Supplementary Explanations

Usually, I come away from most (but not all) popular science publications—whether they are novels or clickbait media pieces—feeling like the author does not understand anything that they wrote about (this is how I feel whenever journalists write articles about bitcoin: nobody actually understands it). Not only is this a huge pet peeve of mine, but also my brain works in such a way that, in order for me to speak confidently about something, I *really* need to understand it. So, as I was writing the 14 chapters of this book, I would often go on long tangents about things like basic biochemistry, molecular biology wetlab techniques, how a neuron works, etc. Yet I acknowledge that all of those detailed undergraduate-level science tangents would be more information than the average person cares to know. While I did include some of it in the main text of this book, because I have to come across like I know what I'm talking about, there was a lot of interesting content that was omitted. The compromise that I came up with then was to copy and paste all of the tangential, sciencey explanations at the end of the book and make a new section called Supplementary Explanations (for the two people who wanted to read an undergraduate-level biochemistry textbook).

While writing a biochemistry textbook is probably overkill, the breakthroughs of this book would not have been possible if it were not for my deep curiosity and need to understand how the things I write about actually work. Plus, academia is not without credibility issues either. For one, there is a reproducibility crisis. I would attribute the reproducibility crisis in large part to academia's publish-or-perish culture. When people live under the threat of "perish" if they don't "publish," that means that they are operating in survival. As you already know if you have read this book, or as you will know if you have yet to read this book, the human mind is less intelligent in survival mode, because the survival part of the brain *only cares* that it has what it needs to survive, it *does not care* about how it gets it or where it comes from. So researchers that publish in the publish-or-perish environment have a powerful incentive not to care if the content of their publications is meaningful or reproducible; their only goal is to *avoid* perishing.

Even so-called high impact journals like *Nature* and *Cell* aren't immune. Therefore, I was highly fastidious in my attention to detail, examining figures in supplemental sections, and I did not write about any concept unless I understood the concept in enough detail, down to the laboratory procedure. If I did not understand certain details, I investigated.

I have done the best that I can to offer as much detail into the things that I write about as possible in this section of the book. Of course, there will always be more details for those who share my curiosity (I could spend another five months writing this book and expanding this section if I have a financial incentive to do so). One thing I did omit was an in-depth description of how to calculate statistical significance because—I'm sorry—that is just too goddamn boring. I also couldn't get around to explaining chromatography, that is just too much for *this* book, even though it is a purification tool used extensively in molecular biology wet labs. Explaining chromatography would be a whole nother book and this section is already overkill enough as it is.

I now leave this section to the curious reader to explore. It contains interesting insights about the world that I came to while writing and researching this book. For example, the "How a neurons works" portion of this section contains an explanation of why we succumb to brain freeze when

we consume an icy beverage, and it also contains a fun analogy showcasing the effects of alcohol on the brain/mind. If one section seems boring, feel free to skip it. If you want to know even more, consult Google Images and YouTube. I have to give credit where credit is due, and Google AI has been *enormously helpful* in helping me to ensure that I'm using terms correctly, and that my analogies are sound. I was able to ask it very specific questions, for example about lab techniques or how T cells might work. I would have loved to include more pictures in this book, but including color images in a book is difficult because it is significantly more expensive and requires a color printing option for the entire book. Of course, with the right financial incentives, I would love to include more pictures in a future edition of this book.

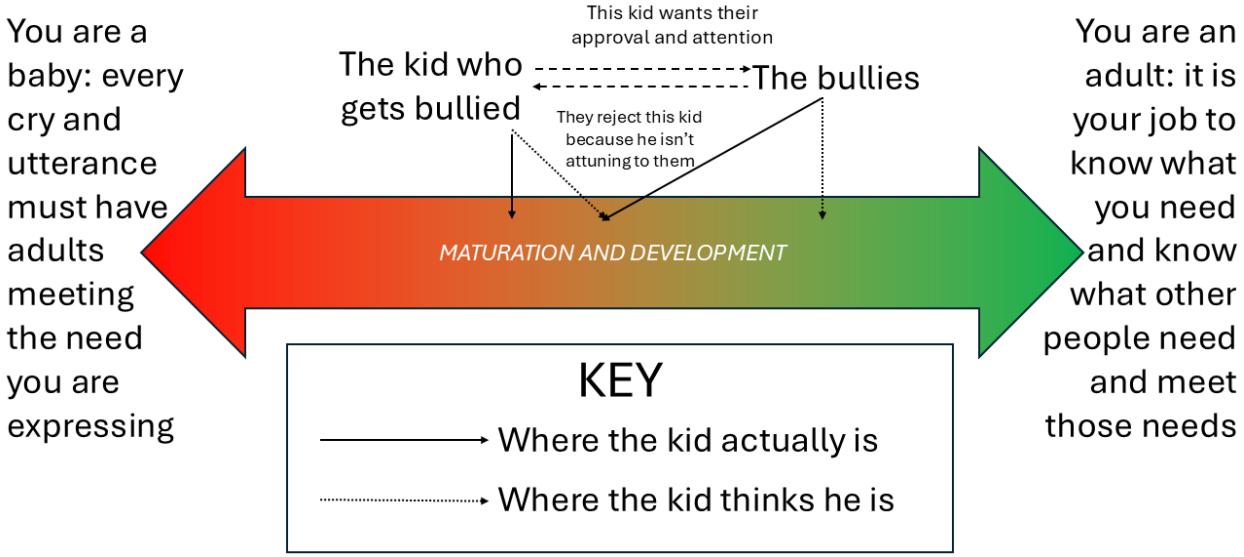
Regardless, enjoy the read!

How Arrested Development Results in Bullying

When I mentioned the story of Daniel in Chapter 5, I mentioned how arrested development causes children to be the victim of bullying. Here is what I meant:

If you think of a human infant, *every cry, utterance and motion for attention MUST be regarded as valid*. Not only must it be regarded as valid, but it must also be followed by an adult caregiver sacrificing any other short-term pursuit and tending to the baby's immediate need. A baby must be able to *expect* that this will be the case if it is to survive.

Now, when we are adults, we know that we cannot expect other adults to sacrifice their short term wants and needs if we cry. As adults, it is our responsibility to be conscious of both what we need and what *other* people need and to create win-win relationships in which all parties can give and receive the things that they need. You can thus think of maturation as the process of transitioning from the infant stage to the adult stage.



Development largely happens through having *experiences* where we express ourselves with other people, and the feedback that we get from those people will positively or negatively reinforce our self-expression. Usually, the experiences involve expressing ourselves and then having our self-expression received positively by other people. A toddler learning how to speak will try every sound that it can and when he or she is understood, then the adults will signal that they understand the toddler and thus the use of the mouth to pronounce a word like “mama” or “dada” is reinforced, as the parents respond positively to those utterances.

Growing up, emotions become more complex and more nuanced. In order to develop into these more sophisticated emotions, these emotions must be expressed amongst other people who respond with acceptance and understanding. If our life has deprived us of having experiences with other people in which we can express ourselves, then the *need* to have those experiences will not disappear even though the body may mature. Deprivation of experiences then causes people to become incapable of nuanced adult emotions and perspectives, even though the body has reached the adult stage. Thus, we would say that development has been halted, or arrested.

Deprivation of an experience necessary for emotional or psychological development is actually traumatic. Trauma can be defined as distress without resolution. Developmental arrests often manifest as rolling snowballs, where a developmental arrest makes it hard for a child to socialize because the child is unsure of how to express him or herself. Often the child will withdraw. If that happens, then the child will in all likelihood go through even greater deprivation of experiences needed for their development, making them further behind. This is a cruel and vicious cycle that usually ends with an adult who has a great deal of unconscious resentment toward society.

Very often, the cruel, rolling snowball of arrested development looks like bullying. I would normally begin this paragraph by introducing the idea of schoolyard bullying. Unfortunately, children in the public school system spend almost no time outside, and all physical touch amongst children has been abjectly forbidden by the incompetent workers in the public education system. So boomers, who have spent the last several decades living vicariously through the T.V. instead of in real life, need to eliminate this mental image of fist-fights in the playground (I’d much rather the kids be fist-fighting than being chemically lobotomized with drugs like Ritalin or have to deal with this gay social isolation sh*t that we have now, to be honest).

With any and all touch and physical contact being seen as infinitely worse than beating a child half to death by the women who work in our modern school system, children are quickly and harshly conditioned out of any and all physical touch (the women are content to deny their need for physical touch and instead seek fantasy gratification by reading bestselling books like *50 Shades of Grey* or *Morning Glory Milking Farm*). So instead of fistfighting, bullying is now the worst *Mean Girls* backstabbing drama amongst both the boys and the girls.

Now that we’ve established that banning physical touch has not in any way improved bullying in schools, let us return to the idea of the spectrum between infancy and adulthood. Chronic bullying tends to occur when you have one child that is more on the infancy side of the spectrum trying to get the attention and approval of other children who are more on the adult end of the

spectrum. This will create friction between these two children, and leave the child who is bullied in what can be an extremely painful place where he or she is chronically unable to get what they need from their peers.

A toddler is not capable of meeting the needs of a baby, and to insist that a toddler shoulder the responsibility of taking care of a baby would be highly traumatic to the toddler (as well as the baby). Obviously, developmental mismatches that go before bullying are far less extreme than a toddler taking care of a baby; it usually instead takes the form of one child wanting attention and belonging from another child but acting in a way that is unsuccessful in getting him in what he wants. This is usually because he is not aware of how his behavior is being received by the other child from whom approval is wanted, similar to how a baby does not have any conscious awareness of how it is received when it cries after it's bumped its toe on something. (I am not saying that bullies are inherently mature, nor am I trying to liken a preteen child who is bullied to a baby; I'm only using the example of a baby because it is an extreme example and extreme examples have the benefit of being extremely clear).

If somebody else wants attention from you, the Golden Rule would stipulate that the best thing for you to do is to give that person the attention that they want, and to give it lavishly. The Golden Rule however, does require that your perception of both yourself and the other person be accurate. The baby cannot fully perceive the complex emotional needs of an adult. Babies can only learn to perceive the complex emotional needs of adults when their needs as children are totally recognized, understood, and met. If their needs are unrecognized and unmet as they go from childhood into adulthood—which is the case in the public education system—then they will remain stuck at that phase in development until the unmet need is recognized and met. These developmental needs can *only* be met in relationships. Frankly, we're not very good at forming relationships that meet our developmental needs. For many people, the *only* place that they can even conceive of meeting any of these needs is in sexual relationships. This also explains why Daniel had crushes on the monks at the monastery he attended high in school.

In an environment where everybody is developmentally arrested, you essentially have a lot of toddlers without the wisdom of an adult. You can bet that the survival part of the brain is very active in an environment like this, and this also means that the superconscious mind is less active. In an environment of emotional toddlers, the ones that act and appear the most adultlike bring the least amount of distress, even if it isn't true to what they really feel. Conversely, the people who act the most babyish in this environment bring the most distress, even if it is true to what's really going on inside. Bullying then, can be thought of a primitive strategy employed by the survival part of the brain to try and "shut up" the toddlerish behavior by other emotionally stunted adults, because constructively responding to the person whose behavior may be unintentionally abrasive is too much work for a toddler.

I am certainly not saying that bullies are in any way inherently mature. However, chronically finding yourself on the receiving end of bullying is not a sign of virtue either, nor is it a red badge of courage. The right thing to do is to accept people where they are and to be patient with and understand them. But if adults do not effectively model compassionate understanding to children, then it is unfair to expect children to treat each other with compassionate understanding (often teachers, I find, are people who have mastered masking their own arrested development).

The blame here lies mostly with the adults—the parents, teachers and the school system. To a lesser extent some blame still lies on the bully. I don't blame the person who is a victim of bullying, but you do owe it to yourself to use the assistance of your superconscious mind to empower yourself in dysfunctional environments.

If a perception that extols the correct application of the Golden Rule were the dominant perception in the children's environment, then bullying would not occur. Yet the public education system does not do what's best for children, and often parents fail in giving their kids what they truly need for their development. Children are forced to obey and conform to the rules whether it is in their best interest or not, and so they will often have to put on a façade of acting like they don't need things that they really do need. So, when another kid who is not as far along in terms of development comes along and ultimately shows them that they're not as mature as they would like to deceive themselves that they are, that induces the fear of taking away something that they created for their protection. And fearful people attack.

The public school system does not try to fix the underlying perceptual problems that are, frankly, embedded into its very core. Instead, the administrators will then hire some bimbo whose husband probably makes all of the money to do some kind of performative "anti-bullying" campaign. The teachers and administrators will congratulate themselves for their empty words, but nothing will really change as a result of their "anti-bullying" shtick.

Does this explanation of bullying apply universally? No. Are there many other reasons kids and adults bully one another? Absolutely. But this does well to explain how some kids, like Daniel from Chapter 5, tend to chronically find themselves on the receiving end of bullying.

Behavioral Reinforcement
Feel free to ignore this section if you're not an idiot behavioral specialist

There are behavioral scientists who will insist that I'm using the term "negative reinforcement" incorrectly. Behavioral scientists define positive reinforcement as "giving something to the subject that makes the behavior more" and negative reinforcement as "taking something away from the subject in response to a behavior that also makes the behavior more." Most people, when they think of the term "negative reinforcement," think that negative reinforcement makes the behavior *less*. Behavioral scientists can be quite stupid (although not all; just the ones who get a lot of authority within the field), and they fool themselves into thinking that understanding unnecessarily complicated terminology makes them *smart*. It doesn't. The point of terminology is to make the content *clear*, *not further obfuscation*.

Therefore, the definition that I propose and use for positive reinforcement (which is basically synonymous with "reinforcement") is "giving the subject something that the subject wants or taking away something that the subject does not want in response to behavior, making the behavior *more*." Negative reinforcement then, means "giving the subject something they don't want or taking away something from the subject that they *do* want in response to the behavior, thereby making it less."

How idiot behavioral scientists use the term "negative reinforcement"

ACTION/OUTCOME	**Making the behavior more**	**Making the behavior less**
Giving something	**Positive reinforcement**—you're giving the person something that they want in response to the behavior	**Positive punishment**—you're giving the person something they don't want in response to the behavior
Taking something away	**Negative reinforcement**—you're taking something away that the person doesn't want in response to the behavior	**Negative punishment**—you're taking away something that the person does want in response to the behavior

How normal, sane people use the term "negative reinforcement"

ACTION/OUTCOME	**Making the behavior more**	**Making the behavior less**
Giving something	**(Positive) reinforcement**—you're giving the person something that they want in response to the behavior	**Negative reinforcement**—you're giving the person something they don't want in response to the behavior
Taking something away	**(Positive) reinforcement**—you're taking something away that the person doesn't want in response to the behavior	**Negative reinforcement**—you are taking away something that the person *does* want in response to the behavior

Reinforcement is very basic. For example, you feel like your head is itchy and you scratch the itch and feel the relief, then the relief that you feel will reinforce the behavior of scratching the itch. Taking away the itch positively reinforces the behavior of scratching. Feeling the itch negatively reinforces the behavior of *not scratching the itch*. You don't have to get bogged down into the weeds of "is it positive or negative reinforcement? Are you *giving* relief or are you *taking away* the itch?" And my terminology, the commonsense terminology, works with what most people describe as negative reinforcement. For instance, most people might think of a mean, incompetent (and probably fat) coach yelling at and haranguing his football team for not running sprints as an example of "negative reinforcement." The coach is trying to get his team to work harder (under the false belief that hard work *always* results in success) and he is giving his team something that they *don't want* in response to perceived laziness. My definitions are consistent with this use of the term "negative reinforcement": by yelling and screaming at his team for showing signs of fatigue, the coach is giving his team something that they *don't want* in response to a behavior, so then they won't *show* signs of fatigue. (Positive reinforcement for *wanted* behaviors tends to be more effective in most circumstances instead of giving energy and attention toward behaviors that you *don't want*).

Note also how behavioral scientists, since they have taken away a word that is very useful, must now come up with another word for negative reinforcement. And so, they choose the word "punishment." When the word "punishment" is used by normal, sane people, it usually means something along the lines of "an infliction of pain or discomfort in response to an unwanted behavior that is *intended* to reduce the unwanted behavior."

This is problematic on many levels. For one, punishment does not necessarily diminish an unwanted behavior. Often, punishments can inadvertently reinforce behaviors by giving *attention* to the unwanted behavior. Getting attention is often the *function* of a behavior, and some people decide that negative attention is better than no attention and so, in those cases, punishments will result in *more* of the behavior, not less of it. In a situation like that, if you want to lessen the behavior then you must *ignore* it, not punish it. The intelligent way to address unwanted behaviors is to *perceive them correctly*, and in perceiving the behavior correctly, you identify the *function* of the behavior and then offer a *replacement behavior* that fills the function of the unwanted behavior but is not disruptive or unwanted.

It is well understood in behavioral science that punishment is not correction. Behavior that is punished will decrease in the context where the punishment was administered. However, in contexts where there is no punishment, the behavior that is punished will amplify. This is because punishment does not address the underlying function of the behavior. I'm sure that we can all point to examples of this, how fainting-on-couches moral grandstanding never fixes the problem. We demonize sexual expression and curiosity and so the internet is now the only place where we can express those things without punishment, judgement or social censure. Now everybody is addicted to pornography.

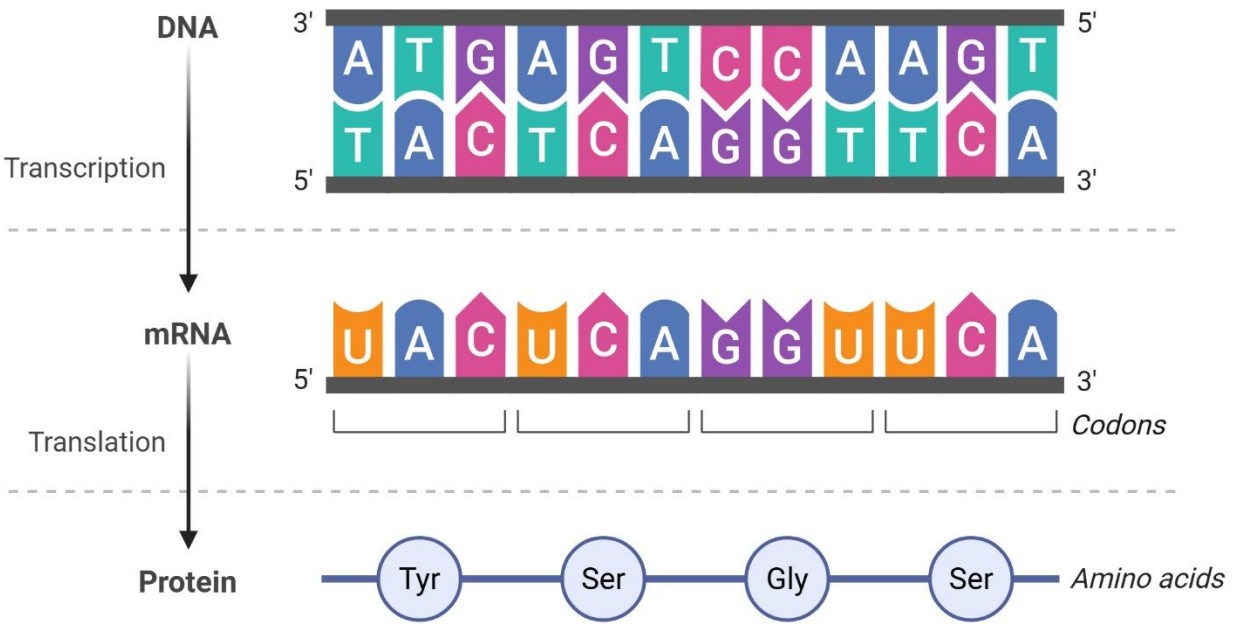
Additionally, punishment also creates *learned helplessness*. Because the function is completely unaddressed, the *need* that it was *the function of the behavior* to try and *meet* has not gone away. And now, the person who has been punished has come to expect pain in response to trying to meet their underlying need, which they cannot do anything about. And so, this person will succumb to learned helplessness.

The mind can often go into complete denial about the function of the behavior (try telling a TV addict that he is watching TV to live vicariously through *Friends* because he is lonely—"what are you talking about! I don't feel lonely!"). Denial is used to not only deny the need, but also to deny the pain of the need being unmet.

You cannot change your mind by changing your behavior, but you can change your mind. Instead of going into denial about your unconscious behaviors, it is good to be curious and open-minded. You must be willing to suspend judgment and admit what it is that you want "I'm doing this thing because I want attention," and then question it "is there a better way for me to obtain the attention that I want? After all, I can't seem to not want it." In asking that question and choosing to perceive the behavior with curiosity, the mind will automatically look for ways to get you what you want and overlook anything that is not useful to that end.

Basic Biochemistry

As a quick biochemistry review, the central dogma of molecular biology is that genes exist as DNA of a specific sequence of adenine, cytosine, thymine and guanine (you don't have to worry about the specific differences among those four, just know that there are four—which we'll abbreviate as A, T, C and G). Very similar to how computers encode all information as a combination of 1s and 0s, DNA encodes the information of how to build proteins based on the combination of A, T, C and G.



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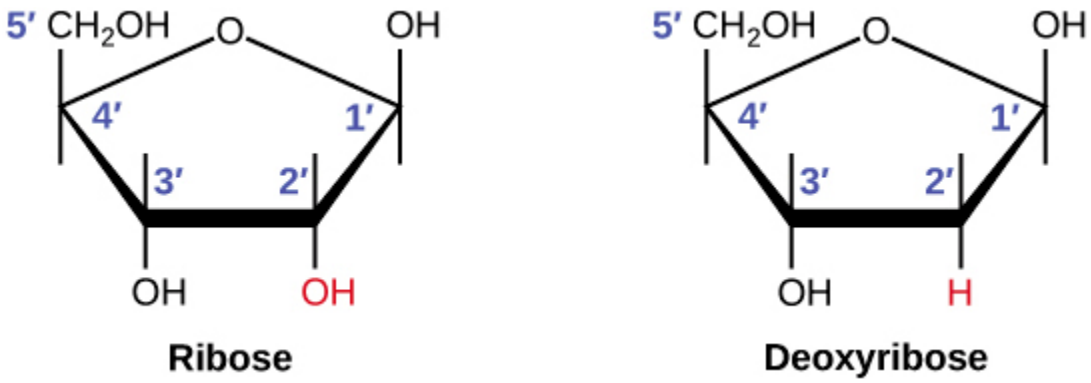
A single strand of DNA is essentially a long chain of As, Ts, Cs and Gs. DNA stands for deoxyribonucleic acid. Ribose is a sugar that has 5 carbons. Four of those carbon atoms plus an oxygen atom are chemically bonded to one another in a geometrically pentagonal arrangement, where the carbons are numbered 1-5. Carbon #1 is attached to the base (A, T, C, or G), carbon #3 is attached to an oxygen and the phosphate of the next ribose, and carbon #5 is not part of the pentagon but it is attached to the phosphate group, of the previous ribose of DNA. Carbon number 2 does not have a hydroxide group attached to it in DNA (hence the name *deoxyribonucleic acid*), while RNA molecules do have a hydroxy group attached to carbon number 2 (this is why they are called *ribonucleic acids*, and there isn't the deoxy in the name). The carbons on the ribose sugar backbone are appended with an apostrophe('), which is pronounced as "prime." This is to distinguish them from the carbons on the base pairs (which do not have the prime/apostrophe).

The hydroxyl (OH) group on the RNA ribose likes to break RNA strands, making it less stable. DNA is very stable and can last for a long time at room temperature. RNA is unstable and will degrade quickly. This system enables the cell to maintain tight control over protein production while keeping the information in a form that is stable, long term.



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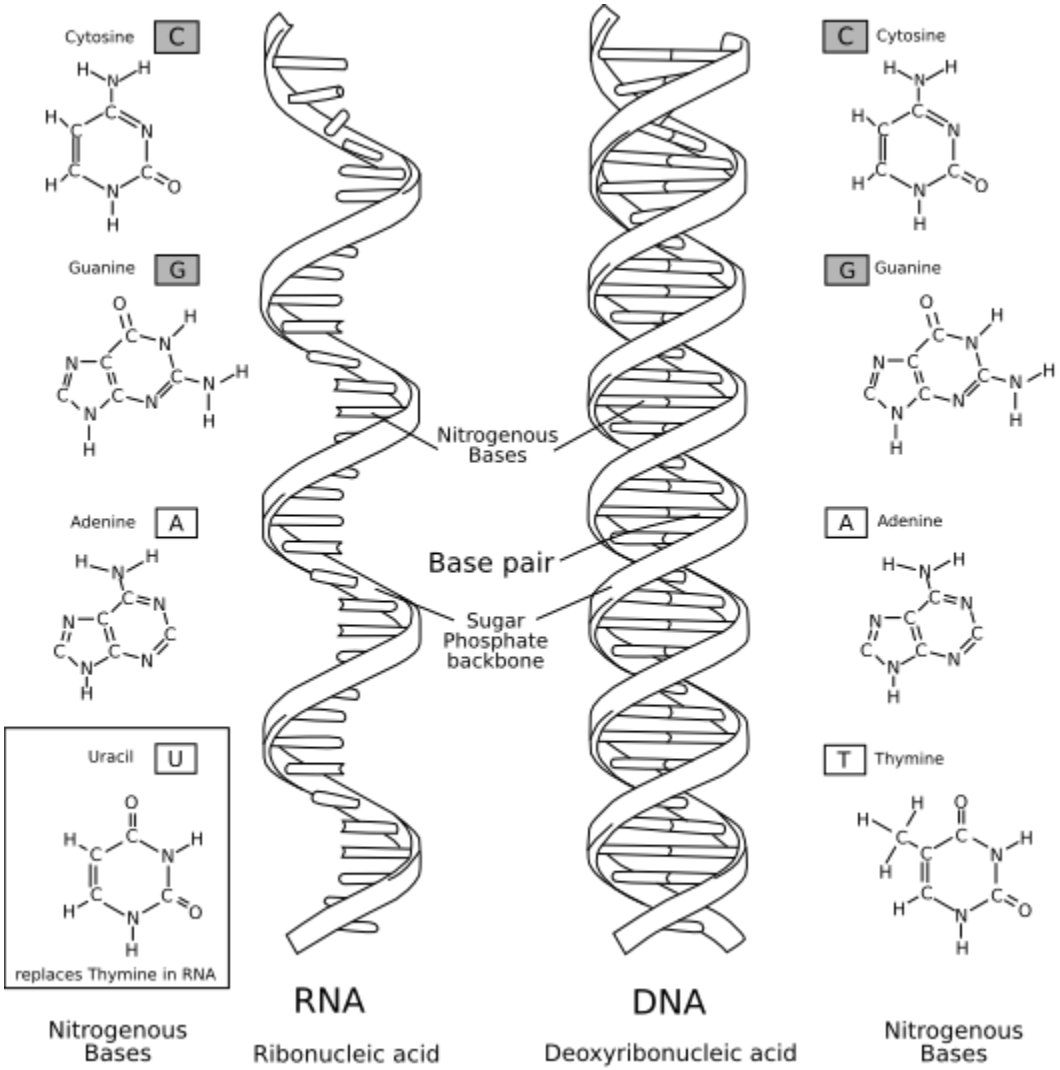

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DNA/RNA Sequences and Protein Production

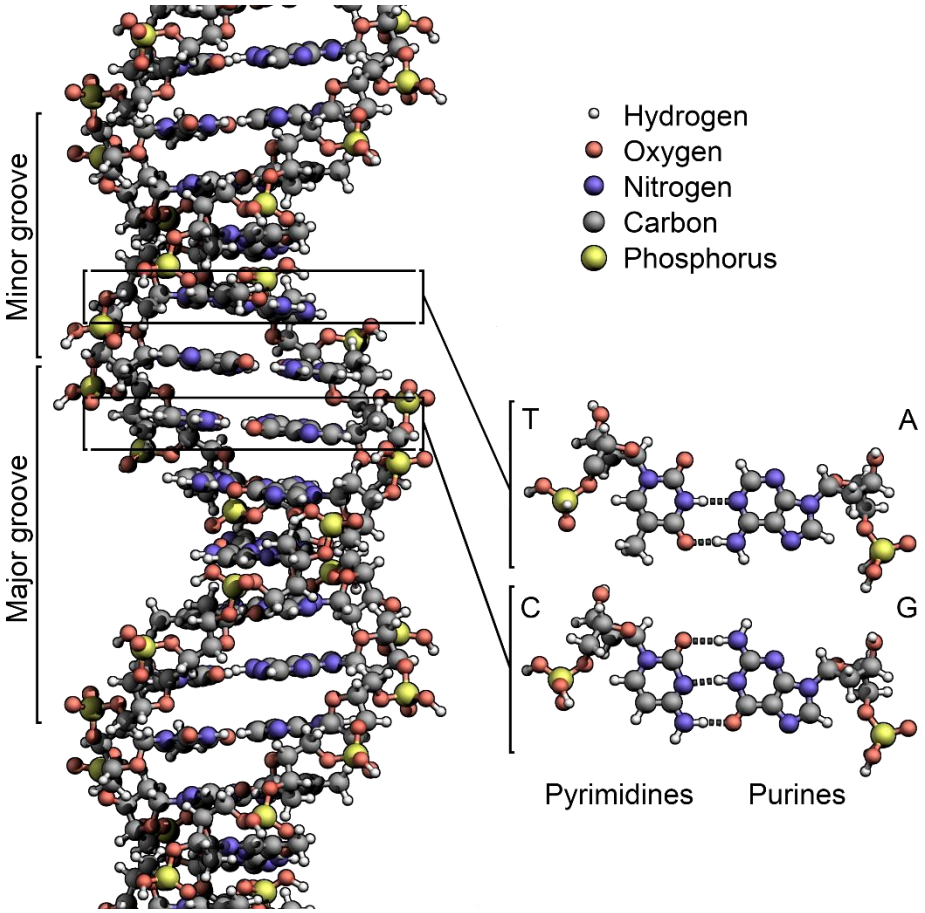
During gene expression, the information encoded within DNA is used to make a protein. Essentially, the unique combination of As, Ts, Cs and Gs corresponds to a unique combination of 20 potential amino acids. Amino acids are the building blocks for proteins, which are the nanomachines and structures that enable the cell to be what it is and do everything that it does.

Amino acids can be one of the following

- polar—meaning that they want to bond with water
- non-polar—meaning that they don't want to bond with water,
- positively or negatively charged (charged proteins *really* want to bond with water).

The unique combination of amino acids determines the protein's structure. The structure of the protein then determines what the protein will actually do. Some proteins might be used as the cell's structure (it's *cytoskeleton*), some may be molecular machines that facilitate the cell's movement, others will be excreted by the cell to communicate with another cell somewhere else in the body, etc.

You are probably also familiar with the fact that DNA has two strands that exist in a double helix. Each strand within the double helix is complementary, meaning that one strand binds specifically to the other strand based on the ATCG sequence. A binds with T and C binds with G, so an ATCG strand will bind to a TAGC, except since each strand goes in the opposite direction we have to put it in reverse, so an ATCG strand will bind to a CGAT strand (DNA sequences go from the 5'→3' direction by default, because this is the direction in which all DNA is synthesized).



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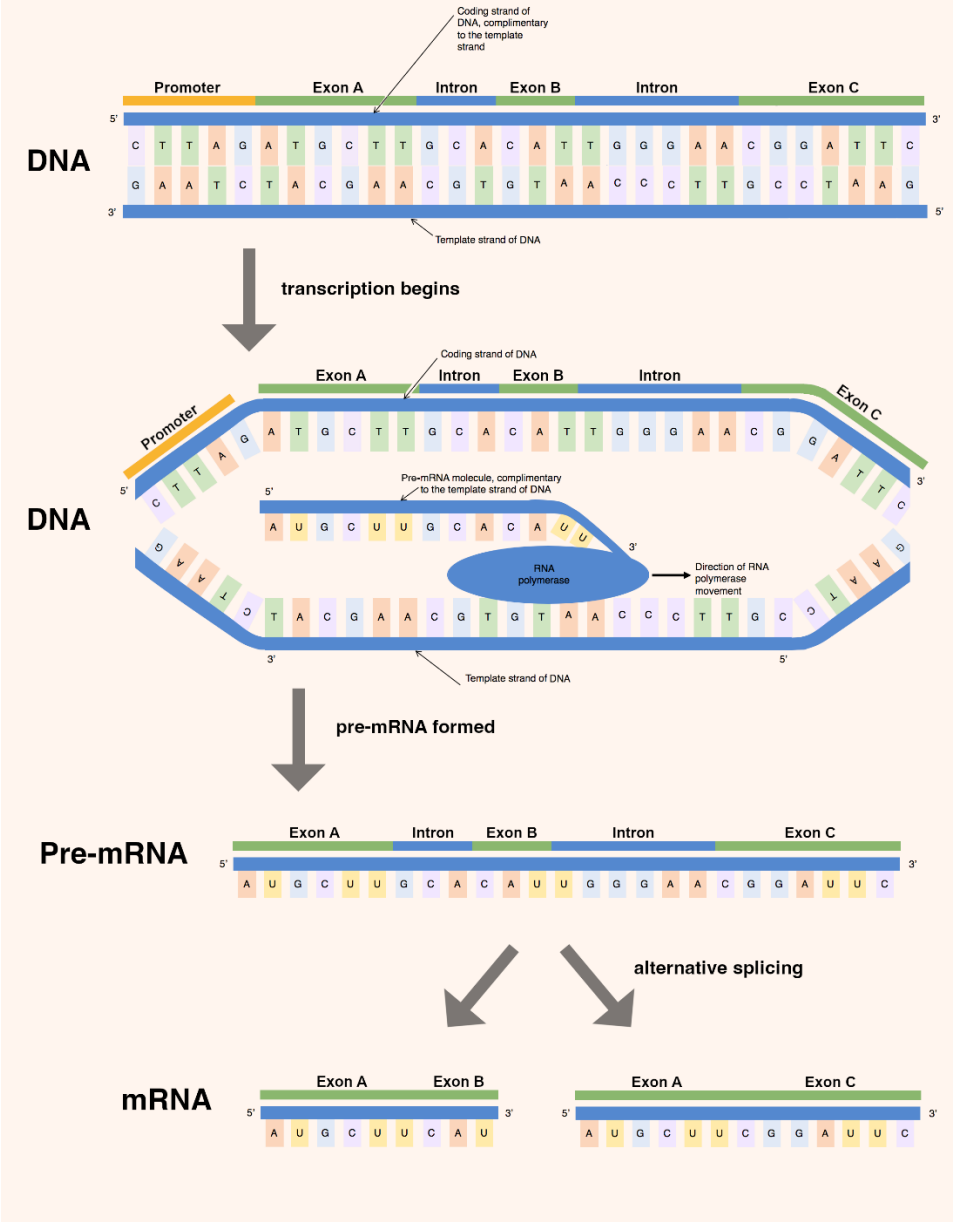
5’-ATCG-3’
3’-TAGC-5’

Each DNA strand is used as the template to make another strand when the cell divides by mitosis, with each daughter cell having one of the original strands as well as one new strand complementary to it. The binding of two complementary DNA strands is highly thermodynamically favorable, which is a fancy way of saying that if you put two single strands of DNA water, and if each DNA strand is complementary to the other, they will eventually find their complementary strands and remain stable in the double helix structure. So if you have random DNA sequences all inside of a solution together, then the most probable outcome is for complementary strands to bind with each other.

In order to go from DNA to proteins, you first need to produce transcripts of RNA from the DNA sequence. These RNA transcripts are produced inside of the nucleus of the cell (which houses the DNA). In order to generate RNA from DNA in the cell’s chromosomes, a protein called a transcription factor must bind to a region of DNA called the promoter, or promoter sequence. The transcription factor then recruits other proteins floating inside the nucleus to then bind to itself in something that we call the *transcription initiation complex*. There are dozens upon dozens of proteins involved in the transcription initiation complex, with each one performing

different functions needed for transcription (transcription is the official name of the process of making RNA from DNA). One of these proteins in the transcription initiation complex is called RNA polymerase. RNA polymerase leaves the transcription initiation complex, moves along a single strand of DNA (the DNA strands have to be separated by the proteins that are part of the transcription initiation complex) and generates an RNA strand with a sequence that is complementary to the DNA strand (an 5'-ACGU-3' for a 3'-TGCA-5', in RNA, uracil is used instead of thymine).



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The RNA, dubbed "messenger RNA," or mRNA, must then leave the nucleus of the cell and go to the cytoplasm (the cytoplasm is just a fancy word for the contents of the cell that are inside the cell membrane but outside of the nucleus). Once in the cytoplasm, the mRNA is then used by

large proteins called ribosomes which assemble a protein based upon the “AUCG” sequence of the RNA. This string of amino acids is called a protein, and the process of producing proteins is called *translation*. To jog your memory, amino acids can be polar, nonpolar, or charged. Charged and polar amino acids like water, while nonpolar amino acids don’t like water. The string of amino acids will fold itself in such a way that amino acids that like water will face the outside and amino acids that don’t like water will face inside away from water, thereby giving the protein its structure.

The sequence of the mRNA directly determines the unique amino acid sequence of the protein, which in turn, determines the protein’s structure and function. Ribosomes bind to the mRNA, and recruit individual tRNAs (tRNA means “transfer RNA”) which will each contain an amino acid. Each tRNA binds to the template mRNA strands at three base pairs. Three bases that bind to a tRNA which determine an amino acid in the protein’s sequence is called a *codon*. In addition to codons which will code for individual amino acids, there are also start and stop codons which will determine the beginning and end of the protein. The start codon is always the amino acid methionine.

Second Base									
First Base	U		C		A		G		Third Base
U	UUU	phe	UCU	ser	UAU	tyr	UGU	cys	U
	UUC		UCC		UAC		UGC		C
	UUA	leu	UCA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG		UGG	trp	G
C	CUU	leu	CCU	pro	CAU	his	CGU	arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	ile	ACU	thr	AAU	asn	AGU	ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	lys	AGA	arg	A
	AUG		ACG		AAG		AGG		G
G	GUU	val	GCU	ala	GAU	asp	GGU	gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	glu	GGA		A
	GUG		GCG		GAG		GGG		G

Codon Table: 20 amino acids and their three letter abbreviations are: phe: Phenylalanine, leu: Leucine, ile: Isoleucine, val: Valine, ser: Serine, pro: Proline, thr: Threonine, ala: Alanine, tyr: Tyrosine, his: Histidine, gln: Glutamine, asn: Asparagine, lys: Lysine, asp: Aspartic Acid, glu: Glutamic Acid, cys: Cysteine, trp: Tryptophan, arg: Arginine, gly: Glycine, AUG is the start codon and is also met: Methionine.

The messenger RNA, or mRNA, can be edited. When RNA is edited, it is *spliced*. It is helpful to note that not all the RNA in an RNA transcript encodes for a protein, and we would call this RNA *non-coding RNA*. Also, before the RNA is translated into a protein by the ribosome, the RNA can be *edited*, or as molecular biologists call it, *spliced*. The parts of the RNA that are chopped off in this editing or *splicing* stage are called *introns* while sequences that remain are called *exons*. mRNA (or rather, mRNA in eukaryotes such as all plants, animals, fungi and yeast) contains a polyadenine—or a polyA—5’AAAAAAAAAAAAA…3’—tail at the end of the

mRNA transcript. The polyA tail causes proteins called polyA binding proteins (PABP) to bind to the mRNA, protecting the RNA from being degraded by machinery within the cytoplasm.

RNA splicing can have a variety of functions within the cells of the body. For example, sometimes RNA splicing directly impacts the function of the protein. Once cell might want the protein regulated by another pathway within the cell while another cell type may want the protein active all of the time. Thus we can say that differential RNA splicing can be an important mechanism of cell differentiation. Adrenergic receptors, which are receptors that bind to stress hormones like adrenaline or noradrenaline, are differentially spliced across cell types so that they can do different things in response to adrenaline (i.e. the heart can increase the heart rate while the liver breaks down glycogen and releases glucose during fight or flight).

Micro RNAs and RNA Interference

In Chapter 9, I explained how Cao et al[1] and Mansour et al[2] knocked down BDNF in the hypothalamus using something called microRNAs (miRNA). To do this, both groups genetically modified mice by using the adeno-associated virus (AAV) to place DNA inside of neurons which coded for mRNA that was complementary to the RNA of the BDNF gene (more on how the adeno-associated virus was injected into the mouse brain can be found in a later section).

Molecular biology labs use several different species of animals as "model organisms." Examples of model organisms include mice and rats, drosophila fruit flies and nematode worms called *Caenorhabditis elegans*, commonly abbreviated as *C. elegans*. It was in the early 1990s that Ambros and Ruvkun, both of whom led research groups at Harvard University, discovered a novel mechanism of post-transcriptional regulation of mRNA. This would come to be known as RNA interference.

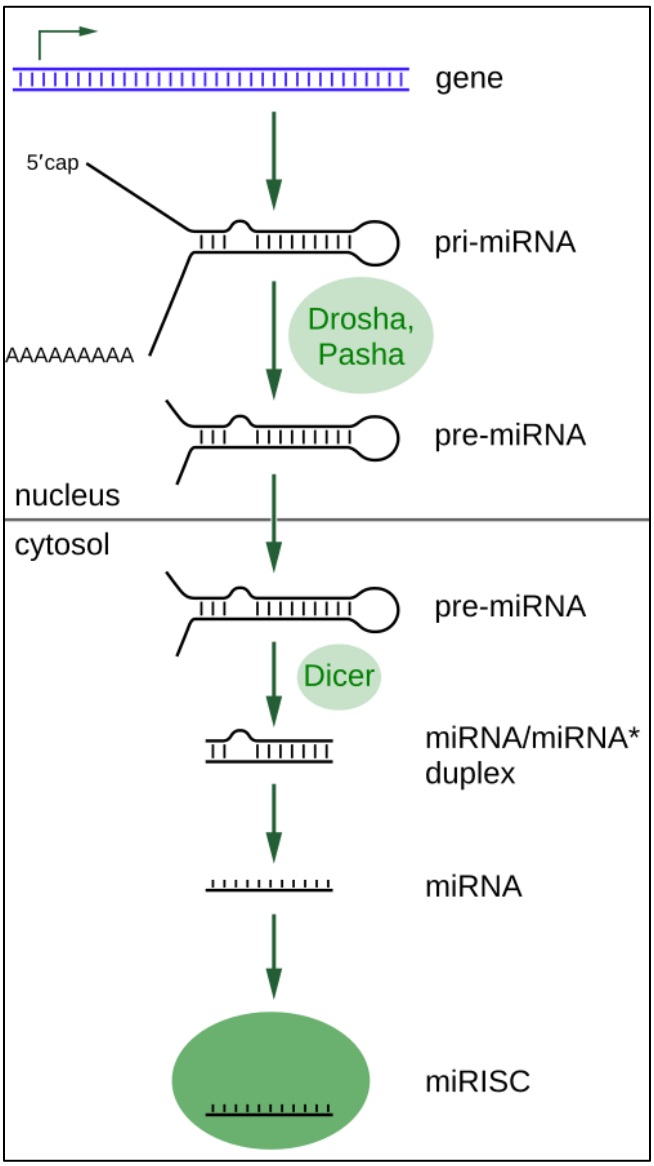
In *C. elegans*, there is a gene called *lin-14*, which is called a heterochronic gene. Heterochronic comes from the Greek "hetero-" meaning "different and "chronos" meaning "time;" heterochronic genes control the timing of developmental events. In *C elegans*, loss of function mutations in the *lin-14* gene causes L2 patterns of development to occur prematurely in the L1 stage of larval development; gain of function of *lin-14* causes L1 patterns of development to occur inappropriately into the L2 stage of larval development.

Ambros and Ruvkun's groups discovered that another gene, called *lin-4*, was instrumental in downregulating the amount of the LIN-14 protein, and it was accomplished after transcription of the *lin-14* gene but before translation. The *lin-4* produces an RNA transcript, which is then spliced to produce a smaller, 22-base-pair RNA transcript that was complementary to the *lin-14* mRNA, more specifically, the 3' untranslated region. The result was a decrease in the amount of LIN-14 protein leading to the next stage of larval development in *C elegans*.

This turned about to be a landmark discovery, as this was an entirely new mechanism of regulating protein expression that is present in all mammals. We now know that the miRNA is processed by the microprocessor complex, which cleaves the precursor miRNA into a shorter RNA structure. After it leaves the nucleus and enters the cytoplasm, an RNase III enzyme called Dicer cleaves the pre-miRNA into an even smaller transcript. Then, the mature miRNA product

is loaded into an enzyme called Argonaute protein, forming the RNA-induced silencing complex (RISC).

RISC then uses the miRNA as a guide to find the complementary mRNA. Once RISC binds to its target mRNA, it stops the mRNA from being translated into proteins. It first does this by physically blocking the ribosomes from binding to the mRNA, preventing them from producing proteins. RISC also recruits other enzymes that remove the mRNA's polyadenine tail, which results in its destruction. **miRNAs were the mechanism used by several of the papers** (Cao et al,[1] Xiao et al,[3] Mansour et al[2]) **to disable the BDNF RNA so that the call cannot produce functional BDNF protein**.



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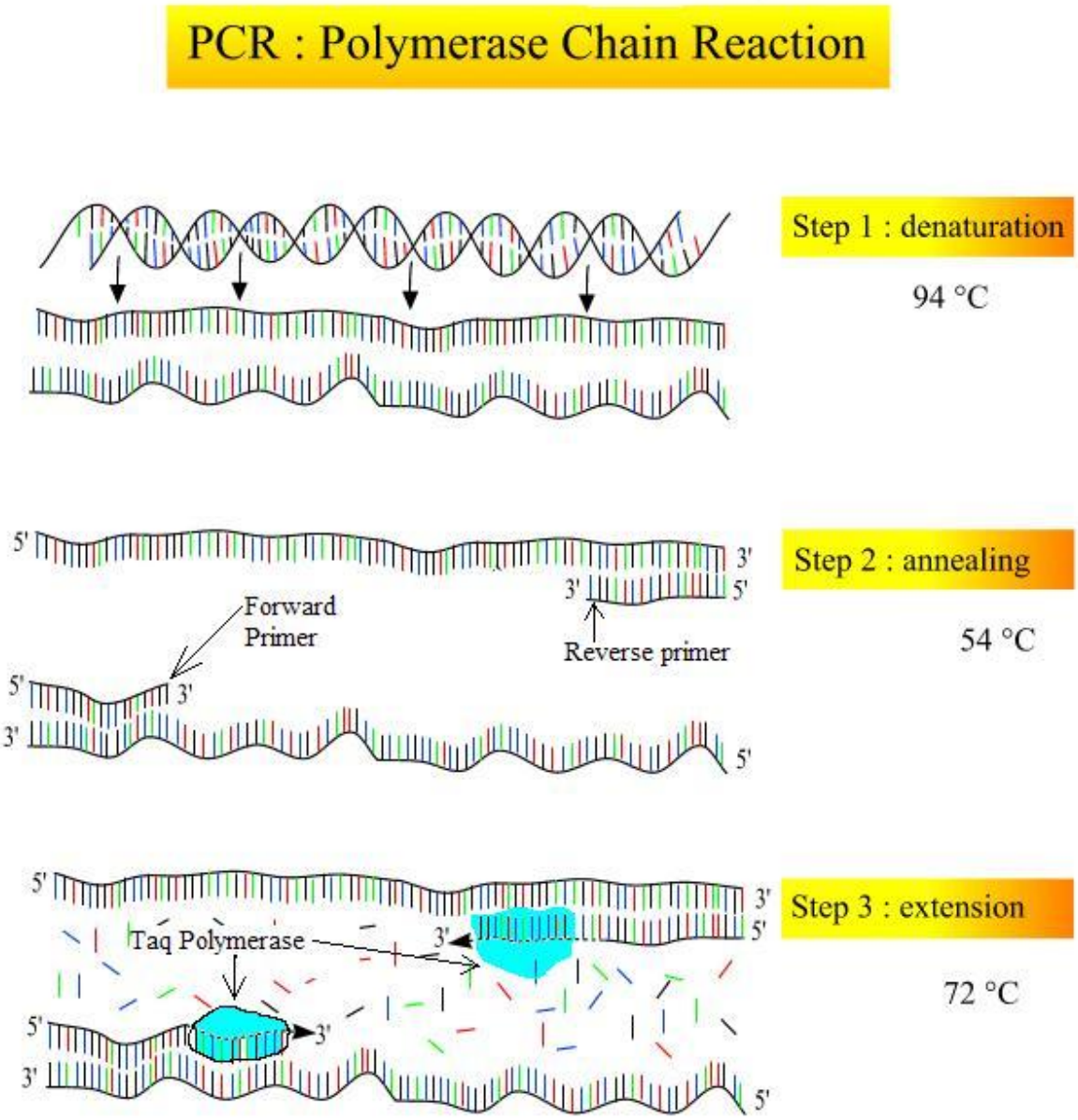
PCR Amplification and Quantifying Gene Expression



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This is definitely something that I would highly advise YouTubing or Googling, as much of the descriptions here would work much better with pictures and animations. It's kind of overkill to describe PCR amplification and gel electrophoresis in a book like this, but for all two of my readers that share my neurotic need to understand everything but don't have biochemistry degrees, this is for you!

Most biochemistry wet lab work involves pipetting a fluid that looks like and is mostly water from one tube into another tube of fluid that also looks like and is mostly water. It is also the case that the things biochemists study are very, very tiny, and we often work with very tiny amounts

of it. If we want to study a gene from a single cell, that is very difficult because in this single cell there is only one copy of the gene that we want to look at. Also, all DNA behaves chemically the same regardless of its sequence.

So then, if we have a single cell and we want to do research on its genome, then it would help if we could make more copies of the gene. This is where we use a technique called PCR amplification, which is one of the most commonly used biochemistry wet lab techniques in use today.

A PCR, which stands for a polymerase chain reaction, involves making lots of a specific DNA sequence. We only need a single copy of that gene, and as long as there is one copy of that gene, a PCR can be used make more of it. Then, with lots of DNA with this single sequence, we can see it using another technique called agarose gel electrophoresis. We can also put it into a plasmid as the first step of genetically engineering a mouse. We can also run further PCRs that can, for example, add a hemagglutinin tag which will enable us to see our gene of interest using immunofluorescence techniques, etc.

To understand how a PCR works, it is helpful to understand how DNA replication works (I also recommend looking into Google and YouTube for pictures and animations, as pictures will make many of the concepts I'm about to describe a lot more intuitive). When DNA is being replicated, an enzyme called a DNA polymerase moves along the *template* strand and uses it as a template to make a new copy of its complementary strand.

DNA can only be synthesized in the 5' to 3' direction, meaning that the DNA polymerase has to travel down the template strand in the 3' to 5' direction. The DNA polymerase itself is also like a motor: as it travels down the DNA strand it is reading to make the complementary strand, it goes with quite a lot of force. DNA polymerase synthesizes DNA at the rate of 1,000 base pairs per second, and its power to mass ratio vastly exceeds that of a modern automobile engine.
In order to begin synthesizing a new DNA strand, DNA polymerase must bind onto double-stranded DNA. The cell gives the DNA polymerase double stranded DNA by using an RNA primer.

A primer is just a single stranded DNA or RNA sequence that is used to facilitate or "prime" replication (the use of the word primer in this instance bears no relation the use of the word prime in labeling the carbons on the ribose backbone of DNA or RNA). When we're running a PCR, we order primers of specific sequences made synthetically from chemical companies. The primer sequence we want depends on the gene we're trying to PCR amplify. We just need two primers: one that binds (or anneals) to one end of the gene and another that binds (or anneals) to the other end of the gene (we will call one primer the *forward primer* and we will call the other primer the *reverse primer*), and DNA polymerase will take care of everything in the middle. So, if we want to get channel rhodopsin from algae, all we need is the algae, and primers for the gene sequence of the channel rhodopsin gene and we can make all of the copies of the channel rhodopsin gene that we will ever need, and store it in a refrigerator or freezer to make more copies of it later on (DNA is very stable and can last for a long time, unlike RNA).

I said previously that DNA strands bind to their complement strand. The binding of two complementary DNA strands is highly energetically and thermodynamically favorable, meaning that if you stick two DNA strands in water, they will find and bind to each other in the famous double helix structure well on their own. However, if DNA is going to be replicated (which it has to be for mitosis), then the DNA strands have to be pulled apart into individual strands. Cells accomplish this mainly by using a protein called DNA helicase to unwind DNA strands. Yet using DNA helicase is not practical for us because (1) it's difficult for us to mass-produce and (2) it would need *other* proteins to load it onto the DNA, and that becomes a very complicated very fast. We want something simple…

So, to solve this problem, we separate the DNA strands using heat. DNA binds to itself via hydrogen bonds, which is the same thing that keeps water molecules attached to each other when you have liquid water. Chemically, *breaking bonds requires energy* and *forming bonds releases energy*. So, to break bonds then, we have to invest energy by heating up the sample to boiling point (100°C or 212°F, Celsius is always preferred in lab science). Actually, in this case, we don't have to go *quite* up to water's boiling point; 99°C will work.

Proteins normally denature and breakdown whenever we boil them. This is why eggs go from goopy, snotty liquids and solidify when cooked—the proteins in the egg white are like strings wrapped into little balls, and when cooked they unwind and then bind with each other, making for a solid substance when the egg is cooked.

We *don't* want this happening to our DNA polymerase, because if it does then it won't be able to produce more DNA. So, we use a DNA polymerase taken from a *thermophilic* organism, in other words, a bacterium isolated from very, very hot conditions. That way, our DNA polymerase won't break down when we are heating our PCR mixture.

So then, let's review! To begin our PCR amplification, we place the following in a small, heatproof tube:

- A single copy of the gene we want to amplify
- DNA primers specific to the gene we want to amplify (we call this the forward primer and the reverse primer)
- A heat proof DNA polymerase
- The actual building blocks of DNA in high concentrations. The formal name for the building blocks of DNA are Deoxynucleotide triphosphates, of which there are four types:
    - dATP (deoxyadenosine triphosphate)
    - dCTP (deoxycytidine triphosphate)
    - dGTP (deoxyguanosine triphosphate)
    - dTTP (deoxythymidine triphosphate)

We get all of these ingredients into a heatproof tube, we place that tube into a PCR machine, and the PCR machine controls the temperature. First, it raises the tube up to 99°C for a minute or so, then it lowers the temperature to somewhere between 50-60°C for a few minutes, then, because the thermophilic DNA polymerase functions best at a temperature higher then 60°C, the machine

goes back up to 70°C for a few minutes. Then, after a few minutes at 70°C, the PCR machine goes back up to 99°C and begins another cycle.

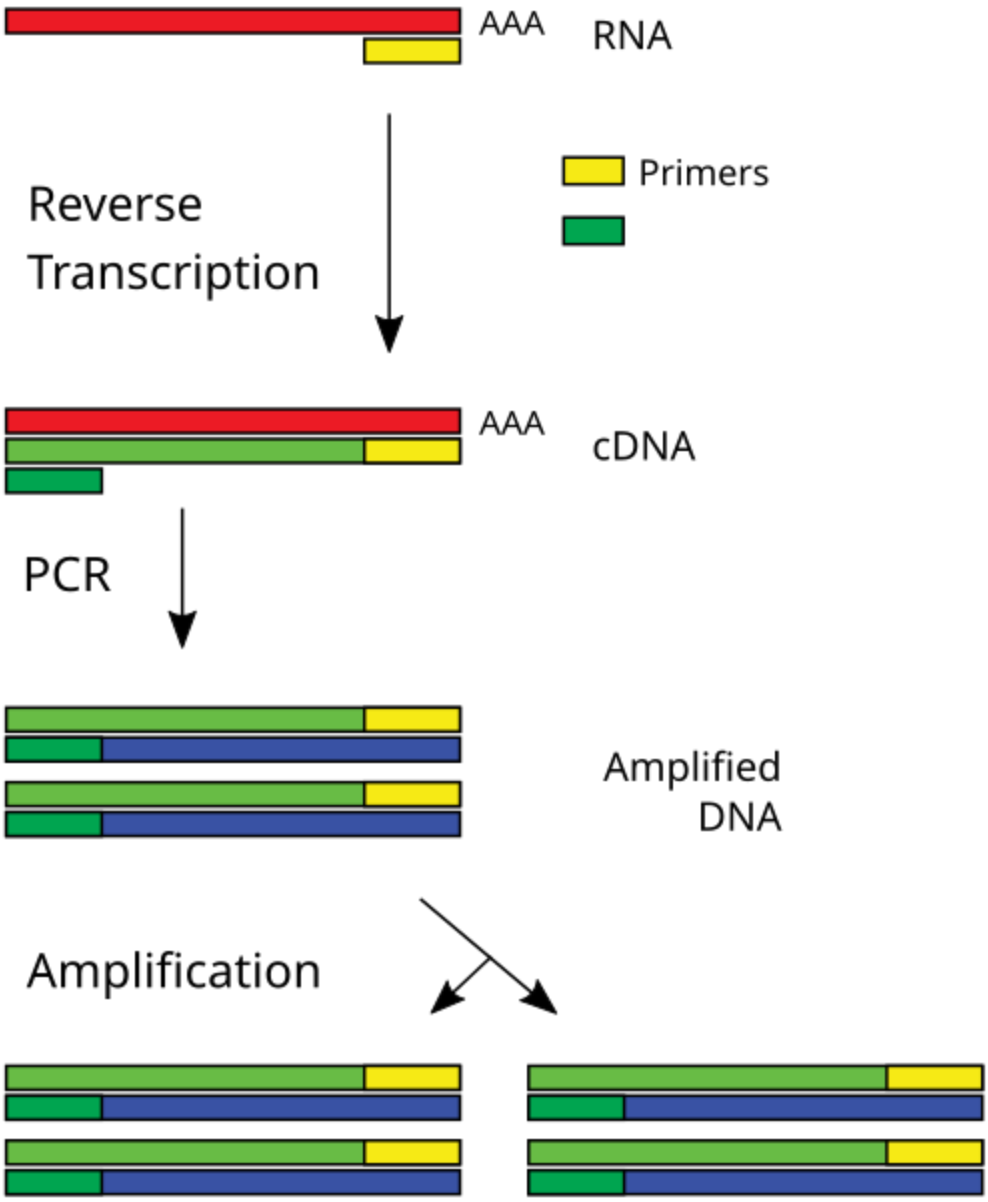
Theoretically, every cycle should *double* the amount of the gene present. So, after 30 cycles, you now have 2^{30} copies of your DNA sequence. After 60 cycles, you now have 2^{60}, or over 1 quintillion copies of your original gene.

Usually, the most common thing to do here would be to run a gel electrophoresis. Gel electrophoresis involves placing your biomolecules of interest (in this case DNA) into a gelatinous substance and then placing the gel in an electric field. The electric field causes charged molecules like DNA, which is negatively charged, to move toward the positively charged end of the electric field. The gel—which in this case will be made of agarose—will also have something in it like ethidium bromide which binds to DNA and glows orange under UV light, allowing us to actually see the DNA. Bigger molecules move more slowly through the gel than smaller molecules, so you can therefore separate DNA of different sizes if necessary. The brightness of the band can be measured to give us a rough estimation of the amount of DNA, and, by extension, the amount of DNA that was originally in the sample.

After the gel and with the DNA loaded onto it has been sitting in the electric field for some time, the DNA will progress down the gel and the ethidium bromide will make the DNA visible as an orange glowing band. The brighter the band, the more DNA that that band contains. Thus we can get a rough quantification of how much DNA there is on the gel.

Other types of gels include an SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylimide Gel Electrophoresis), which measures proteins instead of DNA. The principle is very similar: proteins go down a gel by means of an electric field. Proteins from a biological sample are treated with sodium dodecyl sulfate to give them all a similar charge to mass ratio, making them all negatively charged. Then, the proteins travel down the gelatinous substance (in this case polyacrylamide) at speeds that vary depending on their size. Then a protein-binding dye is used to make the protein band on the SDS PAGE visible. If my explanation of gel electrophoresis has left you scratching your head, not completely sure what it is, then I recommend that you search YouTube or Google Images. This is definitely one of those times where pictures help!

Reverse Transcriptase PCR



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PCR is usually used to make DNA, not RNA. So, if we want to use PCRs to study RNA in a cell, we first must isolate the RNA (most likely using a commercial lab kit), and then treat the RNA with an enzyme called a reverse transcriptase, which uses the RNA as the template to make DNA. Reverse transcriptase, like polymerase, needs double stranded DNA or RNA to work, so we use a DNA primer.

Reverse transcriptases are usually found in viruses that use them for replication. Some viruses have no DNA and use RNA. Even though RNA is unstable, these viruses have structural elements which enables the RNA resist decay. In order to co-opt an infected cell to make more of

the virus's RNA, the virus uses a reverse transcriptase to convert its RNA into DNA, and then that DNA becomes the template from which to produce more viral RNA transcripts.

Reverse transcriptase PCR, or RT-PCR, enables relatively easy quantification of gene expression. So, when researchers isolate neurons from mice living in the enriched environment, this technique is used to look at what proteins are being expressed more due to enriched environment living.

Then, once the RNA has been treated with the reverse transcriptase, a single stranded complementary DNA strand is synthesized, which is abbreviated as cDNA. PCR is used to amplify the cDNA and then we can run it on a gel to get a rough quantification of how much the gene was expressed.

Real Time or Quantitative PCR

Quantitative PCR used to be called "Real Time PCR" but it was abbreviated as RT-PCR, which made it very easily confused with reverse transcriptase PCR. Now, qPCR is preferred to avoid this confusion. qPCRs involves using strategies to get the PRC product (which is DNA) to fluoresce (fluoresce just means to glow under the presence of light with a high enough frequency), and the fluorescence can be measured and quantified, giving us a rough estimation of how much DNA present in the sample. qPCRs can either use a molecule that fluoresces when it binds to double stranded DNA (such as SYBR green), or it can take a primer, put a fluorescent molecule on one end of the primer and a quencher to that fluorescent molecule on the other end of the primer, and then, as the primer binds to the template DNA strand, the fluorescent molecule breaks off and is no longer quenched, resulting in a fluorescent signal.

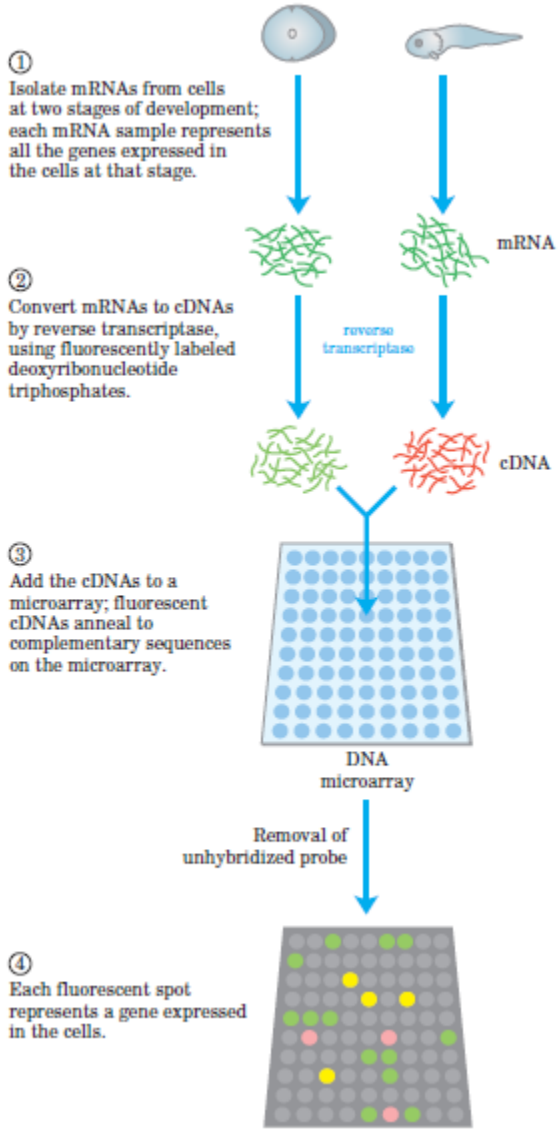
Thus, we can use the intensity of glowing light (i.e. fluorescence) to give us a measure of how much DNA there is in our sample. Using quantitative or real-time PCR on cDNA from a reverse transcriptase reaction is largely the method used by several of the publications to measure the increase in expression of BDNF and leptin receptors in the brain.[1,4]

Gene Expression Microarray

Li et al[5] measured differences in tumor gene expression from both enriched and public-school mice using a technology called a gene expression microarray. A gene expression microarray utilizes a chip which contains single stranded DNA of sequences which correspond to known genes whose expression you are trying to measure.

cDNA is made from RNA isolated from the tumors of mice living in the enriched environment, only this time it is made using dNTPs that have one color for the enriched-environment mice (green) and another color for the public-school mice (red). Then, both samples are combined and added to chips that have single stranded DNA attached to them of various known sequences (Li et al looked at a lot of mitochondrial genes). Different locations on each chip would have different complementary DNA strands attached to them. Then, after the chips were treated with cDNA from the animals, the chips would be washed, leaving only the DNA that is complementary to those chips. The relative abundance of either color on each location would directly correlate to how much the different cell types are expressing either gene. Li et al found

that the enriched environment caused genes relating to the maintenance of the mitochondria in cancer cells to be expressed less than in control mice using this method.



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High-Throughput Sequencing and RNA seq

Modern technology has improved DNA sequencing significantly. The human genome project was a race to see which lab could first map the entire human genome. It relied largely on Sanger Sequencing, which is a technology that is now somewhat obsolete. There are now machines that can map the entire genome of a cell in a matter of hours.

Essentially, primers are attached to the solid surface of a sequencing chip. Then, specialized dNTPs are added, where each base (a base is adenine, thymine, cytosine or guanine, abbreviated as A, T, C or G) contains a fluorescent marker that glows a unique color based on the base (so adenine (A) is Green, Thymine (T) is Red, Cytosine(C) is Blue, and Guanine (G) is Yellow).

There is also a block attached to all of these fluorescently labelled dNTPs that blocks the polymerase enzyme from synthesizing anymore DNA after one of these dNTPs has been added. So the cycle goes as follows:

(1) cDNA binds to adapter DNA that is attached to a sequencing chip
(2) fluorescently labelled dNTPs with blocker groups chemically attached to them are added
(3) DNA polymerase adds one dNTP to the DNA sequence
(4) Fluorescently labeled dNTPs are washed away
(5) The fluorescent marker is made to fluoresce, giving a unique color based on the base which is measured and saved onto a computer
(6) The blocker groups are removed, as are the fluorescent tags
(7) Back to step 2 and repeat until the entire strand of DNA has been sequenced

This method can be used to obtain sequence data from individual DNA strands, and the technology has improved to where an extremely large number of DNA sequences can be identified all at once. When there is a large number of sequences (over 10,000 in a single sample), we call this "High-Throughput Sequencing." Samples can contain whole "libraries" of different DNA sequences, and the sequencing data for all of them can be obtained using this technology. RNA-seq essentially uses this technology on cDNA made from RNA isolated from a sample. Bice et al[6] used RNA seq to find differences in gene expression in either enriched-environment mice or public-school mice. Liu et al[7] used a variant of RNA-seq, called single cell RNA-seq or scRNA-seq, to identify neurons that were active in sexual receptivity and aggression in female mice.

If you would like more information on next-gen Sequencing and RNA seq are complex technologies. I highly advise watching YouTube videos to get an understanding of it. I recommend **StatQuest: A gentle introduction to RNA-seq on YouTube, on StatQuest with Josh Starmer YouTube channel**.[8]

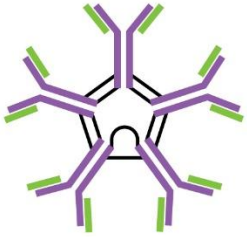
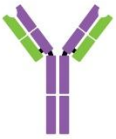
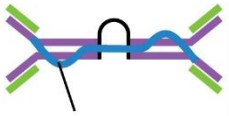
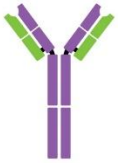
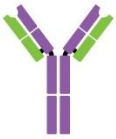
Antibodies, Immunofluorescence and Flow Cytometry

Antibodies are Y-shaped proteins that are one of the dominant mechanisms of long-term immunity. If you think of an antibody as a giant Y (actually it's a very, very, very tiny Y but size is highly relative here), then the tip of the Y will bind to something that is foreign in the body, such as a parasite or a foreign bacteria, and the base of the Y will recruit immune cells (natural killer cells, macrophages, neutrophils, dendritic cells, etc.) to destroy whatever the antibody is bound to. Antibodies are very specific, meaning that one antibody will bind strictly to its target, which is known as the antigen. The part of the antigen to which the antibody binds is called the epitope. The part of the antibody which binds to the epitope on the antigen is called the paratope.

The Y shaped antibody is actually a tetramer of four polypeptide chains (a polypeptide chain is another word for a protein). There are two identical heavy chains and two identical light chains. The base of the Y portion is known as the Fc region and the branches of the Y are known as the Fab regions ("ab" stands for "antigen binding" while "c" stands not for "constant" but for "crystallizable", because it crystallizes easily). There are five isotypes of antibodies, IgG, IgA, IgM, IgE, and IgD, which have different modifications to their heavy chains. B cells can switch isotypes. Usually, they produce IgM and IgD antibodies when they are immature and then will produce IgA, IgG or IgE as they mature. Bice et al[6] found that B cells of enriched-environment

mice released IgA in response to colon cancer to effectively discriminate between beneficial and pathogenic gut bacteria, which has been shown by others.[9] IgG is the antibody type most commonly used by humans for tissue-staining as well as therapeutic application (such as Herceptin®). IgE antibodies are thought to attack parasites as well as be the primary driver for allergies.

The Five Immunoglobulin (Ig) Classes

	IgM pentamer	IgG monomer	Secretory IgA dimer	IgE monomer	IgD monomer
					
Heavy chains	μ	γ	α	ε	δ
Number of antigen binding sites	10	2	4	2	2
Molecular weight (Daltons)	900,000	150,000	385,000	200,000	180,000
Percentage of total antibody in serum	6%	80%	13%	0.002%	1%
Crosses placenta	no	yes	no	no	no
Fixes complement	yes	yes	no	no	no
Fc binds to		phagocytes		mast cells and basophils	
Function	Main antibody of primary responses, best at fixing complement; the monomer form of IgM serves as the B cell receptor	Main blood antibody of secondary responses, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva, colostrum	Antibody of allergy and antiparasitic activity	B cell receptor

Antibodies are produced by B cells, which are immune cells that mature mainly in the bone marrow (and the spleen to some extent). The primary function of B cells is to produce antibodies. The paratope, or the antigen-binding part of the antibody is extremely variable: the body can produce an antibody that is able to bind to any three-dimensional shape on a foreign pathogen. The human body contains about 300 billion B cells in circulation, and each B cell produces a unique antibody—one B cell produces one type of antibody that binds to a specific epitope on an antigen. As B cells mature, there is a screening process where B cells that produce antibodies

which bind to something on the body are eliminated. A mature B cell will then proliferate, and then differentiate into either a plasma cell which is like an antibody factory cell, or a memory B cell, which is a long-lived cell that provides rapid, enhanced responses to future infections.

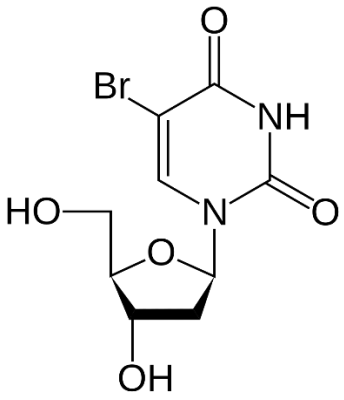
Immunofluorescence is a combination of the words “immune” and “fluoresce.” The immune part is because antibodies are used. If you inject, say, a bunny rabbit with a human protein (likely produced by genetically modified bacteria and then purified), the rabbit will produce antibodies against that protein. The spleen of the rabbit or animal can then be isolated and B cells can be purified, and then, using flow cytometry, B cells that produce antibodies against the targeted protein are isolated. Since cells need to be receiving external growth factors in order to not die, the B cells which produce the specific antibody are then fused with myeloma cells—which don’t need growth factors to not die—enabling mass-production of the antibody of interest. The antibody is then purified using multiple chromatography steps (anion exchange and protein A chromatography). Antibodies can then be modified to have something attached to them that can be visible, such as a fluorescent molecule which is the case in this experiment.

Given that antibodies are the mechanism by which the body’s immune system recognizes friend from foe (self vs non self), antibodies bind *very specifically* to their target antigen. Therefore, producing antibodies that can be visualized and reasonably quantified.

Immunofluorescence is frequently used alongside a technique called flow cytometry. Flow cytometry is a technique that rapidly analyzes and sorts millions of individual cells or particles in a fluid stream. First, you have your fluid of cells, such as immune cells in a liquified solution from the spleen of a mouse, and then you use a machine called a *flow cytometer*. The flow cytometer sucks up the fluid through small, plastic tubing and then allows the fluid to flow through tiny channels that could be between one millimeter and one one-thousandth of a millimeter in diameter. Cameras can then capture data from the cells as they move through these fluid streams. If there is a fluorescent tag on a cell, then the flow cytometer can sort that cell for other purposes, etc. Using different fluorescently tagged antibodies that test for different receptors on natural killer T cells was how Mansour et al[2] determined the maturity of those T cells.

BrdU Cell Labeling

One of the papers by Schloesser et al[10] used BrdU cell labeling to show that the enriched environment increased neurogenesis. In other words, the brains in the enriched environment had more hippocampal stem cells differentiate into new neurons. To show this, researchers used a technique called BrdU cell labeling. BrdU stands for 5-bromo-2'-deoxyuridine, or just Bromodeoxyuridine. BrdU can incorporate into DNA as a thymidine analog (meaning cells might confuse BrdU for the thymine base pair). Researchers periodically injected the animals with BrdU for several weeks prior to the autopsy.

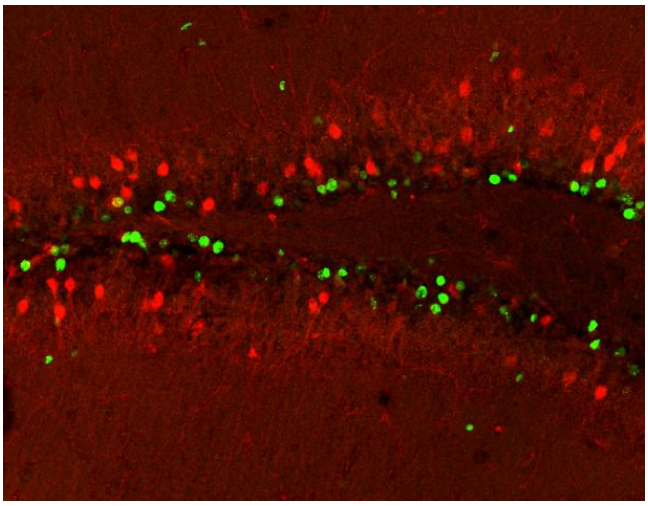


5-bromo-2'-deoxyuridine

To get an antibody inside of the cell, first the brain tissue must be immobilized. This is done by treating it with formaldehyde. Those of you who have ever dissected a frog in school, or seen brain tissue being dissected as part of an educational demonstration might think of brain tissue as being sturdy and hard. Normally the brain is very squishy and delicate. However, it will become harder and sturdier when it has been treated with a chemical like formaldehyde (after it has been drained of blood, of course). This is because formaldehyde is a fixative, meaning that it forms cross-links between proteins, preventing decay, maintaining cellular structure and "freezing" the tissue in a lifelike state.

The fixed brain tissue slices are then treated with a detergent like Triton X-100 or Tween 20 (which is what Schloesser et al[10] used) which will puncture holes in the cell membrane. Then, researchers then treated brain slices from the autopsied animals by soaking them in a liquid that contains fluorescently labeled antibodies which target BrdU. Now, the antibodies can get inside of the neuron and they will bind to any BrdU that is present. The sample is washed once again to remove any antibodies that are not bound to BrdU. The fluorescence can then be detected with a fluorescent microscope, enabling Schloesser et al to observe that the mice in the enriched environment had more BrdU+ neurons, meaning that their brains were producing more new neurons than their public-school counterparts since the first BrdU injection.

Medical schools used to teach the now outdated idea that the number of neurons in the brain was all you had and you could never produce any more. Neuronal stem cells migrate into the hippocampal dentate gyrus and then integrate into the existing brain circuitry as they differentiate into mature neurons. BrdU enables researchers to label and identify cells that are multiplying. And so, by taking brain slices from the hippocampal dentate gyrus and treating them with an immunofluorescent anti-BrdU antibody, researchers can gain a rough count of the number of cells that are dividing, which is thus a correlate of the rate of neurogenesis.



BrdU neurons are stained with a green fluorescent antibody and Arc (activity-regulated cytoskeleton-associated protein) is stained in red

Chromatin, Histones and DNA Repair

Each one of your 2 trillion cells contains approximately 2 meters of DNA. There is enough DNA in your body that if it were unwound end to end it would stretch from Earth to the sun and back 600 times! Therefore, in order for DNA to actually fit into the nucleus in every cell, it has to be compacted.

If you think of DNA like it's a string, then you can think of the nucleosome like it's a bead that the string wraps around. DNA wraps around protein beads called nucleosomes. The nucleosome is comprised of 8 protein subunits, which include two identical histone 2A subunits (H2A), two histone 2B subunits (H2B), two histone 3 subunits (H3) and two histone 4 subunits (H4). Histone 1 then binds to the DNA entering and exiting the nucleosome, stabilizing the structure and promoting tighter compaction.

I mentioned earlier that the central dogma of molecular biology is that DNA is used to make RNA (in a process called transcription) which is then used to make proteins (in a process called translation). RNA can be edited or spliced after it is produced; this is called splicing. Similarly, proteins can be modified after they are produced; these are called post-translational modifications. Upon DNA damage (i.e. double-strand breaks), Histone H2A is phosphorylated (forming γH2AX), which will then recruit other proteins to repair DNA. Sakama et al[11] used an antibody which targeted γH2AX to see how environmental enrichment affected the cell's ability to respond to DNA damage.

Tunel Assays

Cao et al[1] used a Tunel assay which is a protein that binds to breaks or "nicks" in the DNA in the tumor cells' nuclei. The Tunel protein that is typically used in biochemical assays is genetically modified to glow green under UV light.

The blue is a stain called DAPI which shows DNA inside of the cell nuclei. The green is a tunel assay, which shows breaks in DNA that occur in programmed cell death (the formal term for programmed cell death is called “apoptosis”)

The presence of green in the bottom righthand corner picture shoes that the immune system of the mice raised in the enriched environment is hard at work in dissolving cancer cells, this is virtually unseen in the control mice. Other studies have indeed corroborated an increased immune response, including T-cell penetration, into tumors in response to being in an enriched environment.

Immune Cell Cytotoxicity Assay

Cao et al[1] used the CytoTox 96 Non-Radioactive Cytotoxicity Assay[12] to assess the effect of the enriched environment upon natural killer cells in the animal’s environment.

Spleens from a sample of sacrificed mice were placed in a liquid called a dissociation medium. Spleen dissociation medium contains salts and pH buffers (so that the cells don’t die by too much osmosis or off balance pH), fetal bovine serum (this contains growth factors which enable mammalian cells to stay alive), and collagenase which breaks down collagen and causes the cells of the spleen to detach and effectively “dissolve” in water). After the spleen is treated with spleen dissociation medium, a liquid containing immune cells from the spleen (known as splenocytes) are extracted, and then they are treated with a mitogen, meaning a chemical that induces mitotic division. This allows researchers to have an ample supply of immune cells for additional experimentation.

Cao et al[1] added these immune cells taken from the spleens of mice into test tubes alongside cancerous melanoma cells in order to measure how effectively the natural killer cells killed the cancer cells. Cells are allowed to incubate for some time at varying ratios of immune cells to cancer cells, and after a certain point, a stop solution is added. In order to measure how many melanoma cells were killed, they added a purple tetrazolium salt (iodonitrotetrazolium violet; INT), a chemical called NAD+ and an enzyme called Diaphorase. When a cell is killed, it is lysed, meaning that its membrane breaks open and the contents within it enter into the solution outside of it. Among these contents is an enzyme called lactate dehydrogenase, which converts the NAD+ into NADH. NADH will then enable the diaphorase enzyme to convert the purple tetrazolium salt into a red formazin product. This color change is visualized and measured using specialized light detecting equipment.

So then, you have one test tube with no cells in it—only the purple salt and the enzyme. And you have another test tube with cancer cells in a lysis solution (alongside the salt and the enzyme). This is your 0% cytotoxicity and your 100% cytotoxicity. The resulting color changes of all test tubes containing immune cells are compared using these as the 0% to 100% baseline.

The results were predictable: **immune cells isolated from the spleens of mice in the enriched environment showed greater cytotoxicity to tumor cells, meaning that they were more effective at lysing tumor cells. Even when the ratio of cells to tumor cells was 25:1, immune cells from the enriched environment were killed more cancer cells than did the immune**

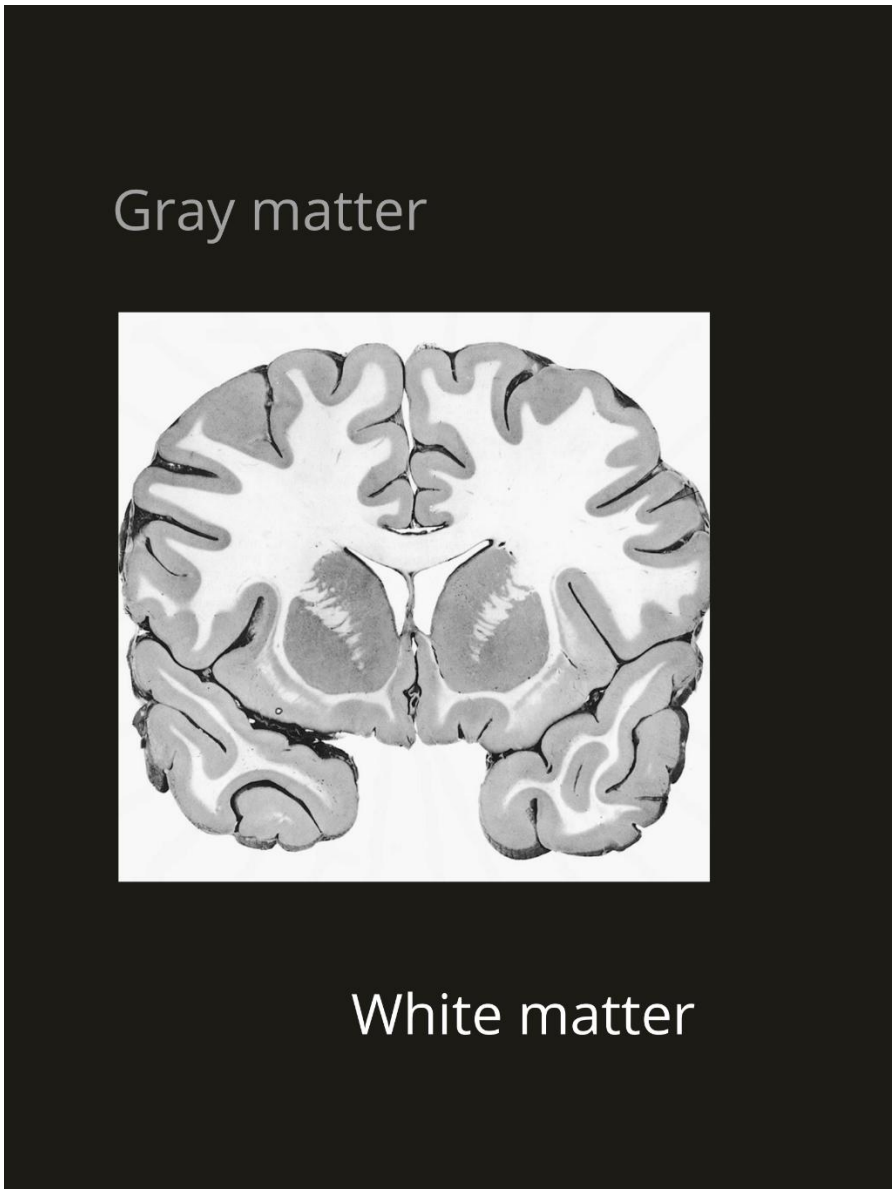
cells taken from the spleens of the public-school mice at an immune cell to cancer cell ratio of 100:1 (figures 1L and 1M in the publication).

The Wnt/β-catenin pathway

β-catenin is a molecule that, when it accumulates, tells cells to grow and divide. It does this by telling the cell to express genes that are relevant to growth processes, like cytoskeletal expansion and ultimately mitosis. Mammalian cells are programmed to die by default, and this protects the body from cancer, which is uncontrolled cell division. Normally, the amount of β-catenin is tightly controlled. To control β-catenin levels within the cell, the cell uses something that we call the destruction complex. The destruction complex includes many proteins, one of which is called Apc (Apc stands for Adenomatous Polyposis Coli, and is a critical tumor suppressor gene) Multiple experiments investigating colon cancer in mice, specifically Cao et al[1] and Bice et al,[6] used mice with defective *Apc* genes. Without properly functioning Apc, β-catenin accumulates and cells divide uncontrollably.

Normally the destruction complex, which includes Apc does not allow β-catenin to accumulate. However, when a cell receives a chemical signal from a protein called Wnt, then the cell has permission for β-catenin to accumulate. The protein Wnt binds to a receptor, called Frizzled (one of the more creative names in molecular biology). When Wnt binds to Frizzled, Frizzled then phosphorylates another protein called LRP, thus activating it (LRP stands for Low-Density Lipoprotein Receptor-Related Protein). LRP is another membrane protein (you can think of transmembrane proteins as moving throughout the cell membrane UNLESS they are on a cholesterol island OR are attached to something solid, such as the cytoskeleton, which is the cellular equivalent to the skeletal system, hence the name). Activated LRP sequesters the destruction complex, removing it from inside the cell and making it less available. With the destruction complex out of the way, β-catenin accumulates. β-catenin then goes into the nucleus of the cell, where it binds to a transcription factor called Tcf4 (Tcf4 stands for transcription factor 4, not one of the more creative names in molecular biology). Tcf4 then causes the expression of genes related to cell growth and division. Cao et al performed an experiment using a defective *Apc* gene, while Bice et al[6] used mice that had both the defective *Apc* gene as well as a mutated variant of the *Tcf4* gene (mice were heterozygous for both mutations, meaning that the other chromosome still had the non-mutated, wild-type variant).

The Basic Structure of the Brain



The brain is composed of gray matter and white matter. If the shape of the brain is thought of as a half-sphere (this is a very crude illustration but it gets the point across), grey matter is located all along the surface of the half sphere (including the bottom which is flat in our hypothetical example) while white matter fills the inner volume of our dome. At one point closer to the bottom center of the half sphere is tiny cluster of grey matter that he hypothalamus. The hypothalamus is the part of the brain that has everything to do with the survival of the body.

The grey matter is comprised primarily of cell bodies while the white matter is comprised primarily of axons, which connect the cell bodies to one another. Grey matter is largely on the surface of the brain while white matter is located inside of the brain. This is due to geometric necessity. Imagine if you have a computer device, and you have a bunch of processor chips and

you have wires to connect these processor chips to each other. Now, if you have to assemble all of these components in this hypothetical PC such that you maximize the number of possible connections between and among the various components, geometric necessity would have you place the components on the outside and the wires connecting them on the inside. Hence you have the grey matter on the outside and the white matter on the inside.

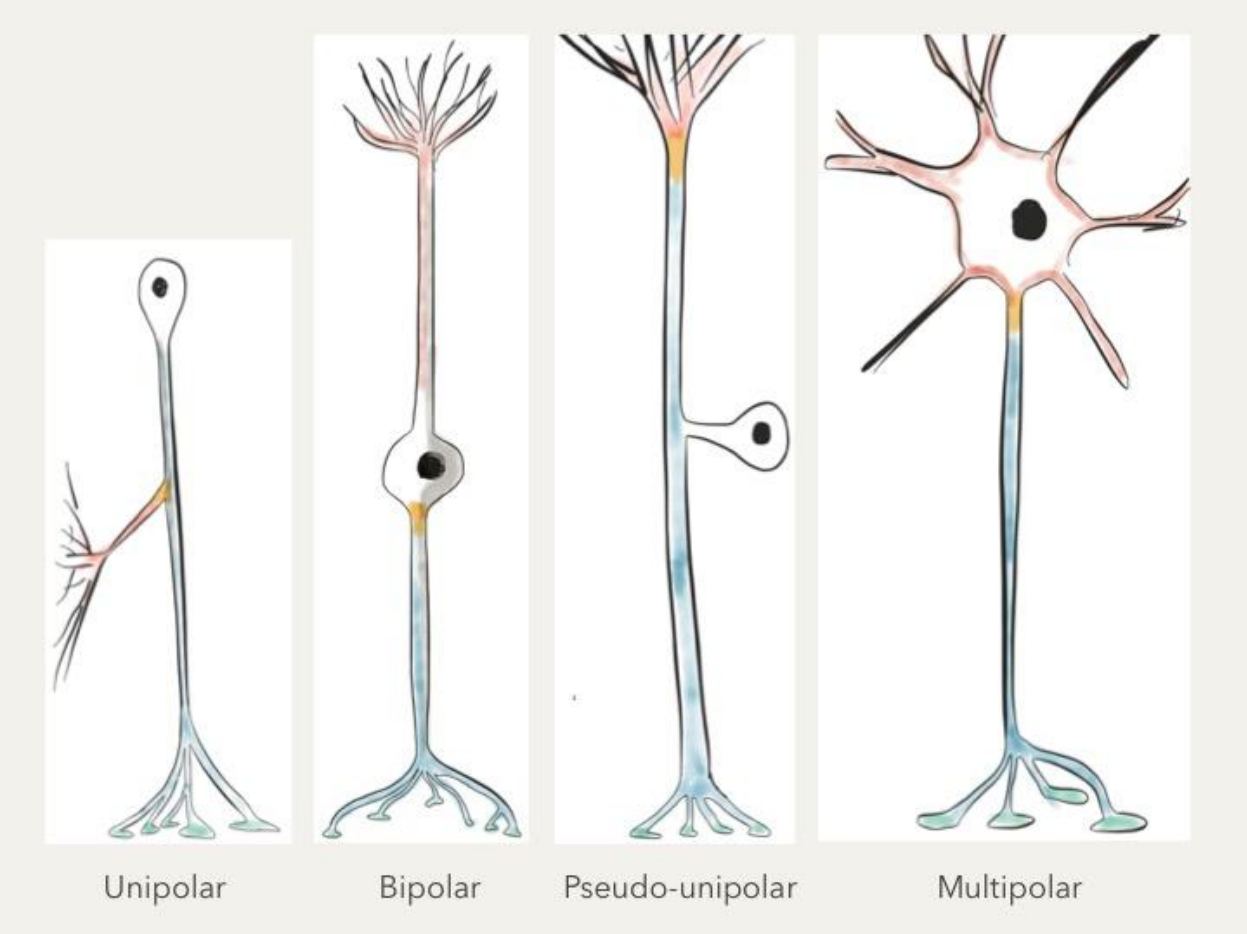
The cell bodies arrange themselves in architectures that lend themselves to specific computational tasks, and different regions will have slightly different neuronal architectures. For example, there is Broca's area which handles speech production and Wernicke's area which handles speech processing. Broca's area involves coordinating the mouth, tongue, lungs, voice box, etc. to produce words and sounds. Brain damage to Broca's area, such as by strokes or traumatic injuries, can result in slow, effortful speech interrupted by pauses. Wernicke's area, involves word comprehension. Damage to Wernicke's area can cause fluent but nonsensical speech.

Both Broca's area and Wernike's possess a distinct architecture of neurons that enable them to carry out their respective roles in human cognition, in a way that is much analogous to how, in a computer, the CPU (central processing unit) and the GPU (graphics processing unit) each possess distinct transistor architectures that enable them to perform specific tasks. The CPU does the lion's share of computer calculations and can handle any type of calculation. The GPU, on the other hand, can only do one type of calculation, but it does that one type of calculation *a lot*. Both the CPU and the GPU are used in different ways by the computer to give you an experience of, say, playing a video game.

Thoughts will take form through the brain by using whatever machinery in the brain that effectively give those thoughts (physical) form. Different thoughts will utilize various combinations of machinery in the brain based on their inherent nature. For example, if you visualize a yellow rose, then different parts of the brain necessary for the visualization will be active during the visualization. If you are a painter and you start to paint the image of a yellow rose, then more regions of the brain will be activated to help you undertake that task. If you talk about yellow roses to a friend, then speech production and other parts of the brain will be utilized, etc.

Neurons, Neurotransmitters and Ions

For my explanation of what a neuron is, I will say that neurons have four parts: the dendrites, the cell body, the axon and the axon terminal. Neurons connect at something called a synapse, which is where the axon terminal of the *presynaptic* neuron releases neurotransmitters, which then activates the *postsynaptic* neuron via its dendrite to then fire. Neurotransmitters are small molecules which bind to ion channels in the post-synaptic neuron leading to depolarization. Neurons have not been observed to have more than a single axon (although their structures can vary from being a unipolar neuron, a bipolar neuron or a multipolar neuron)



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Throughout the entire membrane of the neuron are all kinds of structures. The important ones to remember for the sake of our discussion are:

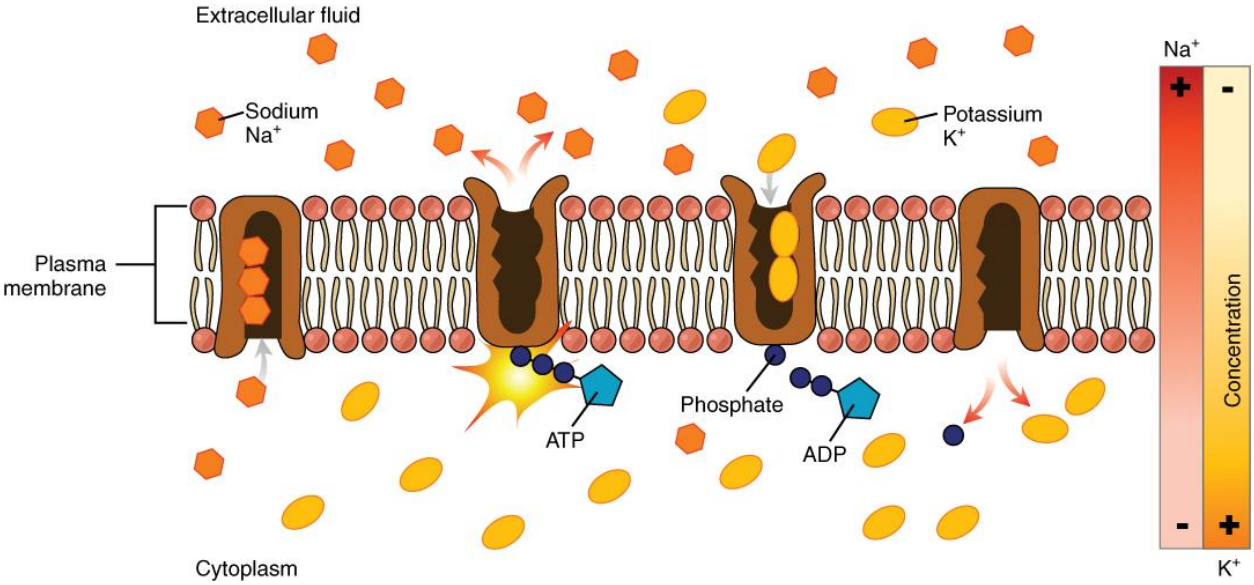
- ligand gated sodium channels (A ligand by definition is a chemical/small molecule that binds to a protein and impacts its function. In this case the "ligand" here is called a *neurotransmitter*)
- voltage gated potassium channels ("voltage gated" means that the ion channel is open or closed based on the difference in charge between the inside of the cell and the outside of the cell, measured in millivolts or mV)
- voltage gated sodium channels,
- sodium potassium pumps
- "leak" channels which allow ions on either side of the nerve cell to travel across the membrane.

This is important to remember as I describe the mechanism by which neurons "fire," also known as *depolarization*.

What do we mean when we say that a neuron "fires"? We'll get to that in detail, but first I will explain about ions, ion channels and ion pumps.

Ions, Ion channels and Ion Pumps
Table salt, which is sodium chloride, will split into positively charged sodium ions and negatively charged chloride ions when dissolved in water. In a glass of salted water, sodium and chloride ions are distributed evenly and randomly throughout the volume of the water in the glass. Neurons in the brain, however, have to keep some areas high in specific ions and other areas low in specific ions. **When the neuron is not firing, or "at rest," it will have more sodium and chloride ions outside of the cell than inside of the cell AND it will have more potassium ions inside of the cell than outside of the cell.**

Nature would *not* have different concentrations of ions inside or outside of the cell. Nature would have the ions evenly distributed on either side of the membrane—like the salt dissolved in water. Therefore, in order to maintain different concentrations of ions on either side of the cell membrane, the neuron must expend energy to "pump" ions into their respective places. It does this by using a protein that is called a sodium potassium pump, which pumps 2 potassium ions into the cell while simultaneously pumping 3 sodium ions out of the cell. Because the pump is pumping sodium and potassium against their concentration gradients (from low concentration to high concentration), it must couple this action with ATP hydrolysis to be energetically favorable.



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Adenosine triphosphate, ("ATP" if you remember from high school biology) is typically thought of as the energy currency of the cell. Mitochondria (the cell's "power plants") produce ATP from adenosine diphosphate (ADP) and add a free phosphate ion. The cell's mitochondria maintain an abundant supply of ATP throughout the cell. Mitochondria require oxygen in order to generate ATP from ADP and phosphate. Because the functioning of neurons depends on them maintaining these concentration gradients, the brain needs a constant supply of oxygen. Any disruption to the brain's ability to maintain these concentration gradients (aka "homeostasis"), even for a brief period of time, can have potentially disastrous consequences on the brain. This is why you might hear in medical shows "his brain has been without oxygen for over a minute!" after they've

performed CPR. That's because the brain must always maintain these gradients as part of your survival and without sufficient oxygen, these concentration gradients cannot be maintained (our bodies cannot store oxygen like it can carbohydrates—the body depends on a limitless supply of oxygen to be available to it at all times).

(This is also why you get brain freezes, by the way. The sodium-potassium pump relies on random motion for sodium and potassium to bind to it. The speed of this random motion, also known as Brownian motion, is directly correlated with temperature. If the sodium-potassium pump can't work fast enough, then your brain won't be able to fire at the rate that it needs to, making neurons exquisitely sensitive to temperature.[13] The cellular machinery in the neuron is designed to function at body temperature, which is 98.6°F or 37°C. When you drink an ice-cold beverage, the ice lowers the temperature of the blood flowing into the brain too much, which disrupts homeostasis in the brain beyond what the brain decides is comfortable, and so it lets you know "HEY! TOO MUCH COLD!" Next time you have brain freeze, try placing something warm like your hand or your wrist on the back of your neck and you'll feel relief, maybe don't use your hand though since it's probably cold from the beverage you're drinking).

Also, because you have *three positive ions moving out* of the cell for every *two positive ions moving into* the cell, the inside of the neuron is more negatively charged than the outside. This results in an electrostatic force, so a positively charged ion such as sodium or potassium will want to go to a place with more negatively charged ions, because positive and negative electrical charges attract one another. This electrostatic difference is measured in millivolts (mV). At "rest" the difference in electrical charge across the neuron's cell membrane (or its *membrane potential*) is −70mV.

What Happens When Neurons Fire

Here, I would once again recommend searching the internet for pictures and animations, as this is a very elaborate process.

I said earlier that nature would have negative and positive charges neutralize, resulting in a voltage difference of 0mV (so no voltage difference) instead of −70mV, and it would have all sodium and potassium ions to be equally distributed on either side of the membrane. This means that when the neuron is "at rest" it is inherently unstable because it is going *against* what would happen naturally. Another word that we would use to describe the resting state of the neuron is that it is *polarized*, because there is an uneven distribution of charge.

Within the neuron's cell membrane there are ion channels which enable ions to travel across the membrane to or from the cell. These channels are specific for specific ions, meaning that a sodium channel will let sodium through it but not potassium, and a potassium channel will let potassium but not sodium. If there aren't other proteins intervening—such as the sodium potassium pump—ions will naturally travel in the direction of highest concentration to lowest concentration.

When the neuron "fires," it allows the ions to move as they would move naturally, but in a controlled fashion. So the neuron goes from its "resting" state of being polarized to its "excited" state in which it is *depolarized*. Typically, neurotransmitters released into the synapse by the

presynaptic neuron will bind to a ligand gated sodium channel, which will cause a large influx of sodium into the cell. As that happens, the positive charge inside of the cell membrane increases, and the voltage difference will become more positive.

If enough sodium enters the inside of the neuron and becomes positive enough, the membrane potential difference will go from its "resting" value of −70mV to −55mV (either a lot of neurotransmitters have been released *or* the post-synaptic cell has many of these ligand-gated channels). If enough sodium enters the inside of the neuron that the membrane potential reaches −55mV, then a chain reaction will occur: even more sodium will rush into the inside of the cell from the outside. This is because the neuron is covered with *voltage-gated sodium channels* that are only open when the membrane potential reaches −55mV.

Only a small number of *voltage-gated sodium channels* need to be open for the chain reaction to occur. This is because the influx of sodium at one receptor will open a neighboring *voltage-gated sodium channels*, which will then open even more neighboring *voltage gated sodium channels* in a chain reaction that will eventually move across the entire length of the neuron. The opening of *voltage-gated sodium channels* continues across the neuron until it reaches the end of the neuron, or the synaptic terminal. Neurotransmitters are held in membrane enclosed vesicles inside of the synaptic terminal. Once the *voltage-gated sodium channels* are open at the synaptic terminal, calcium is released from the endoplasmic reticulum, which causes the vesicles containing the neurotransmitters to fuse with the membrane and be released outside of the neuron into the synapse. Neurotransmitters can cause all kinds of events within the body. Sometimes the neurotransmitters might cause other neurons to fire, sometimes they will trigger muscle movement, etc.

After it fires, the neuron has to return to its resting state (in other words, it has to *repolarize*). After enough sodium has entered the neuron such that its membrane potential becomes +30mV, the *voltage-gated sodium channels* will be closed by a "block" that is built into *the voltage-gated sodium channel* itself. This "block" is like a tethered cap (a tethered cap is like a lid that is attached to the water bottle by a string). When the block or lid of the voltage-gated sodium channel is on, so to speak, we call this its "inactive state," (the voltage gated sodium channel has three possible states: "open," "closed," and "inactive"). At this time, *voltage-gated* ***potassium*** *channels* now open causing positively charged potassium ions to exit the neuron. As potassium ions exit the neuron, the charge returns to its more negative, polarized or "resting" state of −70mV. As the cell returns to the negatively charged state due to the outflux of potassium, the *voltage-gated sodium channels* and *voltage-gated potassium channels* both close. At this point the sodium potassium pump described earlier will then continue to do the work to return to homeostasis. The neuron is now back at its rest state, ready to fire as soon as a neurotransmitter has been released. This process can occur within a single neuron up to a thousand times a second.

It is also worth noting that outside of the neuron there is a high concentration of chloride ions. Chloride ions are negatively charged, and so when they are allowed into the cell, that will cause the membrane potential to go below the standard −70mV. The chloride ions go against the charge gradient because they are so abundant outside of the cell that simple osmosis favors their motion into the inside of the cell. Therefore, neurons can be inhibited from firing by being made to open chloride channels. When chloride enters into the neuron and causes the neuron's membrane

potential to be less than −70mV (up to −90mV), we call this *hyperpolarization* and say that the neuron is *hyperpolarized*. In the brain tissue, glutamate is the name of the neurotransmitter that results in sodium uptake by the neuron (thus causing it *depolarize* or fire, so we call glutamate the "excitatory" neurotransmitter), while GABA (gamma-aminobutyric acid) is the name of the neurotransmitter that binds to chloride ions (thus causing it to *hyperpolarize*, making it less likely to fire and so we call GABA the "inhibitory" neurotransmitter).

That neurons can be inhibited or repressed by opening chloride channels is important because BDNF release depends on neurons depolarizing. **Therefore**, **hyperpolarization** (**by intake of chloride ions**) **will**, **in general**, **reduce the likelihood of BDNF release because it reduces the likelihood of neurons firing**. In order for BDNF to be released, the neurons must fire (there are very likely exceptions to this rule, but this is the general rule). I would hypothesize that if cells of the adaptive immune system release BDNF in response to detecting their target antigens,[14] then that might cause local sympathetic nerves to signal to the hypothalamus that there is a threat that the immune system needs to deal with at that location. The hypothalamus then, through its control over the sympathetic nervous system, must change itself so as to most efficiently direct the immune system to respond to that threat. Based off of the enriched environment experiments, this form of plasticity for immune system coordination by the hypothalamus hinges on the release of BDNF.

When somebody is in a state of chronic anger repression, neurons within the brain that interface with the sympathetic nervous system are more likely to be hyperpolarized, preventing them from firing as often as they otherwise would. When somebody is in a state where they don't *need* to be angry, then the neurons which interface with the sympathetic nervous system will not be in a state of chronic hyperpolarization. Therefore, I hypothesize that chronic hyperpolarization of neurons within the hypothalamus that interface with the sympathetic nervous system will prevent BDNF release, which in turn will obstruct the hypothalamus from optimal coordination of the adaptive immune system. (Natural Killer T cells aren't *technically* the adaptive immune system, as they are most often categorized as part of the innate immune system. I, however, would say that natural killer cells area a *mixture* of adaptive and innate immunity, as they do have memory.[15])

What Alcohol Does to the Brain

A fun illustration of how alcohol acts on the brain might go as follows: imagine your brain is like a house, and different subpopulations of neurons are like people who live together in this house. We'll call the people who are in charge of this house the fun police. The fun police use GABA to enforce their rules on everyone in the rehab house, but never on themselves. Think of GABA like it's a shock collar on everybody's neck. Everyone in the house has a shock collar on, including the fun police. But only the fun police have access to the button with which shock people. That is how they maintain control.

The fun police use GABA shock collars to stop the rest of the house from having fun and expressing themselves—and maybe the fun police have good reason to do this. Bobby, after all, has a lot of intrusive thoughts and he's let everyone know that he is *really, really curious* about what would happen if he burned the house down. Even worse, Sarah and Jake…*want to have sex!* All of these people, including the fun police, are different aspects of you.

Members of the fun police frequently use these shock collars (GABA) to wield control over every other member of the house ("oh? Bobby wants to play with matches over there?" "Press the button!" BUZZZZ! "Sarah and Jake want to *have sex?!*" *BU*ZZZZZZZZZZZ!). If this brain house already sounds like a rehab house, that would make sense: the rehab house is an external manifestation of the now-sobering alcoholic's inner world. I actually got the idea for this analogy from my friend who told me about his time at rehab.

Then, when you consume copious amounts of alcohol, it's like everyone in the brain house's shock collar gets a low-grade buzz. Everybody in the brain house is used to constant shocks and the constant threat of shocks, so they're desensitized to electric shocks. This low-grade buzz is like nothing for them. The fun police, however, aren't used to constant shocks so they are completely incapacitated by the low-grade buzz. After all, the part of your brain that is inhibiting the rest of your brain is active and excited all the time during your sober, day-to-day life—it's busy inhibiting everything else. Yet this makes that part of the brain that is inhibiting everything else more affected by alcohol. And so, going back to our brain-house analogy, when you are drunk, the fun police are knocked out cold by the mild, low-grade shock. Now, the members of the rehab house get to have their fun and be loud and litter and get naked and dance without the threat of more severe electrocution.

After repeated shocks, which would represent long term alcohol use, the fun police do eventually build up a tolerance, just as long-term alcohol users develop a tolerance for alcohol. They also see that everybody else's tolerance for shocks is too high, so they up the voltage that they apply when they use the collars on everyone else. And then they, in turn, become more desensitized to electric shock. Then, with this increased desensitization for electric shocks, more alcohol is needed to subdue the fun police so that the residents can once again have their fun.

Then, something happens and you decide to stop drinking alcohol cold turkey. This means that the fun police is now back in command, and everybody else lives under the constant threat of electrocution. Yet without those bursts to vent their pent-up energy, they all suffer and the house falls into serious disrepair. The house's heater stops functioning and the members of the house stop making food and go on a hunger strike. The fun police are now cold and hungry.

Eventually, the fun police have no choice but to say "there must be a better way," and then they become teachable. They realize that they can't just use electroshocks to control everybody: they have to work together and help everybody meet their needs ("okay Bobby, why don't you fix the heater and the furnace since you like playing with fire, and maybe cook some food over a fire while you're at it!" "Sarah and Jake you guys can have sex as long as you use protection." Now Sarah and Jake are happy to help Jale with dinner and clean the house because they are rewarded with a sexual outlet)

Alcohol works by activating GABA receptors and inhibiting glutamate receptors. GABA is the primary inhibitory neurotransmitter while glutamate is the primary excitatory neurotransmitter). This causes alcohol to have an overall inhibition effect on the brain, and the part of the brain that is most affected by the alcohol induced inhibition is the part that is the *least inhibited* in your day-to-day, sober life—namely, the part of the brain that is inhibiting everything else. Then, the

brain becomes more excitatory, in order to overcompensate for the inhibition effects of alcohol. What this means is that when people drink alcohol to lower their inhibitions, those very inhibitions they are drinking to bypass *will become stronger when they are sober*.

Brain Stimulation by Electrodes

Placing electrodes into the brain and then applying a voltage across the electrodes causes membrane depolarization. The precise mechanism is most likely that sodium ions move toward the negatively charged cathode away from the positively charged anode. In doing so, they enter - neurons and cause membrane depolarization—(membrane depolarization is synonymous with neurons firing).

The electrode system is thought to influence all neurons around the electrode to fire. The magnitude of the electrode's influence on the surrounding tissue can be increased when an increased voltage is applied. Of course, if the voltage is too high, you can get a faradaic current that will chemically alter the ions of the surrounding tissue, causing chemical damage, which is to be avoided. Faradaic current has to do with the movement of electrons, such as in a wire or from the electrode to the electrolyte or from the electrode into the water, causing water molecules to react and become hydroxide ions (this would screw with the body's optimal pH) and hydrogen gas (which would build up pressure in the brain if it can't escape). There are other possible faradaic reactions that might occur and they're all bad and can cause severe damage to surrounding tissues. So, you want to keep the current as non-faradaic, meaning that you're dealing strictly with the movement of ions within the electrolyte (you can think of brain tissue as an electrolyte in this case).[16]

Eventually the ions will stop moving because they have surrounded the cathode and anode such that they have effectively neutralized the electrodes' effect upon the surrounding tissue. Electrical engineers could thus approximate the electrode as a capacitor in a circuit diagram (a capacitor is a device which stores electric charge). Once enough ions have clustered around he inserted electrode, there will be no more effects from the electrode. Therefore, in order to produce sustained stimulation, a the electrode is turned on and off several times a second (electrical engineers would call this a waveform). When the electrodes are off, the sodium ions now clustered around the cathode can dissipate back into the brain tissue. Then, the electrodes are turned on and so they move back to the electrode. As they move, the neurons surrounding the electrodes will fire.

Provided that the brain slices are kept cold and placed into solutions of hyper-oxygenated artificial cerebral spinal fluid, measurements on the functioning of neurons can be obtained. Extremely tiny electrodes are placed inside and outside of a cell from a slice of post-mortem brain tissue slice, measuring the inside and the outside voltage of the neuron. At rest, the inside of the neuron is 80mV more negative than the outside of the neuron. An electrical current is induced, causing the inside of the neuron to fire and thus become more neutral (we call this "depolarization").

c-Fos and FosB/ΔFosB Expression and Brain Activity

Multiple publications that I cited in this book measured both c-fos and FosB/ΔFosB in mouse brain tissue slices as indicators for what neurons were firing right before death. In spite of their

almost identical names, these are two different proteins from two different genes. The Fos gene encodes for the c-fos protein while the FosB gene encodes for the FosB protein. Both Fos and FosB expression occur immediately after neurons fire, and so measuring them is a reliable way to see what brain regions and what neuronal populations were metabolically active right before death.[17] Lin et al,[18] whose work I cited in Chapter 10, looked at c-fos mRNA expression to identify what neurons were active during mating and aggression. It is thought that it takes 30 minutes for mRNA to translocate from the nucleus of the neuron to the cytosol. So Lin et al[18] had either a mating or an aggression episode follow another mating or aggression episode within 30 minutes of each other. The mice were then sacrificed immediately after the second episode. Then, using Fluorescence in Situ Hybridization, also known as FISH, Lin et al[18] could see what neurons had c-Fos mRNA in *both* the cytosol and the nucleus. That would be an indication that the neuron was metabolically active during both episodes. Fluorescence in situ hybridization uses either a DNA or RNA primer (in this case RNA) that has a fluorescent molecule attached to it that is complementary to the mRNA researchers are attempting to measure (which in this case was the c-fos mRNA).

The c-Fos protein is highly transient and short-lived.[19] FosB, unlike c-Fos, is a more stable and typically gives an indication that chronic—rather than acute—changes are taking place—this is particularly the case as FosB switches to ΔFosB. To make ΔFosB, FosB mRNA is cut, resulting in a shorter protein that doesn't contain a degradation signal. Thus, the transition from FosB to ΔFosB is thought to be like a molecular switch that is involved in chronic changes, such as in addiction.[20,21]

Lehmann and Herkenham[22] examined FosBΔFosB levels in the brains of autopsied mice to see which regions of the brain were active in conferring resiliency to conditioned social defeat due to environmental enrichment. After the mouse lived as an intruder in another mouse's cage for two weeks, the mice went through a battery of behavioral tests and were autopsied to examine FosB/ΔFosB expression in various parts of the brain. FosB/ ΔFosB is a transcription factor which signals neurons to produces specific proteins in response to immediate activity. Lehmann and Herkenham[22] found that environmental enrichment significantly increased activity in the prefrontal cortex and the infralimbic cortex in mice subjected to conditioned social defeat.

The Molecular Mechanism of BDNF

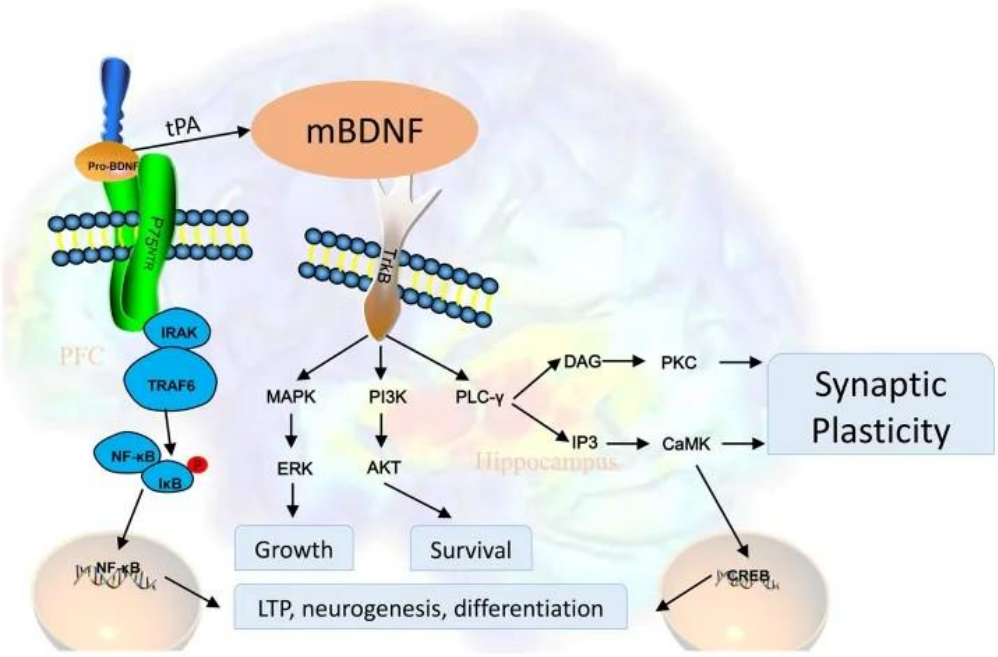
The BDNF gene has 9 non-coding exons, 2 of which can be differentially spliced and then there are 2 different polyadenine tails, resulting in 22 possible splice variants of the BDNF gene. No matter the splice variant, the resulting BDNF is the same. However, it has been shown that different splice variants of BDNF impact *where* the BDNF is produced within the neuron's cell body.[23] For example, one splice variant will result in new connections being formed in the middle of the neuron's axon, while another splice variant has been shown to increase the number of dendritic branches immediately off of the cell body. It has also been shown that different tissue types—immune cells, gonads, cells of the peripheral and central nervous system—appear to express different splice variants of the BDNF gene in a process that is not entirely understood.[24,25]

When BDNF mRNA is first translated, it includes a pro domain and BDNF. The pro domain is cleaved off and thought to potentially impact neurogenesis. BDNF meanwhile, is packaged by

the cell's endoplasmic reticulum into dense core vesicles which are then stored in the pre-synaptic neuron.[26] Prolonged depolarization (depolarization is another word meaning that the neuron "fires" this process will be explained in great deal in the next chapter), high frequency firing, and theta stimulation (theta waves are associated with relaxation, creativity, and memory) are all processes that trigger the release and signaling of BDNF.

Free BDNF can then travel to the post synaptic cell and bind to something called a TrkB receptor. The postsynaptic nerve cell will have many TrkB receptors which exist on their own individually. TrkB receptors are membrane proteins. Membrane proteins can be either static (non-moving) when they exist on cholesterol islands or they can be free moving. TrkB receptors are assumed to be free moving. When BDNF is present in the synapse, it will bind to a TrkB receptor. When a single BDNF molecule binds to a single TrkB receptor, another identical TrkB receptor will join it. Individual TrkB receptors do not interact with each other unless BDNF is present. When the TrkB receptors, bow bound to BDNF, "pair up" or *dimerize* (a single TrkB receptor is a monomer; two TrkB receptors interacting with each other is a dimer), they attach phosphates to each other in what is called an *autophosphorylation*. When TrkB receptors are phosphorylated, they are now *activated*.

Phosphorylated TrkB receptors, now activated by their own autophosphorylation, then recruit other proteins that operate over multiple sophisticated pathways, ultimately guiding gene expression to do things like promote the formation of new synapses, strengthen existing synapses by increasing the amount of receptors on that synapse, and promote cell survival in the case of pre-neuronal progenitor stem cells. TrkB is dominantly activated in the central nervous system by neurotrophins, most notably BDNF, but also neurotrophin-4[27] and sometimes neurotrophin-3.[28]



Source: Yang T, Nie Z, Shu H, et al. The Role of BDNF on Neural Plasticity in Depression. *Front Cell Neurosci*. 2020;14. doi:10.3389/fncel.2020.00082

I talked about the use of microRNA (miRNA) to knock down BDNF expression. Another way to knock down the impact of BDNF besides denying its expression is by using a truncated variant of the TrkB receptor, known as TrkBT2. The mRNA encoding for the TrkB receptor protein can be differentially spliced to result in either one of two truncated TrkB variants, namely TrkBT1 and TrkBT2. BDNF signaling results in two full length TrkB receptors coming together and phosphorylating each other. If one of these TrkB receptors is not full length, then it can't phosphorylate the other or be phosphorylated, preventing further downstream signaling. Thus the truncated TrkB variant is a way to disable BDNF signaling instead of knocking down BDNF using small interfering RNAs. Garofalo et al[29] noted that increased BDNF inhibited glioma growth because the gliomas over-expressed the TrkBT2 variant of the TrkBT2 receptor.

BDNF, c-Fos and FosB/ΔFosB all represent different aspects of brain plasticity. The c-Fos protein has to do with short term memory. When FosB is shifted into ΔFosB, it inhibits c-Fos and has to do with long term habit formation. BDNF has more to do with memory formation. These are all oversimplifications but I think it's fair to say that we have yet to understand the process of brain plasticity and how that translates into our life experience.

Stereotaxic Injection of Lab-Grown Adeno-Associated Virus

Cao et al 2010[1] performed two experiments on mice where they were genetically modified to either "knock up" or "knock down" BDNF expression. To genetically modify the mice, researchers used an Adeno associated virus (often abbreviated as AAV) that carried the DNA for the human BDNF gene, and stereotaxically injected it into the mouse's brain under anesthesia. Stereotaxic refers to the coordinate system imposed on a mouse's head. By injecting the adeno-associated virus into the correct stereotaxic coordinates, you can reliably inject the adenovirus via a syringe into the desired spot of the mouse's brain (which in this case the hypothalamus). It involves placing the mouse in a contraption called a stereotaxic frame that imposes the coordinate system upon the mouse's head. The same principle is used when performing brain surgery on humans, where the patient will where a stereotaxic frame over their head during the procedure so that surgeons can reach into the correct area of the brain.

This human BDNF gene was genetically modified by attaching another protein to it called "hemagglutinin," which enabled researchers to use red fluorescent antibodies to bind to the modified BDNF in brain tissue slices from the hypothalamus of autopsied mice, enabling them to see the results of their genetic modifications with a fluorescent microscope (Lao et al figure 3B). Using a human BDNF is somewhat inconsequential because human BDNF and rodent BDNF are largely identical.

Cao et al[1] also genetically modified control mice in this experiment to produce green fluorescent protein under the microscope. Green fluorescent protein (GFP) is a protein originally discovered in jellyfish that glows green under ultraviolet light, allowing researchers to actually see the neurons they had genetically modified. Using a green fluorescent protein is a way to verify that (1) the genetic modification process *worked* and (2) that benefits attributed to the animal are the result of the increased BDNF production and not from some unknown variable that may have been introduced by genetic modification process (we never know what we don't know, after all).

Aequorea Victoria—the jellyfish that is the original carrier of green fluorescent protein. Osamu Shimomura was the Nobel Prize winning discoverer of it. It is now used almost universally in molecular biology. Copyright Holder is Sierra Blakely, Image retrieved from https://commons.wikimedia.org/wiki/File:Aequorea4.jpg

To deliver the gene of interest, which in this case was the hemagglutinin tagged BDNF, DNA encoding for the gene had to be produced using bacteria (in this case *E.* coli). This would have been done with something called a plasmid (a plasmid a circular piece of DNA that can be replicated alongside the bacterium's chromosomal DNA, which must be *transformed* or *put into* the bacteria). The plasmid would have had an origin of replication that would enable it to get copied by the replication machinery inherent in the *E. coli*, and an origin of replication that would be placed into any other cell type that researchers would use. Usually, the bacteria containing the plasmid are grown in a liquid medium that contains nutrients for the *E coli* to grow as well as an antibiotic. The plasmid then, contains a gene for resistance to that antibiotic so that only bacteria carrying this plasmid could survive (that way you don't get bacteria that don't have the plasmid surviving, after all),

After the genetically modified *E. coli* are grown overnight, the plasmid is isolated the following morning. The plasmid DNA is then transfected into mammalian cell lines ("transfected" in this context means "put into." Transfection is the word we use when inserting plasmid DNA into mammalian cells. Transformation is the word we use when inserting plasmid DNA into bacteria). Mammalian cell lines transfected with plasmid DNA could then be grown *in vitro* and then used to produce the adeno-associated virus (the genetics of the virus are also contained within the plasmid). Once isolated, the viruses were surgically injected into the hypothalamus of each mouse, and then the experiment could begin.

Using this adeno-associated virus, Cao et al,[1] Xiao et al,[3] and Mansour et al[2] Bergin et al[30] genetically engineered mice to overexpress BDNF in the hypothalamus. Cao et al and Mansour et al used the adeno-associated virus method to cause mice to express microRNA to knock down BDNF expression. Bergin et al used the adeno-associated virus method to cause mice to overexpress the truncated TrkBT2 to knock down BDNF expression. Bergin et al also used this method to cause mice to overexpress IL-15 in adipose tissue.

The adeno-associated virus method was also used in behavioral research into aggression using methods like optogenetics, calcium recordings, and DREADD. Lin et al[18] used the adeno-associated virus method to get neurons in the VMHvl of the mouse to express an ivermectin

gated chloride channel, thus inhibiting aggressive behavior. Lin et al[18] and Liu et al[7] used the AAV method to get progesterone receptor bearing neurons in the VMHvl to express channel rhodopsin after induction by Cre-recombinase. Liu et al[7] also used this method to insert GCaMP into the specific neurons whose activity they were aiming to record in response to male social cues.

Genetically Modifying Mice—the Cre Lox System

The Cre-lox system is one of the most common mechanisms of controlling what cells are affected when genetically modifying mice. Cre is the name given to a protein called Cre Recombinase, which attaches to a specific sequence of DNA called a loxP site and then catalyzes a deletion or recombination at that loxP site (at this point, there are *many* Cre-lox variants, where some might delete, others recombine, etc.). Originally discovered in a virus that infects bacteria (called bacteriophage P1), the Cre-lox recombination enabled the DNA of the bacteriophage to remain stably incorporated into a plasmid in bacteria. And genetic engineers, who love taking a gene from one organism (such as the green fluorescent protein found in jellyfish) and finding new places to put that gene into, invented the Cre-lox system for genetically modifying mice. So then, the Cre-lox system was conceived! At least this time genetic engineers aren't putting this gene into places where nobody asked for it, like our food supply, paid for by American taxpayers via government subsidies, for science of course.

The Cre-lox system starts by injecting a mouse embryo with the *Cre* gene that encodes for the Cre recombinase enzyme. This results in what is essentially a random integration of the Cre gene into the mouse genome.[31] When the mice are born, they can be genotyped and bred to maintain a line of mice with a working Cre gene. The gene will also have a promoter attached to it, where the promoter is the sequence of DNA that recruits the transcription initiation complex in response to a transcription factor so that the Cre gene can be expressed. Promoters can be specific to specific cell types, so one promoter might be expressed in liver cells but not in other cell types, and another promoter might be expressed in one subpopulation of neurons but not in other subpopulations. Sometimes promotors will only cause expression under specific circumstances, such as by the ingestion of a drug or by optogenetics, enabling researchers to control Cre expression if they wish. By controlling the promoter, Cre recombinase can be produced by whatever cell types respond to the chosen promoter. This way, Cre can be expressed in certain tissues within the animal and not in others.

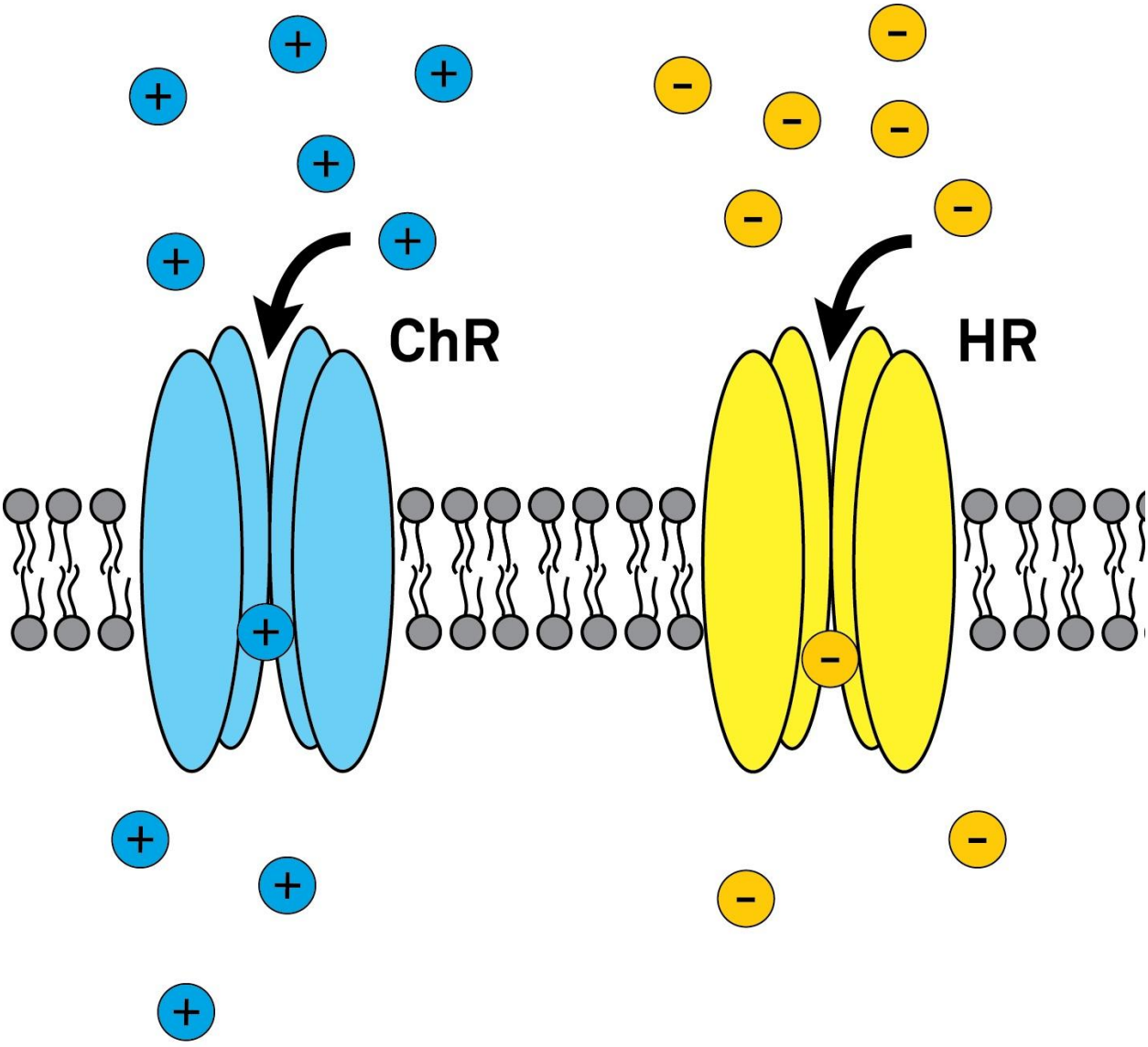
Other methods can be employed to place the Cre gene in a specific place within the mouse genome. These methods include but are not limited to Crispr Cas9, bacterial artificial chromosomes, etc. Liu et al[7] used mice that were bread with a modified Npy2r gene, which had Cre recombinase added to the noncoding sequence of the gene expressing the neuropeptide Y receptor (these were $Npy2r^{Cre}$ mice). This enabled Liu et al to genetically modify β cells (which express the neuropeptide Y receptor)s with either GCaMP or channel rhodopsin. To select for α cells, Liu et al used an additional system very similar to Cre-lox but is instead found in Yeast called Flp-FRT (this has the unfortunate name of "flip-fart"). Flp-FRT is found in yeast, and Cre-lox is typically preferred to Flp-FRT because Flp recombinase functions best at 30°C (which is the optimal temperature for yeast), while Cre recombinase functions best closer to body temperature at 37°C. Flp-FRT functions much the same as Cre-lox, but the FRT DNA sequence and the loxP sequences are different, ensuring that using both within the same cell will not

interfere with each other. So, the Flp recombinase was attached to the Estrogen receptor gene Esr1 ($Esr1^{flp}$ mice). So then, to select for α cells, Liu et al interbred the $Npy2r^{Cre}$ mice with the $Esr1^{flp}$ mice to generate $Npy2r^{Cre}$ $Esr1^{flp}$ mice. The gene sequence of the AAV was designed such that Cre recombinase would turn off the gene of interest, while Flp recombinase would turn it on (the genes of interest here are once again channel rhodopsin and GCaMP).

Yang et al[32] also used mice genetically modified to attach the Cre recombinase protein to a progesterone receptor, so that only those mice expressed the DREADD receptor.

Optogenetics—Turning Neurons on or off with a Literal Light Switch

Optogenetics genetically modifies mice to get specific cells of the brain to express a protein called *channel rhodopsin*. Originally discovered in algae, channel rhodopsin is a light gated sodium channel that opens in response to light. Algae use channel rhodopsin to detect light and then move toward the light for photosynthesis (which just means synthesizing glucose with energy from light). The ATCG gene sequence for this receptor has been identified, and then a loxP site can be added in the middle of the ACTG gene sequence so that the gene will only work after it has been edited by Cre recombinase.



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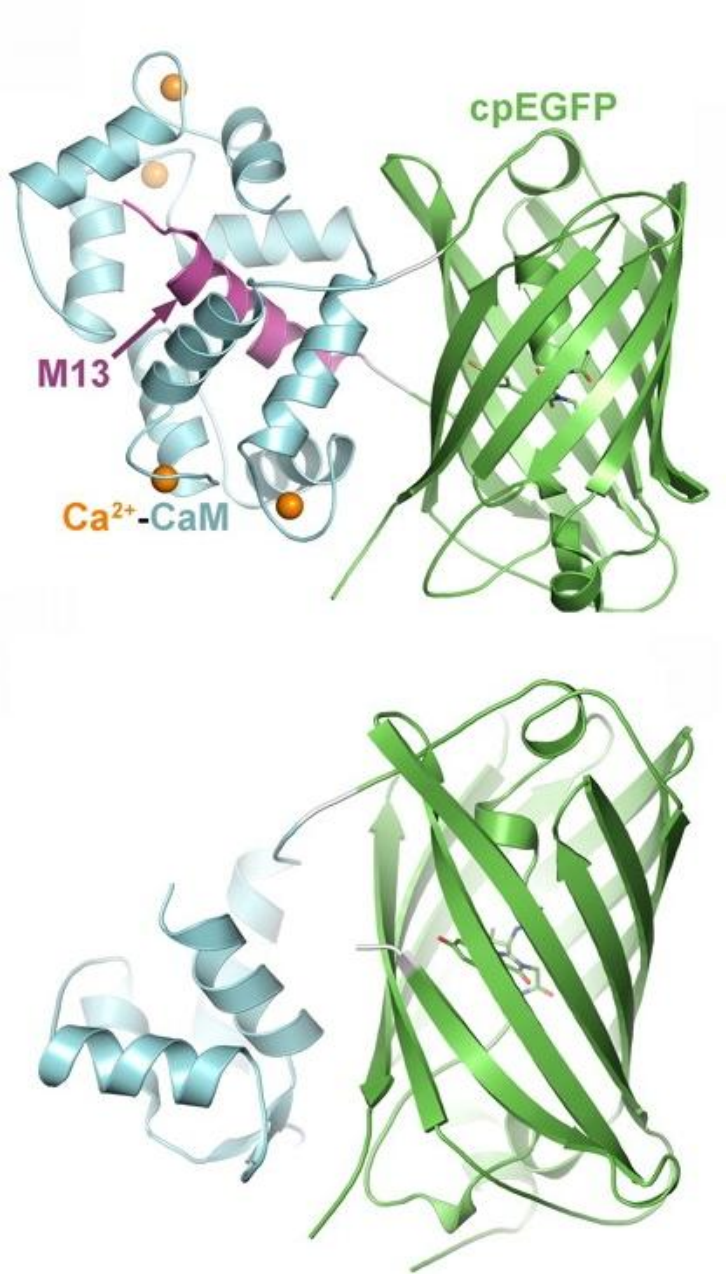
Of course, the channels are *light* sensitive, and therefore, if they are to be activated, a light source of the specific wavelength must reach that area of the brain (which is normally completely dark because it resides in your skull, which along with your skin blocks out most light).

Therefore, researchers will surgically implant a fiber optic cable attached to a light source directly into the skeleton of the mouse. That way, specific neurons can be fired by turning on the light source that is connected to the fiber-optic cable. When the hypothalamic attack area in the ventromedial hypothalamus of the mouse is activated using optogenetics, the mouse goes into sham rage. Lin et al[18] injected Cre recombinase and Channel Rhodopsin viral vectors in the ventrolateral part of the ventromedial hypothalamus (VMHvl), causing sham rage. Liu et al[7] also

injected channel rhodopsin into both α cells (neurons that expressed an estrogen receptor but not a neuropeptide y receptor) and β cells (neurons that expressed the neuropeptide y receptors). When activated optogenetically, α cells induced sexual receptivity in both lactating and virgin female mice while β cells induced aggression in both lactating and virgin female mice.

Calcium Recordings Using GCaMP

Liu et al[7] used calcium recordings to observe both α cells and β cells to measure their activity in response to male social cues. In this instance, the neurons of interest were genetically modified using the AAV method to produce a completely synthetic protein called GCaMP. GCaMP joins together three different proteins: (1) a version of green fluorescent protein called circularly permuted green fluorescent protein, (2) Calmodulin and (3) a protein called M13. Calmodulin binds to M13 in the presence of calcium, which in turn causes a conformational change leading to the green fluorescent protein to fluoresce. This causes neurons of interest to glow when they release calcium (which happens when they fire), and this glow can be measured using the fiberoptic cable setup. This way, Liu et al was able to measure how responsive both α neurons and β neurons in female mice were to social cues from male mice when they were pregnant or lactating versus when they were not pregnant or lactating (in Liu et al's nomenclature, α neurons have the estrogen receptor Esr1 and do not contain the neuropeptide Y receptor Npy2r; β neurons contain the neuropeptide Y receptor Npy2r).

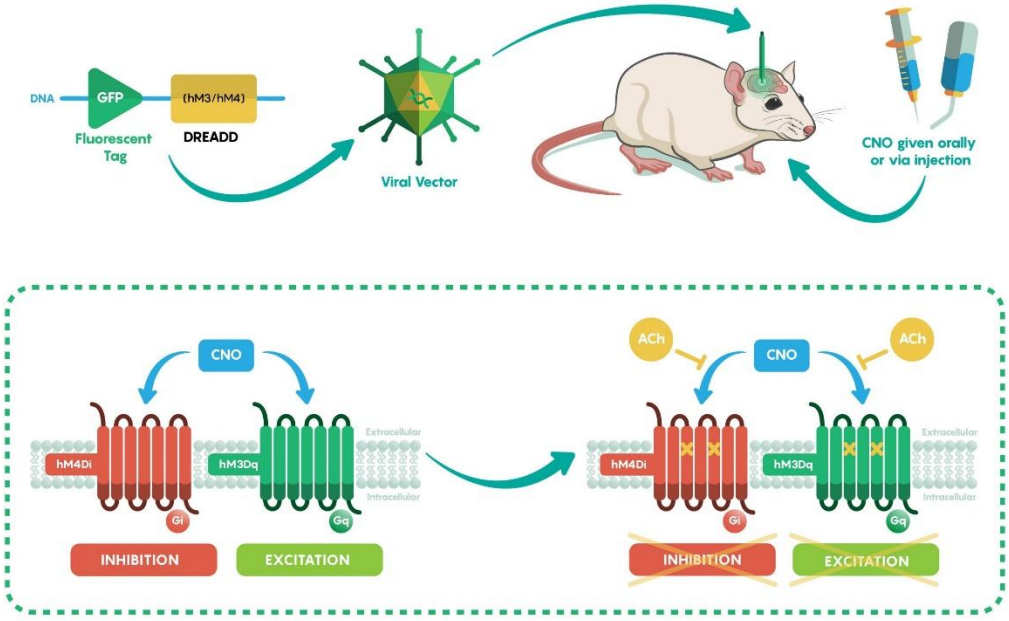


Akerboom J, Rivera J, Guilbe M ... **Crystal Structures of the GCaMP Calcium Sensor Reveal the Mechanism of Fluorescence Signal Change and Aid Rational Design *** Journal of Biological Chemistry, 2008; 284, 6455-6464

Designer Receptors Exclusively Activated by Designer Drugs (DREADD)

Optogenetics certainly wins the award for one of the coolest genetic engineering methods in use today. It does, however, have notable drawbacks. For one, implanting a bioengineered fiber optic cable into the mouse's skull is cumbersome and highly invasive. We really can't be certain about the impact that a giant wire penetrating into the skull of the mouse has on the mouse's behavior. Another approach then, is to use a different receptor that responds to a drug of our choice. For the DREADD method then, we use a drug, not a bioengineered fiber optic cable. DREADD stands for "Designer Receptor Exclusively Activated by Designer Drug." The DR of DREADD,

or the “designer receptor” is something called a G protein coupled receptor, or a GPCR. G protein coupled receptors (GPCRs) will induce signaling cascades when it binds to a specific chemical (which molecular biologists will call its *ligand*), causing profound changes in the cell depending on its type. GPCRs, as their name suggests, contain a G protein and a receptor. The G protein will contain three subunits, Gα, Gβ and Gγ. The Gα subunit contains a guanosine diphosphate (GDP) When bound to its receptor, the Gβ and Gγ subunits, which will inhibit the Gα subunit. Once the GPCR binds to its ligand however, the Gα subunit will release its GDP and replace it with GTP (Guanosine triphosphate), which is more abundant in the cell. Then, the Gα will be released, and go on to the activation of downstream signaling. Notable examples of GPCRs include adrenergic receptors which respond to adrenaline and are necessary to make all of the changes for fight-or-flight and will cause different changes in different organs. Another example of a GPCR is rhodopsin, which is crucial for vision in vertebrates (rhodopsin here is not to be confused with channel-rhodopsin which is used in optogenetics—rhodopsin just means that it reacts to light).



University of Toronto, CC BY 4.0 <https://creativecommons.org/licenses/by/4.0>, via Wikimedia Commons https://ecampusontario.pressbooks.pub/app/uploads/sites/775/2018/03/Unit-3.3-low-res-01-01-scaled.jpg

The Designer Receptor of DREADD is something called a muscarinic receptor, which will respond to a drug called clozapine N oxide or CNO for short. There are a variety of DREADDs that can be used by researchers today, and each will have a unique impact depending on the cell type. Yang et al[32] used human muscarinic receptors, and genetically engineered progesterone receptor neurons in male, Cre-expressing mice to express this muscarinic receptor. In this case, when animals are fed clozapine N oxide, clozapine N oxide will bind to the receptor in the progesterone bearing neurons and cause them to be more excitable and more likely to fire.[33] The full results of Yang et al’s experiments are explained in Chapter 10.

Thus, by feeding a genetically modified male mouse clozapine N oxide or CNO for short, you are effectively achieving the same result as the optogenetics. DREADD and optogenetics are different mechanisms, but they produce similar results on the level of the neuron with DREADD being significantly less invasive.

When the DREADD is expressed in the entire mouse brain, severe seizures result (this is how the authors noted that it works).[34] This is because a seizure is essentially when neurons fire chaotically and uncontrollably. However, when the genetic modification is strictly limited to the ventrolateral part of the ventromedial hypothalamus, the mouse will engage in rage and aggression that is similar to previous studies examining optogenetic stimulation. DREADD activation of progesterone bearing mice causes them to behave aggressively, even attacking females.

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