The effect and functioning of general alcohol anesthetics

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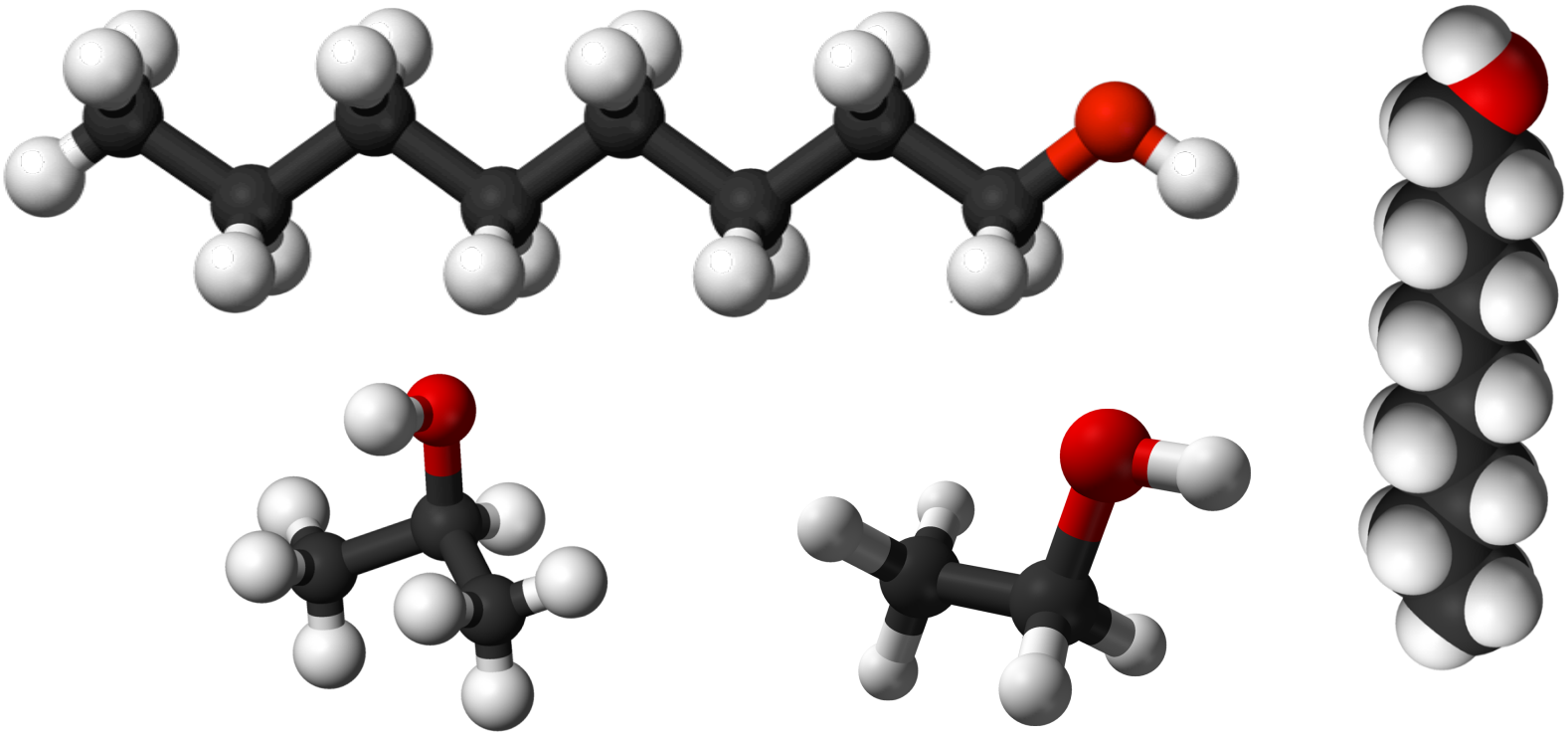


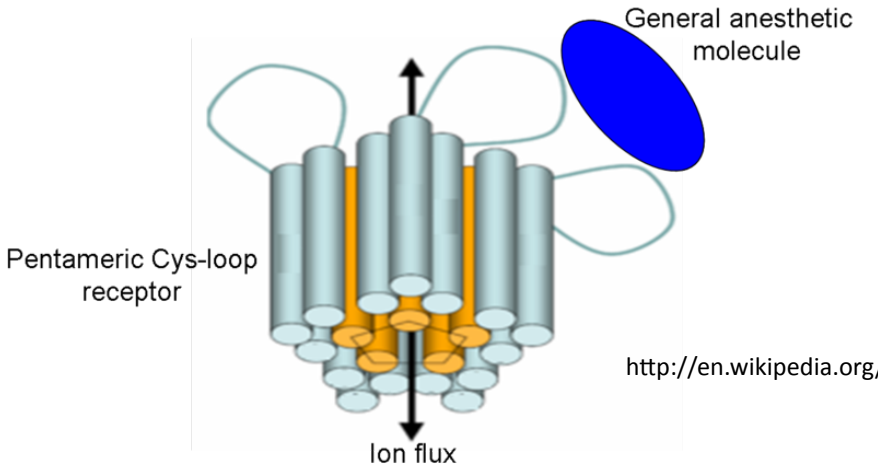
Goals

- To determine if anesthetics function by influencing liquid ordered – liquid disordered membrane lipids
- To determine how anesthetics affect the degranulation signaling pathway

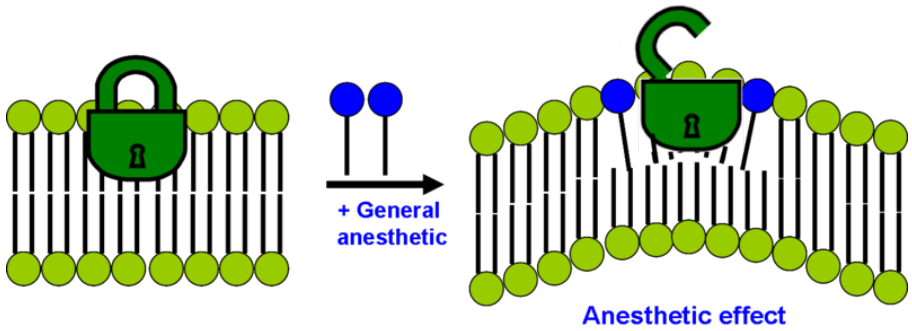
How do anesthetics work?

- Do they directly affect proteins?
- Do they indirectly affect proteins by changing the physical properties of surrounding lipids?
- Even within the field of lipids there is debate

Membrane protein theory of the anesthetic effect



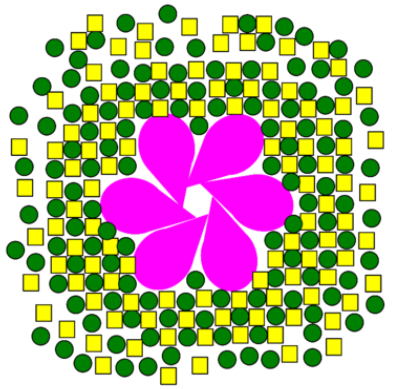
Lipid theory of the anesthetic effect



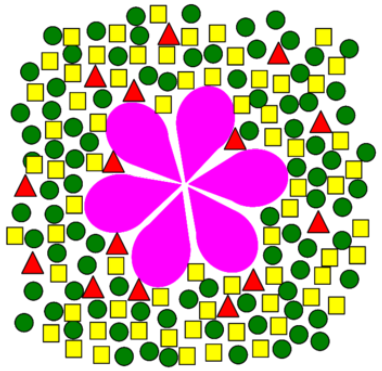
http://en.wikipedia.org/wiki/Theories_of_general_anaesthetic_action

Hypothesis

- Alcohol anesthetics act in a non-specific manner by integrating into the membrane to cause changes to the conformational equilibrium of lipids and proteins in the membrane.
- To test this, we will be seeing if there is a correlation between the anesthetic effect on phase transitioning and a functional signal pathway.



Well-ordered lipid/cholesterol ring around the gap junction connexon is keeping it open



Anesthetic oleamide disrupts this lipid/cholesterol ring, promoting a conformational change in connexon (closure)

http://en.wikipedia.org/wiki/Theories_of_general_anaesthetic_action

How to test hypothesis

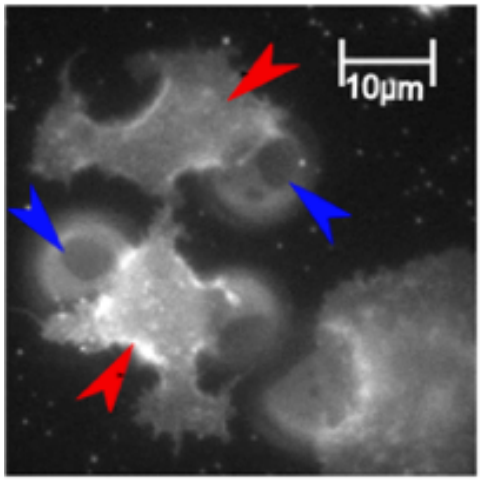
- The model system
  - Using blebs of rat basophilic leukemia (RBL) mast cells (white blood cells responsible for immune responses)
  - Observing phase transition temperature of blebs treated with several concentrations of different alcohols
- The signaling pathway
  - Using the degranulation immune response signaling pathway, as a system that has nothing to do with nerve responses or ion channels
  - Observing how alcohols affect certain stages in this pathway
- Connection between model system and degranulation
  - If alcohols have a similar effect on both experiments, correlations can be made providing evidence that general anesthetics have an indirect effect on proteins via lipid membranes.

Alcohols as liquid anesthetics

- The concentrations that are used in the following experiments are based on a paper published by Michael J. Pringle et. al.
- Determined concentration (AC50) for each alcohol at which 50% of tadpoles lost the righting reflex (the response allowing animals to properly orient their bodies)
- For our purposes, this concentration is termed “1X” and each multiple of this value is “2X”, “5X” etc.

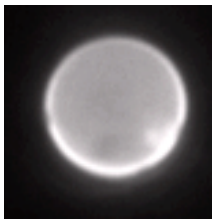
Phase transitions in blebs

- By treating RBL cells with formaldehyde and Dithiothreitol (DTT), the cells release blebs that can be used as more simple model membranes
- Membrane is labeled with DiI-C12, which prefers to partition into liquid disordered regions
- As temperature is decreases, blebs phase separate into distinct light phase (liquid disordered) and dark phase (liquid ordered) regions.

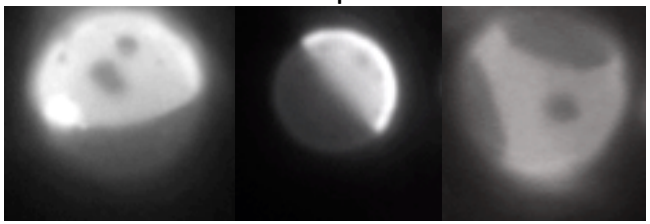


Matcha, Minimal model of plasma membrane heterogeneity requires coupling cortical actin to criticality., **2011**, *Biophys. J.*

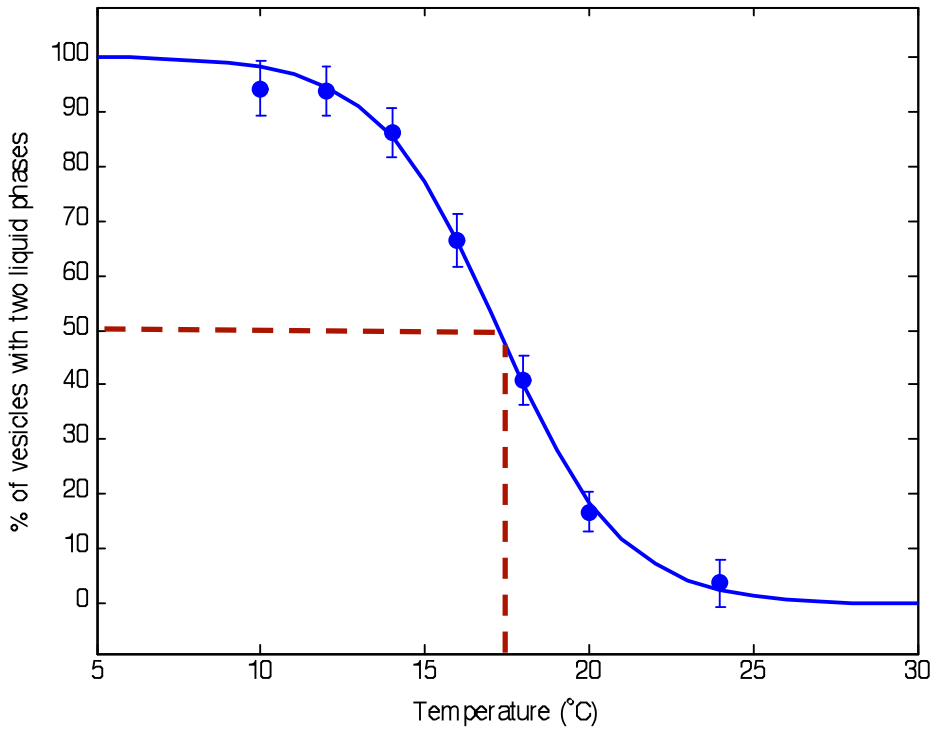
Above transition temperature



Below transition temperature



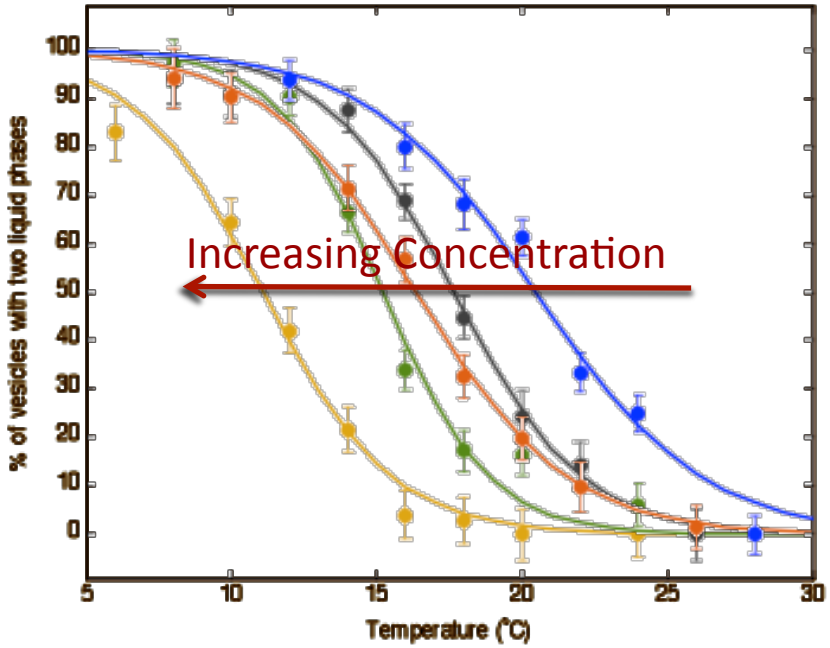
Analysis of phase transitioning



- Although each bleb has a clearly defined phase transition temperature, the population of blebs does not.
- By counting the number of phase separated blebs for each image, a sigmoidal curve representing phase transitioning is produced.
- The temperature at which 50% of the blebs are phase separated is known as the transition temperature.

Applying alcohols

- The blebs are treated with various multiples of a pre-determined concentration (AC50) for each given alcohol (ethanol, propanol, octanol, decanol), which lowers the phase transition temperature.



Control

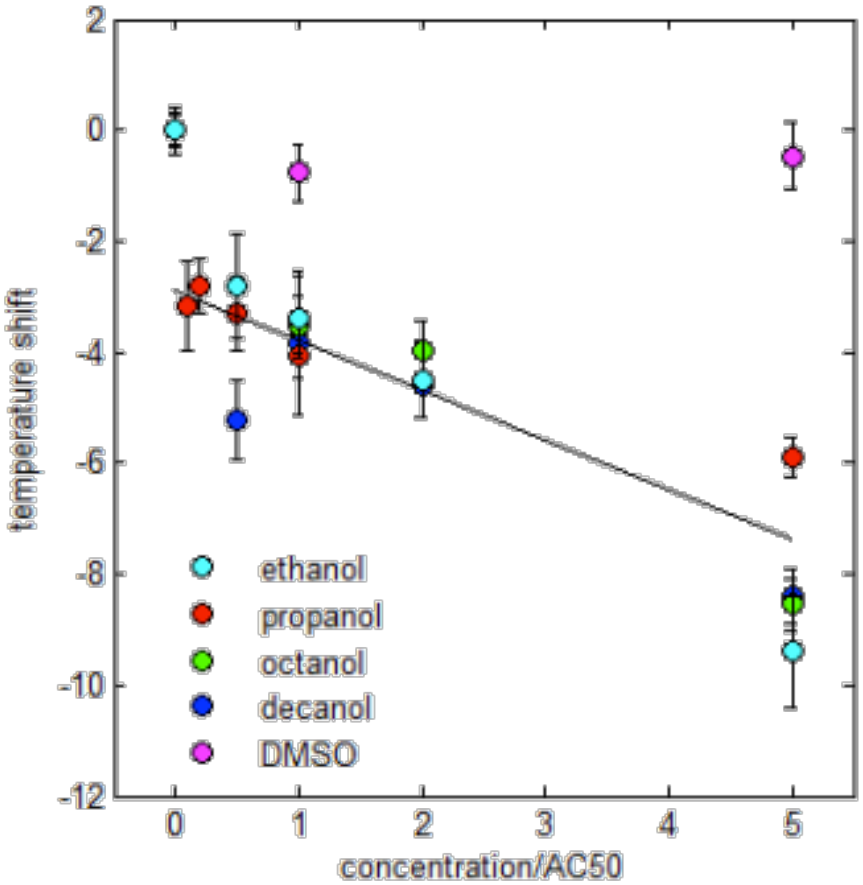
.5X ethanol

1X ethanol

2X ethanol

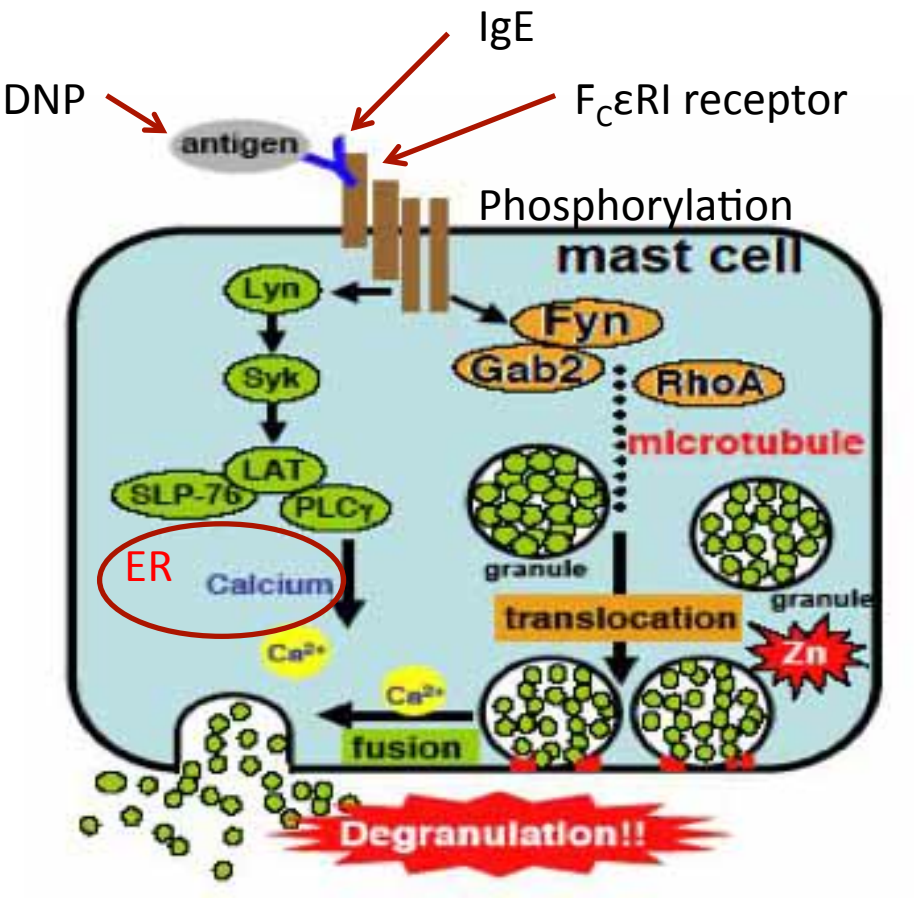
5X ethanol

Plot of transition temperature changes



- Points represent the difference in critical temperature between the experimental (added alcohol) and the control (no anesthetic)
- The decrease in transition temperature as concentration increases indicated the interference in membrane composition when alcohols are added
- When dividing by the AC50, all alcohols tested had a very similar effect
- DMSO does not have a documented anesthetic effect so it is being used as a control
  - Our data shows that DMSO has no effect, indicating that the effect seen by the alcohols is significant

Degranulation Signal Pathway



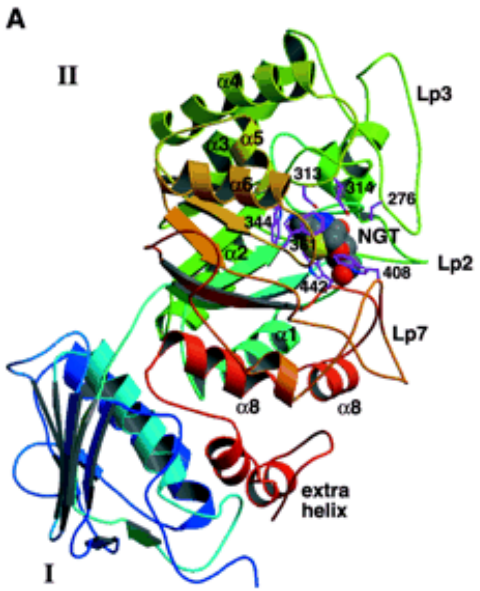
http://web.rcai.riken.jp/en/labo/cytokine/research.html

- Degranulation is an allergic response signaling pathway which is IgE receptor-mediated
- Used as a cell functionality assay

How we are using this signaling pathway

- Well documented that anesthetics have an effect on ion channels
- Demonstrating a clear effect on this unrelated pathway may suggest that it is a physical property of anesthetics that causes their effect rather than a more specific property.

Degranulation

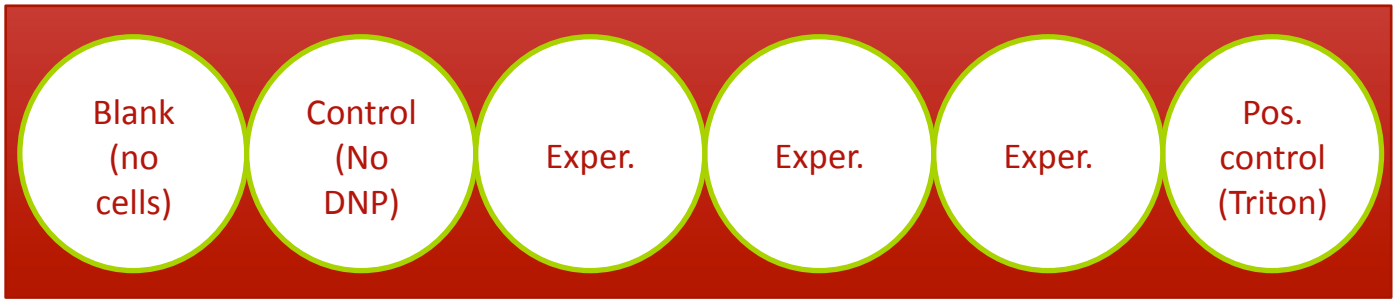


(Mark, Crystallographic Evidence for Substrate-assisted Catalysis in a Bacterial β-Hexosaminidase, 2001, *J. Bio. Chem.*)

- Absorbance assay that detects a specific enzyme (β-hexosaminidase) released in the granules
- Observing a late stage of the signaling pathway

How experiment was conducted

- For each given concentration of each anesthetic, the following set-up was used:



Degranulation data

1. Blank corrected raw data (355, 460)

	Pos. Cont.	Exp	Exp	Exp	Control	Blank	Pos. Cont.	Exp	Exp	Exp	Control	Blank	
A	23850	7049	5813	6349	4176		20398	6871	6712	6978	1534		**0X**
B	22670	5258	4188	4026	499		20757	3058	3735	3163	407		**1X**
C	19949	2477	2036	2497	659		21150	2012	2183	2012	591		**2X**
D	20075	3694	3627	3389	509		20392	758	706	1006	398		**5X**
E	20388	5505	5312	5136	385		21395	5484	5851	5880	561		**0X**
F	21234	5952	5593	4923	450		22471	3985	3270	4397	429		**1X**
G	19848	5619	5441	5235	553		23798	6052	6135	6262	633		**2X**
H	20729	5397	5453	5394	290		21901	1227	1537	1572	827		**5X**

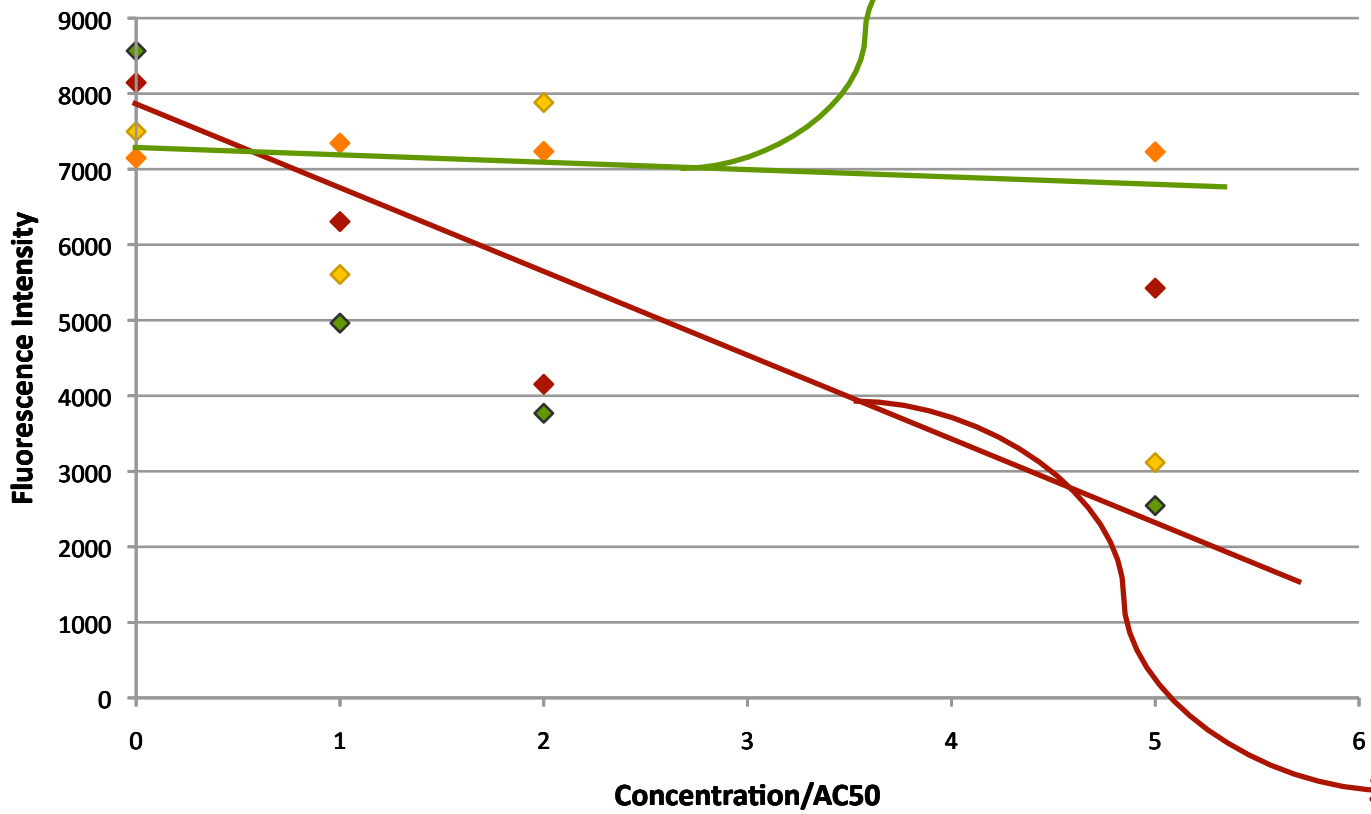
2. Raw Data (355, 460)

	Pos. Cont.	Exp	Exp	Exp	Control	Blank	Pos. Cont.	Exp	Exp	Exp	Control	Blank	
A	25592	8791	7555	8091	5918	1742	22110	8583	8424	8690	3246	1712	**0X**
B	24485	7073	6003	5841	2314	1815	22400	4701	5378	4806	2050	1643	**1X**
C	21764	4292	3851	4312	2474	1815	22849	3711	3882	3711	2290	1699	**2X**
D	21930	5549	5482	5244	2364	1855	22114	2480	2428	2728	2120	1722	**5X**
E	22218	7335	7142	6966	2215	1830	23155	7244	7611	7640	2321	1760	**0X**
F	23090	7808	7449	6779	2306	1856	24192	5706	4991	6118	2150	1721	**1X**
G	21652	7423	7245	7039	2357	1804	25530	7784	7867	7994	2365	1732	**2X**
H	22544	7212	7268	7209	2105	1815	23571	2897	3207	3242	2497	1670	**5X**

ETHANOL
PROPANOL
OCTANOL
DECANOL

Degranulation Data

However, there are several outliers, particularly for decanol, primarily due to the fact that many alcohols are quite volatile



- Ethanol
- Propanol
- Octanol
- Decanol

Most of the points follow a steadily decreasing absorbance amount as concentration increases

What this tells us

- The exocytosis of granules is affected by anesthetic alcohols in a comparable way to how transition temperature is affected
- Provides more evidence that anesthetic may be effecting cell functioning in an indirect manner through the lipid component of membranes

Future direction…

- We can probe earlier parts of pathway
  - Calcium released from ER
  - Activation of receptors in the membrane

Linking phase transition to degranulation

- Using transition temperature of blebs, we showed that alcohols lowered the transition temperatures by similar amounts for each given AC50 value
- Degranulation decreased in a similar manner for increasing concentrations of alcohols.
- Future experiments will provide further evidence for the functioning of general anesthetics
- Making the correlation between degranulation and phase transition provides more evidence that the liquid-liquid phase transitions is key to the functioning of anesthetics and that general anesthetics act indirectly on proteins through this method

QUESTIONS?