates the probability of a sample having a value below (or above) the mean, 2*tcdf to obtain the p-value
        a. Since the data is assumed to be normally distributed, tcdf(-1*10^99,t,df) = tcdf(t,1*10^99,df)

b. Distribution of data for a two-tailed t-test:



4. The confidence interval can also be calculated if desired (although it is not necessary for this experiment)
    a. Formula:

$$Lower\ bound: (\bar{X}_1 - \bar{X}_2) - t_{\alpha/2} * \sqrt{\frac{s_1^2}{n_1} + \frac{s_{12}^2}{n_2}}$$

$$Upper\ bound: (\bar{X}_1 - \bar{X}_2) + t_{\alpha/2} * \sqrt{\frac{s_1^2}{n_1} + \frac{s_{12}^2}{n_2}}$$

5. Conclusion: compare p-value to level of significance (α)

a. If p>α: reject null hypothesis, accept alternative hypothesis → there is a statistically significant difference between the means of the two populations
b. If p<α: cannot reject null hypothesis → there is no statistically significant difference between the means of the two populations

6. Calculate the standard error of the mean (SEM) for each group:
   a. Formula for true SEM using population standard deviation (σ):

$$SE = \frac{\sigma}{\sqrt{n}}$$

      i. σ = population standard deviation
      ii. n = sample size
      iii. Formula for population standard deviation:

$$\sigma = \sqrt{\frac{\sum(x_i - \mu)^2}{N}}$$

      iv. This formula cannot be used, as the population mean (μ) is unknown; the SEM must be estimated instead using an alternative formula
   b. Formula for estimated SEM using sample standard deviation (s):

$$SE_{\bar{x}} = \frac{S}{\sqrt{n}}$$

      i. s = sample standard deviation
      ii. n = sample size
      iii. Formula for sample standard deviation (s):

$$S = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

         1. x i = data point
         2. x bar = sample mean
         3. n = sample size

7. Calculate the range of the error bars for the graph using the SEM
   a. Error bars: sample mean ± SEM

8. Graph the data using the appropriate sample mean and error bars
    a. If the error bars overlap, there is no statistically significant difference in particle density between the two groups
    b. If the error bars do not overlap, there is a statistically significant difference in particle density between the two groups
9. Repeat steps 1-8 for other protein

t-Test and Graphing (Digital):

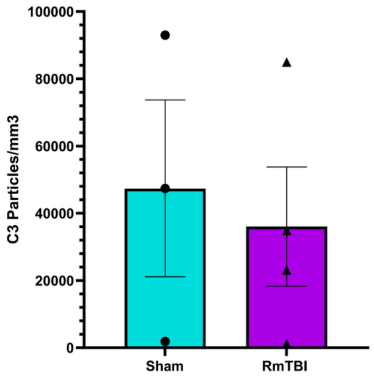
- GraphPad Prism program used

1. Choose: Column (1 independent variables) v.s. Grouped (2 independent variables)
2. Average particle densities for C1q and C3 from each mice inputted into separate sheets
    a. Separate data into RmTBI and sham injury groups for each sheet
3. Normality and lognormality test performed (data passed if at least one "yes" was displayed)
4. Choose: Unpaired v.s. Paired Data
5. Choose: Assume both populations have same standard deviation v.s. Welch's correction (do not assume populations have standard deviation)
6. Confidence level selected as 95%
7. Perform t-test function → results show whether there is a statistically significant difference between the means of the two groups
    a. Example of digital t-test results (C3):

Table Analyzed	C3				
Column B	RmTBI				
vs.	vs.				
Column A	Sham				
Unpaired t test					
P value	**0.7243**				
P value summary	**ns**				
Significantly different (P < 0.05)?	**No**				
One- or two-tailed P value?	Two-tailed				
t	df	t=0.3731	df=5		
How big is the difference?					
Mean of column A	47394				
Mean of column B	36046				
Difference between means (B - A) ± SEM	-11349 ± 30415				
95% confidence interval	-89534 to 66837				
R squared (eta squared)	0.02709				
F test to compare variances					
F	DFn	Dfd	1.649	2	3
P value	0.6576				
P value summary	ns				
Significantly different (P < 0.05)?	No				
Data analyzed					
Sample size	column A	3			
Sample size	column B	4			

8. Graph data with standard deviation (SD) or standard error of the mean (SEM)

a. Example of graph (C3):



Information on Antibodies and Conjugates Used in Experiment (Source: Abcam, Thermo Fisher, and MP Biomedicals)

Primary Antibodies:

- **Rabbit α-C1q** (Abcam)
    - Catalog Number: ab182451
        - Product: https://www.abcam.com/products/primary-antibodies/c1q-antibody-48-ab182451.html
    - Host: Rabbit
    - Isotype: IgG
    - Species Reactivity: Mouse
    - Class: Monoclonal
        - Produced Recombinantly
    - Immunogen: Human Complement C1q
    - Storage:
        - Buffer: 0.01% Sodium azide, 99% PBS, pH 7.2
        - Conditions: store at 4°C short term, store at -20°C long term, avoid freeze/thaw cycles
- **Goat α-C3** (MP Biomedicals)
    - Catalog Number: ICN55730
        - Product: https://www.fishersci.com/shop/products/anti-complement-c3-polyclonal-ab-5-mp-biomedicals/ICN55730
    - Host: Goat
    - Species Reactivity: Mouse
    - Class: Polyclonal

Secondary Antibodies:

- **Donkey α-rabbit AF568** (Thermo Fisher)
    - Catalog Number: A10042
        - Product: https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A10042
    - Host: Donkey
    - Isotype: IgG
    - Species Reactivity: Rabbit
    - Class: Polyclonal
    - Immunogen: Gamma Immunoglobulin
    - Storage Conditions:

        - Buffer: PBS, pH 7.5
        - Conditions: 4℃, in dark
    - Cross Adsorption: against bovine, chicken, goat, guinea pig, hamster, horse, human, mouse, rat, and sheep serum
    - Form: Whole Antibody
- **Donkey α-goat AF 647** (Thermo Fisher)
    - Catalog Number: A32849
        - Product: https://www.thermofisher.com/antibody/product/Donkey-anti-Goat-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32849
    - Host: Donkey
    - Isotype: IgG
    - Species Reactivity: Goat
    - Class: Polyclonal
    - Immunogen: Gamma Immunoglobulin
    - Storage Conditions:
        - Buffer: PBS, pH 7.5
        - Conditions: 4℃, in dark
    - Cross Adsorption: against human IgG, mouse IgG, rabbit IgG, rat IgG, and non-immunoglobulin goat serum
    - Form: Whole Antibody

Fluorophores*:

- **Alexa Fluor 568** (Thermo Fisher)
    - Colour: Orange-Fluorescent
    - Laser Line Wavelength: 568 nm
    - Excitation Wavelength Max: 578 nm
    - Emission Wavelength Max: 603 nm
    - Initial Brightness (per ThermoFisher Scientific): 4
- **Alexa Fluor 647** (Thermo Fisher)
    - Colour: Far Red-Fluorescent
    - Laser Line Wavelength: 594 nm or 647 nm
    - Excitation Wavelength Max: 650 nm
    - Emission Wavelength Max: 671 nm
    - Initial Brightness (per ThermoFisher Scientific): 5

*The emission wavelength is always longer (and thus has a lower energy) than the excitation wavelength, as energy is lost as heat when excited electrons return to their ground state

Glossary:

- **Antibody (Immunoglobulin)**: proteins produced by the immune system to bind foreign molecules in the body to facilitate their elimination
    - Produced by B cells - production mechanism harnessed to produce desirable antibodies in host animals which can then be used to detect molecules of interest in research
- **Antigen**: the foreign molecules which antibodies bind to
- **Epitope**: the attachment of point on an antigen for an antibody
- **Isotype**: classification of antibodies based on the shape of their heavy-chain constant region
- **IgG (immunoglobulin G)**: the most common type of antibody isotype in human serum
- **Animal Immunization**: method of antibody production where target antigen (or antibody) is injected into a host animal
- **Monoclonal Antibody**: antibody produced from different B cells in a host animal; can recognize multiple different epitopes
    - Recovered directly from host serum
- **Polyclonal Antibody**: antibody produced from identical cloned immune cells; only recognize a single epitope (higher specificity)
    - Expressed by monoclonal hybridoma cells (produced by fusing spleen cells from host with immortal myeloma cells)
- **Immunogen**: antigen which is able to evoke an immune response, including the production of antibodies
    - In antibody production, immunogens are created by conjugation of the target antigen with a carrier protein and then injected into the host animal
- **Recombinant Antibodies**: antibodies produced in vitro using synthetic genes; allows for long-term secure supply of identical antibodies
- **Cross Adsorption**: step in secondary antibody purification process where antibodies which bind to non-target immunoglobulins are filtered out

Data Collection

Female

~~Test~~ ***Cohort Summary:***

- ***Animals:*** 9 female adolescent (P48) C57BL/6 mice
  - Mice numbered (EN#): 46, 47, 53, 54, 62, 69, 71, 79, 80
  - 5 mice (EN# 46, 53, 62, 71, 79) assigned to RmTBI group
  - 4 mice (EN# 47, 54, 69, 80) assigned to sham injury group
- ***Injury Delivery:***
  - RmTBI: 50g projectile fired five times over 24 hours at a speed of 5m/s ± 0.2m/s at the heads of the mice
  - Sham injury: same conditions as RmTBI group, but projectile not fired
- ***Cryosectioning:***
  - 40µm-thick coronal slices
  - Serial sectioning used
- ***Immunohistochemistry:***
  - C1q:
    - Primary Antibody: rabbit α-C1q (Abcam, catalog # ab182451)
    - Secondary Antibody: donkey α-rabbit AF568 (Thermo Fisher, catalog # A10042)
  - C3:
    - Primary Antibody: goat α-C3 (MP Biomedicals, catalog # ICN55730)
    - Secondary Antibody: donkey α-goat AF 647 (Thermo Fisher, catalog # A32849)
- ***Image Analysis Parameters:***
  - Brightness & Contrast:
    - Min-Max:
      - C1q: 2-25
      - C3: 0-25
  - Subtract Background:
    - Rolling Ball Radius:
      - C1q: 1.5px
      - C3: 20px
  - Thresholding:
    - Range: 255
      - C1q: 45-~~225~~
      - C3: 20-255
  - Analyze Particles:
    - Size:
      - C1q: 3.5-infinity
      - C3: 3.5-infinity
- ***Data Refinement:***
  - Brain Slice Outliers:

Test Cohort

Female Cohort

        - C1q: None
        - C3: None
    - Mouse Outliers:
        - C1q:
            - RmTBI: None
            - Sham: #79
        - C3:
            - RmTBI: #54
            - Sham: None
- ***Statistical Test Results:***
    - Mean Particles Density:
        - C1q:
            - RmTBI: 23473 particles/mm^3
            - Sham Injury: 13342 particles/mm^3
        - C3:
            - RmTBI: 2860 particles/mm^3
            - Sham Injury: 49456 particles/mm^3
    - t-Test Results:
        - C1q: no statistically significant difference
        - C3: no statistically significant difference

*Underlined information indicates values which may change between different cohorts

		C10										
EN#	Image#	Area (mm2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound	Upper bound	Outlier?	Mouse average
46	1	0.424	33	0.01484	2223.719677						FALSE	
	2	0.424	15	0.01484	1010.781671						FALSE	
	3	0.424	94	0.01484	6334.231806	1617.250674	4278.975741	2661.725067	-2375.336927	8271.563342	FALSE	3189.577718
47	1	0.424	179	0.01484	12061.99461						FALSE	
	2	0.424	94	0.01484	6334.231806						FALSE	
	3	0.424	26	0.01484	1752.021563	4043.126685	9198.113208	5154.986523	-3689.3531	16930.59299	FALSE	6716.082659
53	1	0.424	22	0.01484	1482.479784						FALSE	
	2	0.424	110	0.01484	7412.398922						FALSE	
	3	0.424	102	0.01484	6873.315364	4177.897574	7142.857143	2964.959569	-269.541779	11590.2965	FALSE	5256.06469
54	1	0.424	50	0.01484	3369.272237						FALSE	
	2	0.424	301	0.01484	20283.01887						FALSE	
	3	0.424	386	0.01484	26010.78167	11826.14555	23146.90027	11320.75472	-5154.986523	40128.03235	FALSE	16554.35759
62	1	0.424	350	0.01484	23584.90566						FALSE	
	2	0.424	592	0.01484	39892.18329						FALSE	
	3	0.424	228	0.01484	15363.8814	19474.39353	31738.54447	12264.15094	1078.167116	50134.77089	FALSE	26280.32345
69	1	0.424	767	0.01484	51684.63612						FALSE	
	2	0.424	987	0.01484	66509.43396						FALSE	
	3	0.424	67	0.01484	4514.824798	28099.73046	59097.03504	30997.30458	-18396.22642	105592.9919	FALSE	40902.96496
71	1	0.424	531	0.01484	35781.67116						FALSE	
	2	0.424	274	0.01484	18463.61186						FALSE	
	3	0.424	25	0.01484	1684.636119	10074.12399	27122.64151	17048.51752	-15498.65229	52695.41779	FALSE	18643.30638
79	1	0.424	3428	0.01484	230997.3046						FALSE	
	2	0.424	468	0.01484	31536.38814						FALSE	
	3	0.424	2950	0.01484	198787.062	115161.7251	214892.1833	99730.45822	-34433.96226	364487.8706	FALSE	153773.5849
80	1	0.424	92	0.01484	6199.460916						FALSE	
	2	0.424	173	0.01484	11657.68194						FALSE	
	3	0.424	1058	0.01484	71293.80054	8928.571429	41475.74124	32547.16981	-39892.18329	90296.49596	FALSE	29716.98113

		C3										
EN#	Image#	Area (um2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound	Upper bound	Outlier?	Mouse average
46	1	0.424	79	0.01484	5323.450135						FALSE	
	2	0.424	247	0.01484	16644.20485						FALSE	
	3	0.424	2436	0.01484	164150.9434	10983.82749	90397.57412	79413.74663	-108136.792	209518.1941	FALSE	62039.53279
47	1	0.424	27	0.01484	1819.407008						FALSE	
	2	0.424	20	0.01484	1347.708895						FALSE	
	3	0.424	13	0.01484	876.0107817	1111.859838	1583.557951	471.6981132	404.3126685	2291.105121	FALSE	1347.708895
53	1	0.424	11	0.01484	741.2398922						FALSE	
	2	0.424	20	0.01484	1347.708895						FALSE	
	3	0.424	45	0.01484	3032.345013	1044.474394	2190.026954	1145.552561	-673.854447	3908.355795	FALSE	1707.097934
54	1	0.424	31	0.01484	2088.948787						FALSE	
	2	0.424	773	0.01484	52088.94879						FALSE	
	3	0.424	232	0.01484	15633.42318	8861.185984	33861.18598	25000	-28638.814	71361.18598	FALSE	23270.44025
62	1	0.424	134	0.01484	9029.649596						FALSE	
	2	0.424	213	0.01484	14353.09973						FALSE	
	3	0.424	207	0.01484	13948.78706	11489.21833	14150.9434	2661.725067	7496.630728	18143.531	FALSE	12443.84546
69	1	0.424	61	0.01484	4110.512129						FALSE	
	2	0.424	137	0.01484	9231.80593						FALSE	
	3	0.424	44	0.01484	2964.959569	3537.735849	6671.15903	3133.423181	-1162.39892	11371.2938	FALSE	5435.759209
71	1	0.424	4217	0.01484	284164.4205						FALSE	
	2	0.424	576	0.01484	38814.01617						FALSE	
	3	0.424	32	0.01484	2156.334232	20485.1752	161489.2183	141004.0431	-191020.889	372995.283	FALSE	108378.257
79	1	0.424	2525	0.01484	170148.248						FALSE	
	2	0.424	89	0.01484	5997.304582						FALSE	
	3	0.424	178	0.01484	11994.60916	8995.956873	91071.42857	82075.4717	-114117.251	214184.6361	FALSE	62713.38724
80	1	0.424	11	0.01484	741.2398922						FALSE	
	2	0.424	20	0.01484	1347.708895						FALSE	
	3	0.424	49	0.01484	3301.886792	1044.474394	2324.797844	1280.32345	-876.010782	4245.283019	FALSE	1796.945193

EN#	Injury	C1q	Q1	Q3	IQR	LB	UB	Outlier?
46	0	3189.578						FALSE
53	0	5256.065						FALSE
62	0	26280.32						FALSE
71	0	18643.31						FALSE
79	0	153773.6	5256.06469	26280.32345	21024.25876	-26280.3235	57816.71159	TRUE
47	1	6716.083						FALSE
54	1	16554.36						FALSE
69	1	40902.96						FALSE
80	1	29716.98	14094.78886	32513.47709	18418.68823	-13533.2435	60141.50943	FALSE

EN#	Injury	C3	Q1	Q3	IQR	LB	UB	Outlier?
46	0	62039.53						FALSE
53	0	1707.098						FALSE
62	0	12443.85						FALSE
71	0	108378.3						FALSE
79	0	62713.39	12443.84546	62713.38724	50269.54178	-62960.4672	138117.6999	FALSE
47	1	1347.709						FALSE
54	1	23270.44						TRUE
69	1	5435.759						FALSE
80	1	1796.945	1684.636119	9894.42947	8209.793351	-10630.0539	22209.1195	FALSE

Injury	C1q
0	3189.578
0	5256.065
0	26280.32
0	18643.31
0	
1	6716.083
1	16554.36
1	40902.96
1	29716.98

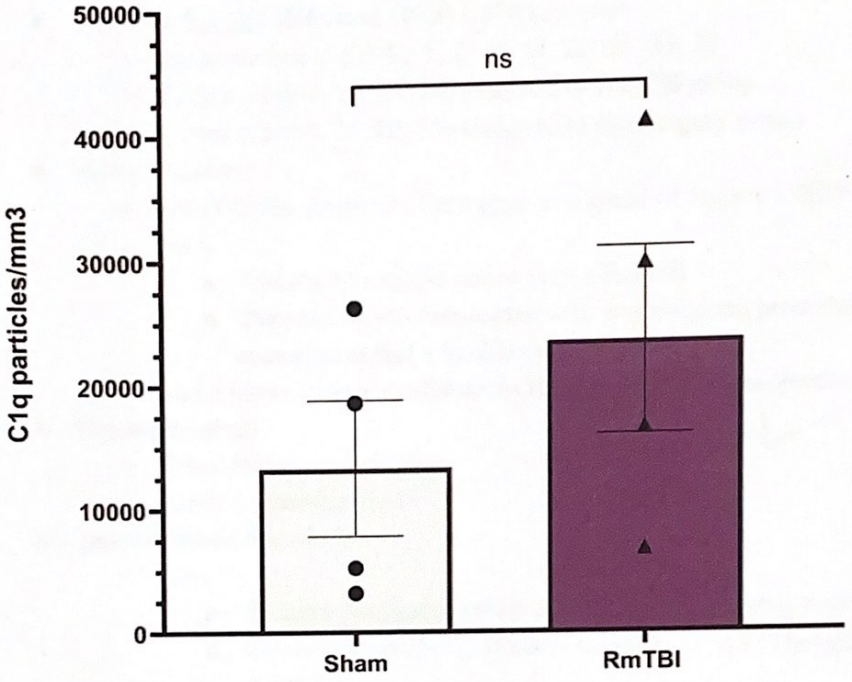
Injury	C3
0	62039.53
0	1707.098
0	12443.85
0	108378.3
0	62713.39
1	1347.709
1	
1	5435.759
1	1796.945

EN#	Injury	C1q	Q1	Q3	IQR	LB	UB	Outlier?
2	0	478.88589						FALSE
11	0	0687.3315						TRUE
20	0	2502.2461						FALSE
35	0	34344.115	26746.40611	43429.91914	16683.51303	1721.136568	68455.18868	FALSE
1	1	3072.7762						FALSE
10	1	7169.8113						FALSE
25	1	4752.9200						FALSE
36	1		18912.84816	35961.36568	17048.51752	-6659.928122	61534.14196	FALSE

EN#	Injury	C3	Q1	Q3	IQR	LB	UB	Outlier?
2	0	341.86882						FALSE
11	0							FALSE
20	0	92969.45						FALSE
35	0	47371.96	24606.91824	70170.70979	45563.79155	-43738.76909	138516.3971	FALSE
1	1	3113.2075						FALSE
10	1	190.47615						FALSE
25	1	84973.04						FALSE
36	1	34905.66	17632.52471	47422.50674	29789.98203	-27052.44834	92107.47978	FALSE

Table Analyzed	C1q				
Column B	RmTBI				
vs.	vs.				
Column A	Sham				
Unpaired t test					
P value	0.3173				
P value summary	ns				
Significantly different (P < 0.05)?	No				
One- or two-tailed P value?	Two-tailed				
t	df	t=1.091	df=6		
How big is the difference?					
Mean of column A	13342				
Mean of column B	23473				
Difference between means (B - A) ± SEM	10130 ± 9289				
95% confidence interval	-12599 to 32859				
R squared (eta squared)	0.1654				
F test to compare variances					
F	DFn	Dfd	1.845	3	3
P value	0.6275				
P value summary	ns				
Significantly different (P < 0.05)?	No				
Data analyzed					
Sample size	column A	4			
Sample size	column B	4			

Table Analyzed	C3				
Column B	RmTBI				
vs.	vs.				
Column A	Sham				
Unpaired t test					
P value	0.1205				
P value summary	ns				
Significantly different (P < 0.05)?	No				
One- or two-tailed P value?	Two-tailed				
t	df	t=1.809	df=6		
How big is the difference?					
Mean of column A	49456				
Mean of column B	2860				
Difference between means (B - A) ± SEM	-46596 ± 25761				
95% confidence interval	-109631 to 16439				
R squared (eta squared)	0.3529				
F test to compare variances					
F	DFn	Dfd	370.9	4	2
P value	0.0054				
P value summary	**				
Significantly different (P < 0.05)?	Yes				
Data analyzed					
Sample size	column A	5			
Sample size	column B	3			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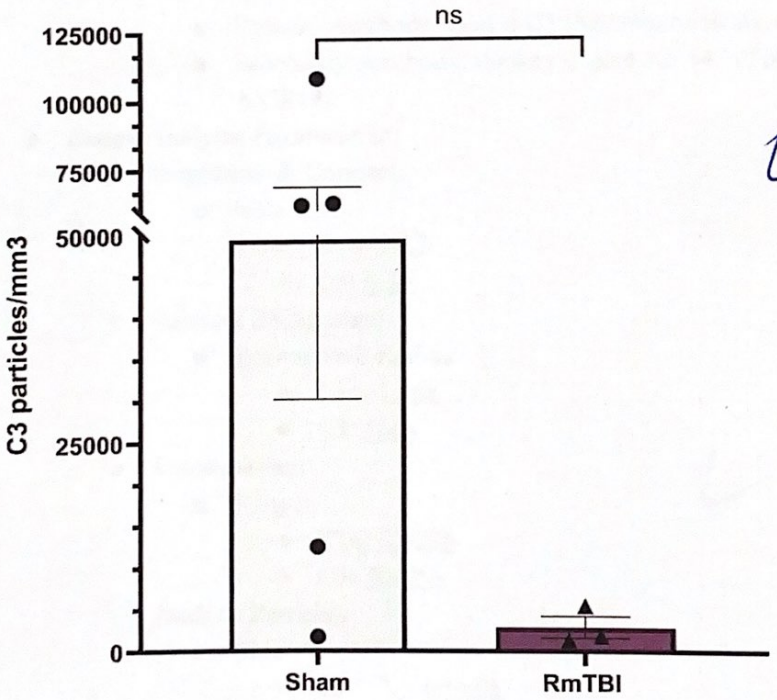
40000
30000
20000
10000
0
C1q particles/mm3
ns
Sham
RmTBI


125000
100000
75000
50000
25000
0
C3 particles/mm3
ns
Sham
RmTBI

Test Cohort Summary:

- ***Animals:*** 8 male adolescent (P48) C57BL/6 mice
  - Mice numbered (EN#): 1, 2, 10, 11, 20, 25, 35, 36
  - 4 mice (EN# 1, 10, 25, 36) assigned to RmTBI group
  - 4 mice (EN# 2, 11, 20, 35) assigned to sham injury group
- ***Injury Delivery:***
  - RmTBI: 50g projectile fired once at a speed of 5m/s ± 0.2m/s at the heads of the mice
    - Simulated a mTBI rather than a RmTBI
    - Purpose was to familiarize with experimental procedures and determine if experiment had a likelihood of success
  - Sham injury: same conditions as RmTBI group, but projectile not fired
- ***Cryosectioning:***
  - 40µm-thick coronal slices
  - Serial sectioning used
- ***Immunohistochemistry:***
  - C1q:
    - Primary Antibody: rabbit α-C1q (Abcam, catalog # ab182451)
    - Secondary Antibody: donkey α-rabbit AF568 (Thermo Fisher, catalog # A10042)
  - C3:
    - Primary Antibody: goat α-C3 (MP Biomedicals, catalog # ICN55730)
    - Secondary Antibody: donkey α-goat AF 647 (Thermo Fisher, catalog # A32849)
- ***Image Analysis Parameters:***
  - Brightness & Contrast:
    - Min-Max:
      - C1q: 2-25
      - C3: 0-25
  - Subtract Background:
    - Rolling Ball Radius:
      - C1q: 1.5px
      - C3: 20px
  - Thresholding:
    - Range:
      - C1q: 45-225
      - C3: 20-255
  - Analyze Particles:
    - Size:
      - C1q: 3.5-infinity

        - C3: 3.5-infinity
- ***Statistical Test Results:***
    - Mean Particles Density:
        - C1q:
            - RmTBI: 28332 particles/mm^3
            - Sham Injury: 25442 particles/mm^3
        - C3:
            - RmTBI: 36046 particles/mm^3
            - Sham Injury: 47394 particles/mm^3
    - t-Test Results:
        - C1q: no statistically significant difference
        - C3: no statistically significant difference

*Underlined information indicates values which may change between different cohorts

		C1q							
EN#	Image#	Area (mm2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound
1	1	0.424	128	0.01484	8625.336927				
	2	0.424	79	0.01484	5323.450135				
	3	0.424	375	0.01484	25269.54178	6974.393531	16947.43935	9973.045822	-7985.175202
2	1	0.424	93	0.01484	6266.846361				
	2	0.424	183	0.01484	12331.53639				
	3	0.424	146	0.01484	9838.274933	8052.560647	11084.90566	3032.345013	3504.043127
10	1	0.424	671	0.01484	45215.63342				
	2	0.424	674	0.01484	45417.78976				
	3	0.424	755	0.01484	50876.01078	45316.71159	48146.90027	2830.188679	41071.42857
11	1	0.424	1070	0.01484	72102.42588				
	2	0.424	886	0.01484	59703.50404				
	3	0.424	1191	0.01484	80256.06469	65902.96496	76179.24528	10276.28032	50488.54447
20	1	0.424	185	0.01484	12466.30728				
	2	0.424	557	0.01484	37533.69272				
	3	0.424	705	0.01484	47506.73854	25000	42520.21563	17520.21563	-1280.32345
25	1	0.424	160	0.01484	10781.67116				
	2	0.424	719	0.01484	48450.13477				
	3	0.424	223	0.01484	15026.95418	12904.31267	31738.54447	18834.23181	-15347.03504
35	1	0.424	655	0.01484	44137.46631				
	2	0.424	314	0.01484	21159.02965				
	3	0.424	560	0.01484	37735.84906	29447.43935	40936.65768	11489.21833	12213.61186

Upper bound	Outlier?	Mouse average
	FALSE	
	FALSE	
31907.00809	FALSE	13072.77628
	FALSE	
	FALSE	
15633.42318	FALSE	9478.885894
	FALSE	
	FALSE	
52392.18329	FALSE	47169.81132
	FALSE	
	FALSE	
91593.66577	FALSE	70687.33154
	FALSE	
	FALSE	
68800.53908	FALSE	32502.24618
	FALSE	
	FALSE	
59989.89218	FALSE	24752.92004
	FALSE	
	FALSE	
58170.48518	FALSE	34344.115

Table Analyzed	C1q				
Column B	RmTBI				
vs.	vs.				
Column A	Sham				
Unpaired t test					
P value	**0.8325**				
P value summary	**ns**				
Significantly different (P < 0.05)?	**No**				
One- or two-tailed P value?	Two-tailed				
t	df	t 0.2256	df=4		
How big is the difference?					
Mean of column A	25442				
Mean of column B	28332				
Difference between means (B - A) ± SEM	2890 ± 12809				
95% confidence interval	-32674 to 38454				
R squared (eta squared)	0.01257				
F test to compare variances					
F	DFn	Dfd	1.564	2	2
P value	0.78				
P value summary	ns				
Significantly different (P < 0.05)?	No				
Data analyzed					
Sample size	column A	3			
Sample size	column B	3			

		C3							
EN#	Image#	Area (um2)	Particles count	Volume (mm3)	Density (particles/mm3)	1st quartile (Q1)	3rd quartile (Q3)	IQR	Lower bound
1	1	0.424	303	0.01484	20417.78976				
	2	0.424	325	0.01484	21900.26954				
	3	0.424	401	0.01484	27021.56334	21159.02965	24460.91644	3301.886792	16206.19946
2	1	0.424	25	0.01484	1684.636119				
	2	0.424	36	0.01484	2425.876011				
	3	0.424	21	0.01484	1415.09434	1549.865229	2055.256065	505.3908356	791.7789757
10	1	0.424	16	0.01484	1078.167116				
	2	0.424	13	0.01484	876.0107817				
	3	0.424	24	0.01484	1617.250674	977.0889488	1347.708895	370.6199461	421.1590296
20	1	0.424	150	0.01484	10107.81671				
	2	0.424	662	0.01484	44609.16442				
	3	0.424	3327	0.01484	224191.3747	27358.49057	134400.2695	107041.779	-133204.1779
25	1	0.424	66	0.01484	4447.439353				
	2	0.424	2567	0.01484	172978.4367				
	3	0.424	1150	0.01484	77493.26146	40970.3504	125235.8491	84265.49865	-85427.89757
35	1	0.424	1457	0.01484	98180.59299				
	2	0.424	345	0.01484	23247.97844				
	3	0.424	307	0.01484	20687.33154	21967.65499	60714.28571	38746.63073	-36152.29111
36	1	0.424	495	0.01484	33355.79515				
	2	0.424	181	0.01484	12196.7655				
	3	0.424	878	0.01484	59164.42049	22776.28032	46260.10782	23483.82749	-12449.46092

Upper bound	Outlier?	Mouse average
	FALSE	
	FALSE	
29413.74663	FALSE	23113.20755
	FALSE	
	FALSE	
2813.342318	FALSE	1841.868823
	FALSE	
	FALSE	
1903.638814	FALSE	1190.47619
	FALSE	
	FALSE	
294962.938	FALSE	92969.45193
	FALSE	
	FALSE	
251634.097	FALSE	84973.04582
	FALSE	
	FALSE	
118834.2318	FALSE	47371.96765
	FALSE	
	FALSE	
81485.84906	FALSE	34905.66038

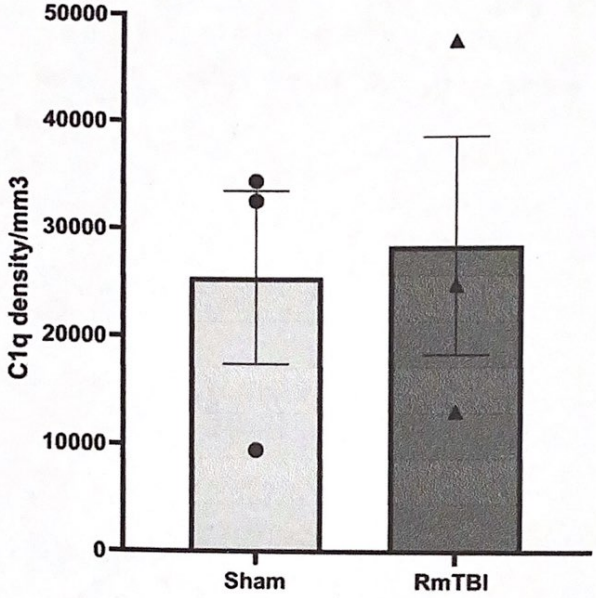
Table Analyzed	C3
Column B	RmTBI
vs.	vs.
Column A	Sham

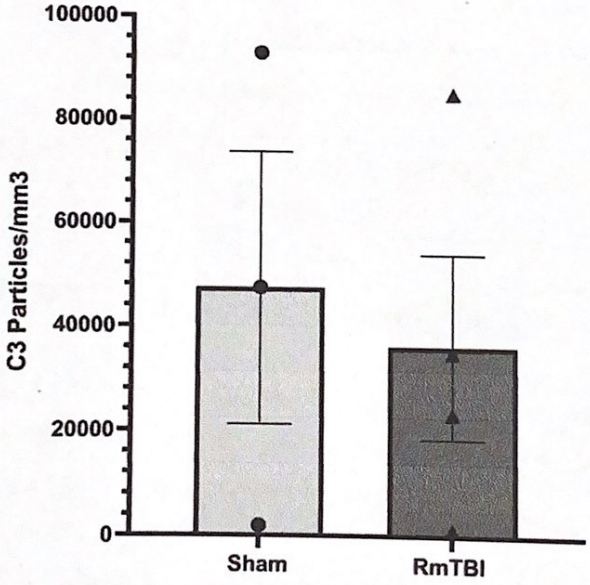
Unpaired t test				
P value	**0.7243**			
P value summary	**ns**			
Significantly different (P < 0.05)?	**No**			
One- or two-tailed P value?	Two-tailed			
t	df	0.3731	df=5	

How big is the difference?	
Mean of column A	47394
Mean of column B	36046
Difference between means (B - A) ± SEM	-11349 ± 30415
95% confidence interval	-89534 to 66837
R squared (eta squared)	0.02709

F test to compare variances					
F	DFn	Dfd	1.649	2	3
P value	0.6576				
P value summary	ns				
Significantly different (P < 0.05)?	No				

Data analyzed		
Sample size	column A	3
Sample size	column B	4


50000
40000
30000
20000
10000
0
C1q density/mm3
Sham
RmTBI


100000
80000
60000
40000
20000
0
C3 Particles/mm3
Sham
RmTBI

Test Cohort t-Test (C19): Two-sample Two-Tailed t-Test

RmTBI (1): 13073, 47170, 24753 $H_0: \mu_1 = \mu_2$

Sham (2): ~~9478~~ 9479, 32502, 34344 $H_a: \mu_1 \neq \mu_2$

$n_1 = 3 \quad n_2 = 3$

$\bar{x}_1 = 28332 \quad \bar{x}_2 = 25442$

$S_1 = 17328 \quad S_2 = 13855$

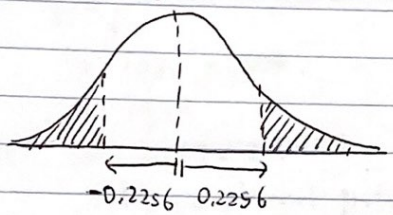
$S_1^2 = 300273540 \quad S_2^2 = 191953286$

$\alpha = 0.05 \quad df = 3+3-2 = 4$

$$t = \frac{28332 - 25442}{\sqrt{\frac{300273540}{3} + \frac{191953286}{3}}}$$

$= 0.22561...$

≈ 0.2256



$P(|t| > 0.2256) = 2 \times tcdf(-1 \times 10^{99}, -0.2256, 4)$

$= 0.83257..$

≈ 0.8326

$P > 0.05 \rightarrow$ Cannot reject H_0 (no significant difference)

Test Cohort t-Test (C3): Two-sample Two-Tailed t-Test

RmTBI (1): 23113, 1190, 84973, 34905 $H_0: \mu_1 = \mu_2$

Sham (2): 1842, 92969, 47372 $H_a: \mu_1 \neq \mu_2$

$n_1 = 4 \quad n_2 = 3$

$\bar{x}_1 = 36045 \quad \bar{x}_2 = 47394$

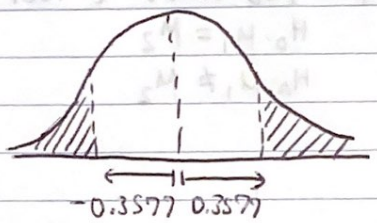
$S_1 = 35484 \quad S_2 = 45564$

$S_1^2 = 1259114256 \quad S_2^2 = 2076078096$

$\alpha = 0.05 \quad df = 3+4-2 = 5$

$$t = \frac{36045 - 47394}{\sqrt{\frac{1259114256}{4} + \frac{2076078096}{3}}}$$

$= -0.35767.. \approx -0.35787$



$P(|t|>0.3577) = 2 \times tcdf(-1 \times 10^{99}, -0.3577, 5)$

$= 0.73517.$

≈ 0.7352

$P > 0.05 \rightarrow$ Cannot reject H_0 (no significant difference)

Test Cohort Graphing (C1q)

RmTBI (1): 13073, 47170, 24753
Sham (2): 9479, 32502, 34344

$\bar{x}_1 = 28332 \quad \bar{x}_2 = 25442$

$n_1 = 3 \quad n_2 = 3$

$$SEM \approx \frac{S}{\sqrt{n}} = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n(n-1)}}$$

$$SEM_1 \approx \sqrt{\frac{(13073-28332)^2 + (47170-28332)^2 + (24753-28332)^2}{3(3-1)}}$$

$= 10004.3037...$

≈ 10004

~~Error Bars: 28332 ± 10004~~ ~~28332 ± 10004~~

~~$= 28332 \pm 30008$~~

Error Bars: $\bar{x} \pm SEM$

28332 ± 10004

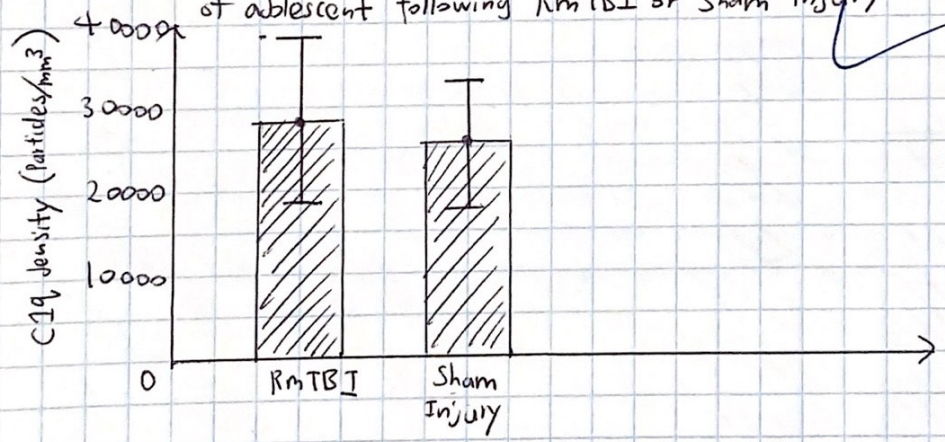
$$SEM_2 \approx \sqrt{\frac{(9479-25442)^2 + (32502-25442)^2 + (34344-25442)^2}{3(3-1)}}$$

$= 7999.02674...$

≈ 7999

Error Bars: 25442 ± 7999

C1q Complement protein density in the motor cortices of adolescent following RmTBI or Sham injury



Error bars overlap → no statistically significant difference

Test cohort Graphing (C3)

RmTBI (1): 23113, 1190, 84973, ~~34905~~ 34905

Sham (2): 1842, 92969, 47372

$n_1 = 4$ $n_2 = 3$

$\bar{x}_1 = 36045$ $\bar{x}_2 = 47394$

$$SEM_1 \approx \sqrt{\frac{(23113-36045)^2 + (1190-36045)^2 + (84973-36045)^2 + (34905-36045)^2}{4(4-1)}}$$

$= 17742.03209...$

≈ 17742

Error Bars: 36045 ± 17742

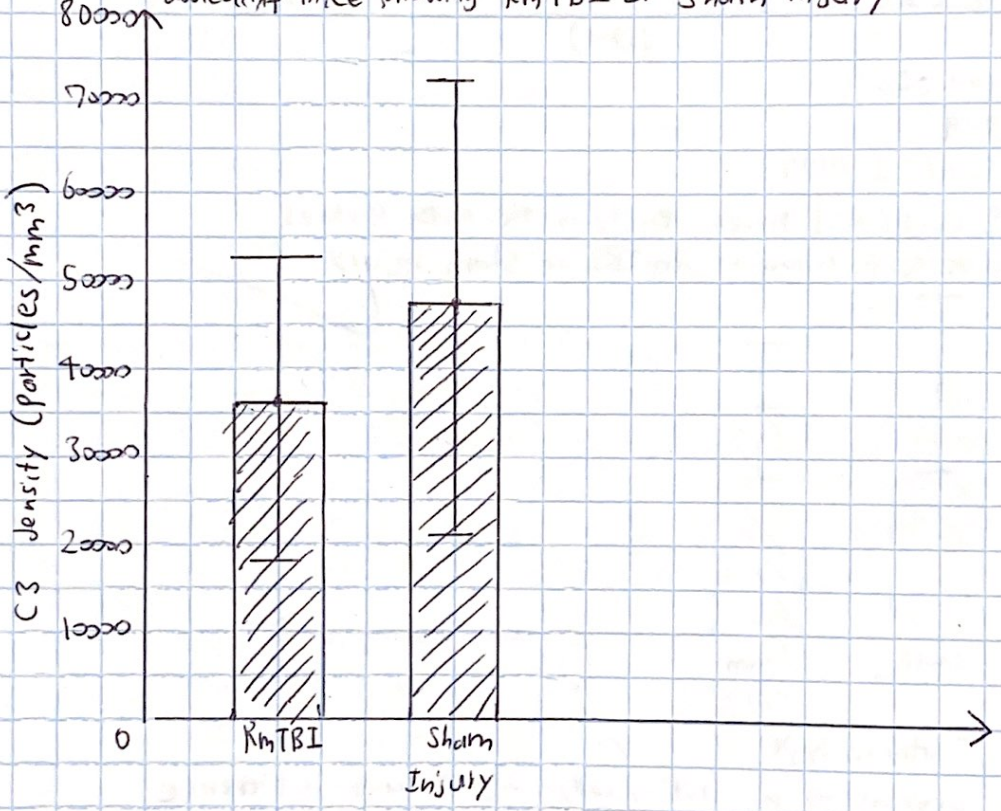
$$SEM_2 \approx \sqrt{\frac{(1842-47394)^2 + (92969-47394)^2 + (47372-47394)^2}{3(3-1)}}$$

$= 26306.10136...$

≈ 26306

Error Bars: 47394 ± 26306

C3 Complement protein densities in motor cortices of adolescent mice following RmTBI or Sham injury

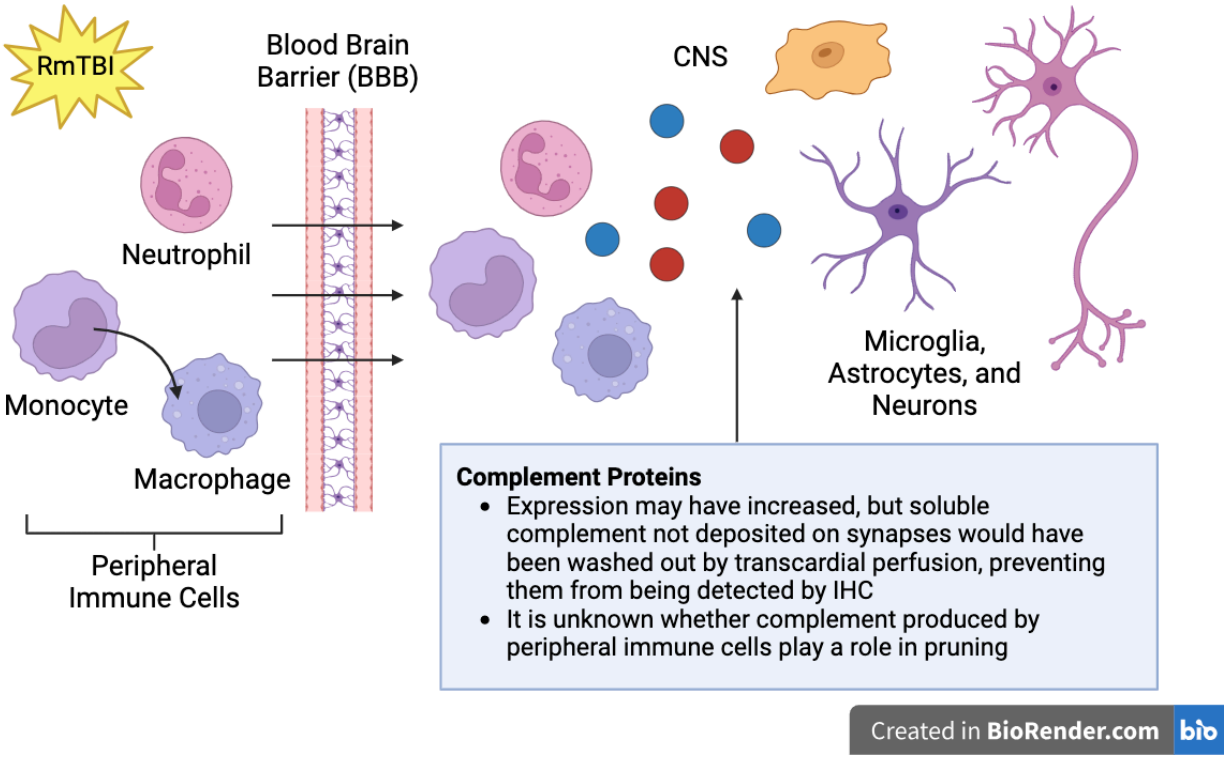


Error bars overlap → no statistically significant difference

Discussion, Conclusion, and Future Directions

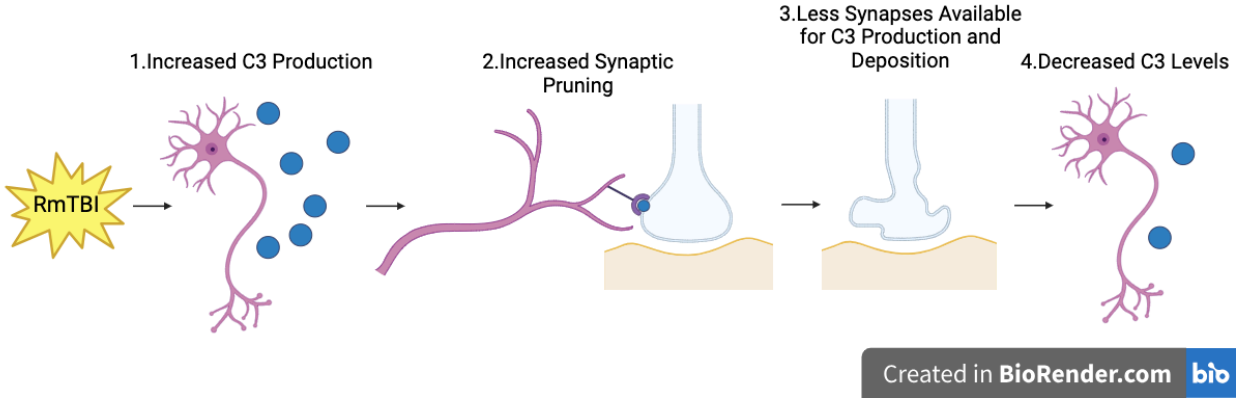
Discussion:

1. Possible Reasons for No Statistical Significance:
    a. **Low power of study**: sample size was too small to yield significant results
        - Sample sizes from experiment:
            - Female sham: n=5
            - Female RmTBI: n=4
            - Small sample size was due to limitations in resources provided for study
        - A good sample size is 10% of the population)
        - Past studies have used:
            - Female sham: n=31
            - Female RmTBI: n=30
            - Male sham: n=27
            - Male RmTBI: n=27
    b. **High variability in complement C1q and C3 expression between individual mice**
    c. **No biological effect**: RmTBIs do not induce significant changes in C1q and C3 complement expression in female mice
        - Results may be different for male mice
    d. **RmTBI cascades are time-dependent**: C1q and C3 complement levels may have differed based on time of euthanization/observation
        - Timeline of synaptic pruning (in particular, at what time after injury pruning peaks) is unknown
    e. **Transcardial perfusion washes out any soluble complement proteins, only allowing IHC to detect membrane-bound C1q and C3 deposited on the synapses**
        - Complement can either be soluble or membrane-bound
        - After RmTBI, brain recruits peripheral immune cells (neutrophil, monocytes/macrophages) into the CNS. These peripheral immune cells can produce C1q and C3. However, while the soluble complement produced by peripheral immune cells, microglia, astrocytes, and neurons may increase, these cannot be detected by IHC and it is unknown whether this is true or not.
        - Soluble complement may have played a role in synaptic pruning in the future, although it is unknown whether peripheral immune cell-synthesized complement play a role in pruning or not.
        - See diagram below:



2. Possible Reasons for Potential C3 Downregulation:
    a. **Neurons downregulate C3 expression in response to injury of inflammation**
        - No concrete biological reason as to why
    b. **Neuronal death after RmTBI decreases C3 expression**
        - Causes of neuron death after RmTBI (part of primary and secondary injury cascades):
            - **Diffuse Axonal Injury (DAI)**: differential velocities of white and grey matter tear neurons
            - **Wallerian Degeneration**: mechanoporation caused by stretching causes depolarization of neuron and apoptosis of the distal neuron segment
            - **Excitotoxicity**: depolarization of pre-synaptic neuron by mechanoporation causes excessive glutamate neurotransmitter release to the post-synaptic neuron, triggering calcium influx and subsequent neuronal death through mitochondrial dysfunction or cytotoxic molecule release
        - Neurons synthesize both C1q and C3 in CNS
            - It is unlikely that this is a cause, as we should also have observed a potential C1q downregulation if this were the case
    c. **Increased pruning of synapses decreases C3 expression**

- C3 expression may initially have increased, causing increased synaptic pruning. However, increased pruning would decrease the number of synapses available for C3 production and deposition (as synapses are part of neurons, which synthesize C3), leading to decreased C3 levels being detected by IHC over time.
- C3 expression may differ based on time after injury when observations were taken, and the certainty regarding the optimal time for observation is again handicapped by lack of knowledge regarding the timeline of pruning after RmTBIs.
- See diagram below:



Conclusion:

There was no statistically significant change in the expression of C1q or C3 complement proteins observed motor cortices of female adolescent mice after RmTBIs, as compared to mice which underwent sham injuries. However, there may have been a potential decrease in C3 expression in the motor cortices of female adolescent mice. My hypothesis that the mice subjected to RmTBIs would show a greater increase in C1q and C3 expression compared to mice subjected to sham injuries was not supported, but my experiment may have yielded different results had the number of soluble C1q and C3 complement proteins been able to be quantified. In regards to the effect of RmTBIs on synaptic pruning, which was the focus of my experiment, although C1q and C3 complement expression was not significantly affected by RmTBIs, changes in microglia density after RmTBIs may still cause changes in synaptic pruning, which may be responsible for the cognitive deficits seen after RmTBIs. It is also possible that C1q and C3 complement proteins (and by extension, the complement system) are not responsible for changes in synaptic pruning after RmTBIs and do not play a crucial role in the neurodegeneration observed after RmTBIs.

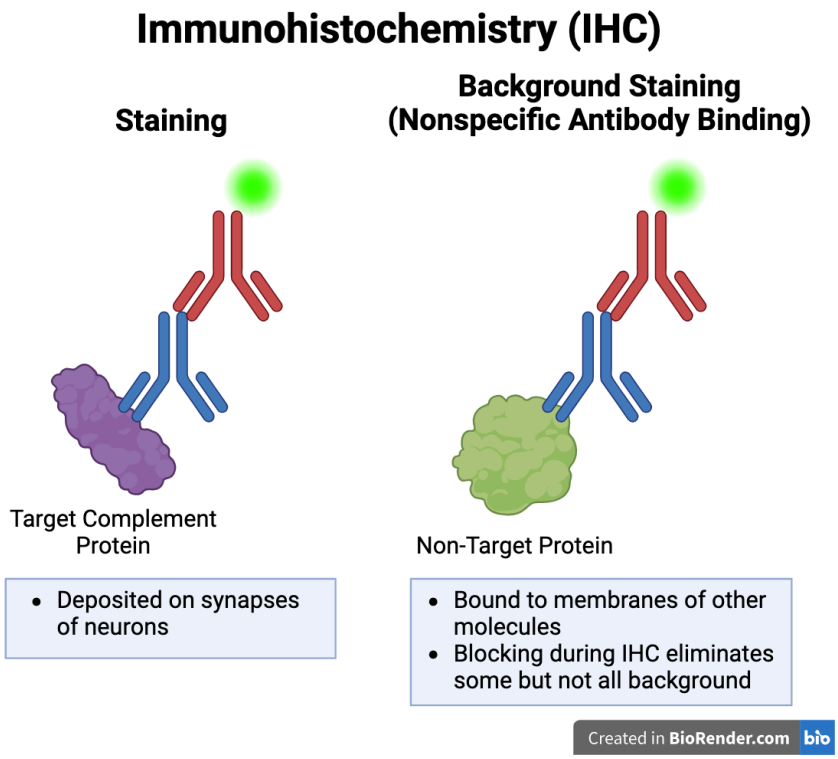
Significance:

1. **Better understand the unique pathophysiology of adolescent and female RmTBIs, especially in regards to synaptic pruning and neuroinflammation**
    a. Prevent the development of long-term cognitive deficits and NDDs in the future
    b. Raise awareness regarding the need for TBI research to be more representative of adolescent and females, who have previously been overlooked compared to adults, the elderly, and males. A lack of accurate representation of population demographics may be responsible for the lack of consistency between TBI studies and interfere with the applicability of their results.
    c. Improve accuracy of TBI education so that the public may be better informed of the potential risks associated with injury.
    d. My results showed that the role of the complement system in adolescent and female TBIs may not be as substantial as previously thought. These findings can be used to direct future studies in the direction of investigating other unique injury mechanisms of adolescent and female TBIs.
2. **Investigate the possibility of using complement inhibitors as a potential pharmaceutical TBI therapy**
    a. Inhibitors of C3 convertase (converts C3 into C3b, which is involved in synaptic pruning) has been proposed as a potential pharmaceutical TBI therapy
    b. Despite being successful in preclinical studies, no pharmaceutical TBI therapy to this date has succeeded in clinical trials. As a result, no FDA-approved pharmaceutical TBI therapy is commercially available.

c. My results indicate that complement inhibitors would not be an effective pharmaceutical TBI therapy, but these findings can be used to direct future studies in the direction of other proposed therapies
    i. For example, estrogen and progesterone (female sex hormones) are suspected to have neuroprotective effects and have been proposed as a potential pharmaceutical TBI therapy

Future Directions:

1. **Repeat experiment using male adolescent mice and.or a larger sample size**
    a. Male mice have been observed to experience more severe motor deficits than female mice. As a result, they have more dramatic changes in C1q and C3 expression in their motor cortices.
2. **Observe complement changes in other regions of the brain**
    a. Other proposed regions: **corpus callosum (CC), thalamus, angular insular cortex (AID)**
        i. Changes in microglia and dendritic spine density observed in these regions after RmTBIs, likely caused by changes in synaptic pruning.
            1. Males: decreased microglia and dendritic spine density in AID, increased dendritic spine density in MC
            2. Females: decreased spine density in AID
3. **Observe mice at different times after injury**
    a. Proposed times:
        i. **6 hours, 1 day, and 3 days after injury**: times of peak peripheral immune cell recruitment - may indicate peak in RmTBI primary and secondary cascades
        ii. **1 week, 2 weeks, and 1 month after injury**: determine long-term changes in complement expression
4. **Use cell markers to tag neurons and compared with IHC results to confirm if all detected particles are bound to neurons**
    a. Although blocking was performed to block all non-specific binding sites/epitopes, background staining may still occur. Background staining occurs when the primary antibodies bind to a non-target protein (bound to the membrane of a random cellular structure). This is in contrast to the principal stain, where primary antibodies correctly tag the C1q and C3 complement proteins (bound to the synapses of neurons). Since all C1q and C3 complement proteins are expected to be bound to the synapses of neurons, comparing images from the neuronal cell marker stains and the IHC would allow me to eliminate any signal that was background staining, improving the accuracy of my results.
    b. See diagram below:



5. **Use cell markers to tag peripheral immune cells, microglia, astrocytes, and neurons**
    a. As these cells are expected to be sources of C1q and C3 synthesis after RmTBIs, their approximate numbers can be used to estimate the number of C1q and C3 complement proteins (both membrane-bound and soluble) produced.