

ASSESSING MORPHOLOGICAL CHANGES
TO THE PERIAQUEDUCTAL GRAY AND
THE CORPUS CALLOSUM IN RESPONSE TO OPIOID
EXPOSURE IN AN ADOLESCENT RAT MODEL

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The risk-taking behavior that occurs during adolescence, as well as the brain development that coincides with this period, makes adolescents vulnerable to trying and becoming addicted to drugs of abuse, such as opioids. Drug addiction causes changes to the brain at the chemical scale (triggering or inhibiting neurotransmitter release), the cellular scale (strengthening or weakening neural connections), and at the gross anatomical scale (i.e., changes detected on brain scans). The goals of this investigation are to 1) refine gross neuroanatomical imaging techniques and 2) apply the techniques to a rat model of adolescent opioid addiction. I used a nascent soft-tissue imaging technique called diceCT (diffusible iodine-based contrast-enhanced computed tomography), which allowed me to digitally analyze small neuroanatomical structures without dissection. First, I tested the efficacy of two diceCT protocols: the standard approach of fixation, staining, and scanning specimens, and another approach that adds a tissue-stabilization step, which is reported to limit the tissue shrinkage that can occur when a specimen is exposed to high concentrations of iodine. MicroCT visualizations from this experiment revealed that tissue stabilization is not routinely necessary to obtain satisfactory brain scans, as elliptical Fourier analysis found no significant *shape* differences when neuroanatomical structures were compared between stabilized and non-stabilized brains. Then, for a second experiment, I used the same adolescent rat brains to determine if opioid exposures during life can be associated with gross anatomical changes to the periaqueductal gray (PAG) and corpus callosum. I found highly significant changes to the cross-sectional shape of both the PAG and the corpus callosum in the opioid-exposed group compared to the drug-naïve group. Whether these anatomical changes translate into functional or behavioral consequences should be the target of future investigations. Potential limitations to this experiment are the low sample size ($n = 18$) and the complicated design of drug exposures. While further research is necessary to overcome these limitations, the implications of this preliminary investigation are troubling, especially given adolescents' increasing access to opioids.

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CHAPTER I

POTENTIAL DRUG EFFECTS ON THE REWARD PATHWAY AND COGNITIVE CENTERS

Introduction

Adolescence is a period of social and cognitive change in which an individual becomes prepared for the independence of adulthood. Perhaps the most notable transition seen during the adolescent years is heightened risk-taking behavior. While these behaviors may be unnerving to parents of adolescents, the neurological causes and consequences of risk-taking during adolescence may be attributable to neurological evolution; that is, brain ontogeny encourages risky behaviors during adolescence, and these risky behaviors allow adolescents to not only practice adult roles but also receive neurological reward—in the form of dopamine—for novelty seeking (Ernst and Fudge, 2009). Adolescent behavior therefore reinforces the brain’s motivational system—a developmental change that promotes reproductive success because it encourages exploration of new food sources and potential mates (Wahlstrom et al., 2010).

Brain ontogeny predisposes adolescents to greater pursuit of rewards, when compared to adults. The parts of the brain that are responsible for pursuing and reinforcing reward—the subcortical regions—mature earlier than the prefrontal cortex, which is responsible for impulse control (Somerville et al., 2010). More specifically, the medial and ventral prefrontal cortices modulate

the balance between approach behaviors (controlled by the ventral striatum) and avoidance behaviors (controlled by the amygdala). Due to interactions with these brain regions, the adolescent prefrontal cortex “encourages” approach behaviors and “discourages” avoidance behaviors, at least compared to a healthy adult (see the Triadic Model described in Ernst and Fudge, 2009). Adolescents are therefore more likely to be motivated to seek novel experiences than are adults, who have more balanced prefrontal cortex control over subcortical regions.

The imbalance that results from prefrontal cortex underdevelopment leads to the risky behaviors that are common to many adolescents (Steinberg, 2004; Casey and Jones, 2010). While it can be adaptive for an adolescent to be willing to take risks during this period that is often associated with greater independence and exploration of adult roles, it can also lead to negative behavioral outcomes (Wahlstrom et al., 2010). A notable implication of adolescent novelty-seeking is an increased willingness to try an addictive drug, even if the risks are clear, compared to either a child or an adult (Winters and Arria, 2011).

Adolescents are more sensitive than adults to positive hedonic effects of drugs and less sensitive to the aversive properties of these drugs (Doremus-Fitzwater et al., 2010). This may be due in part to accentuated dopamine activity during adolescence. Compared to any other developmental period, adolescence is characterized by the highest functionally available dopamine concentrations, the greatest dopaminergic innervation to the prefrontal cortex, and the most dopamine receptors (both D₁ and D₂) in cortical and subcortical regions (Wahlstrom et al., 2010). These data suggest that adolescents have a high level of dopamine activity and therefore may be more incentivized than adults to engage in behaviors that trigger dopaminergic reward. Another consequence of the high level of dopamine activity during adolescence is that—compared to adulthood—drug abuse may be more likely to lead to addiction and relapse (Yamaguchi and Kandel, 1984; Brenhouse and Anderson, 2008). These behaviors are not without consequences: A growing body of evidence suggests that adolescent drug addiction causes lasting cognitive

impairment (Winters and Arria, 2011). Due to the increased drive toward risky behaviors such as drug abuse, as well as the heightened potential for developing addiction and the potential long-term cognitive effects of drug use, adolescents should be included in drug addiction experiments.

Specific Aims

The goals of this thesis introduction are three-fold. I will (1) summarize the anatomy and neurophysiology of the reward pathway and (2) of the cognition areas in the brain; along the way I will provide an overview how drugs of addiction affect these regions. Then, (3) with this background, I will focus on two regions: the periaqueductal gray (PAG) and the corpus callosum. I will show how these two regions are easily analyzable candidates for how exposure to addictive drugs—and in particular, opioids—affects the anatomy of adolescent brain, with possible implications in impaired behavioral responses and cognition.

I hypothesize that opioid exposure during adolescence affects the morphology of the PAG and the corpus callosum. To test this hypothesis, I obtain microCT visualizations of opioid-exposed and opioid-naïve Sprague Dawley rat brains. I then use geometric morphometrics to test for differences in the structure of both the PAG and the corpus callosum between the two groups. More specifically, I analyze the two-dimensional shape of the PAG at a coronal section just dorsal to the ventral tegmental area, as well as the two-dimensional shape of the corpus callosum in a parasagittal plane.

The importance of the PAG and the corpus callosum in opioid addiction are described in more detail below. Briefly, the PAG has been implicated in opioid withdrawal (Bonci and Williams, 1997). It is also involved in pain signaling and pain perception, which are blocked by opioids (Garland et al., 2013). The corpus callosum is a primary marker of cognition integrity (Luders et al., 2007), and drug addiction has been shown to affect many cognition centers (summarized in Gould, 2010). Given that opioids directly affect signaling in the PAG and affect many cognition

regions that use the white matter tracts of the corpus callosum, it is likely that both the PAG and the corpus callosum change in structure as a result of opioid addiction.

How Drugs of Abuse Affect the Reward Pathway

Many of the investigations into how drugs of addiction affect the brain have focused on the reward pathway. This is with good cause, since addictive drugs change the activity of these interconnected regions. Neurobiologists broadly define the *reward pathway* as the parts of the brain that control motivation and salience and that are shaped by reinforcement-based learning (Volkow et al., 2017). The use of some of the terms within this definition vary between fields (e.g., psychology versus neurobiology) and even between sources within a field. I will use these terms as follows. *Motivation* is the tendency to move toward a rewarding stimulus. *Salience* is a measure of the importance of a rewarding stimulus (i.e., how highly the brain ranks it compared to other stimuli). Finally, *reinforcement* is a change in the probability of pursuing a stimulus due to experience; reinforcement is a neurobiological change that results in modified behavior (Volkow et al., 2017). Applying these definitions, we can describe the reward pathway as the brain regions that help an individual learn which stimuli are worth approaching and that motivate an individual to pursue those stimuli. The plasticity of the reward pathway is adaptive to survival because it increases the probability that the individual will repeat the actions that led to a natural reward, such as a pleasurable food.

Drugs of addiction coopt the plasticity of the reward pathway. These strong, artificial rewards can result in *such dramatic changes* in neurochemistry that the reward system loses its ability to function properly. In other words, the adaptive value of the reward pathway is lost (Rogers and Gahring, 2012). This occurs when incentive salience for the drug of addiction is so high that the reward pathway “lowly ranks” previously learned reinforcers, and therefore, motivation to pursue them is reduced. *Addiction* results if the individual pursues the drug reward even when faced with the negative consequences of ignoring natural rewards. For example, a drug-addicted father seeks

out his next high (the artificial reward) rather than nurturing his child (a natural reward), even though the child negligence could have social or legal consequences.

The potential for drugs to become addictive results from their abundant target receptors in the neurons of the reward pathway. These target receptors are spread among the neurons of the ventral tegmental area (VTA), the nucleus accumbens (NAcc), and the prefrontal cortex (PFC), among other regions (Garland et al., 2013). Understanding the functions of these regions helps one to appreciate how drugs become addictive. The VTA, which is in the midbrain, is one of the main centers for the production of dopamine, a neurotransmitter used to signal the presence of a rewarding stimulus (Rogers and Gahring, 2012; Nestler, 2004). Some dopamine-releasing neurons that project from the VTA synapse with neurons in the NAcc, which is responsible for ranking a reward's strength—that is, its relative incentive salience compared to other rewards. Neurons originating in the NAcc, in turn, synapse with the PFC. (The VTA also projects directly into the PFC.) The PFC is responsible for carrying out (or inhibiting) the reward-seeking behavior by weighing the potential costs versus the rewards (Rogers and Gahring, 2012).

A primary pathway to addiction is stimulation of the NAcc with dopamine (Di Chiara and Imperato, 1988). Opioids, for example, which are the focus of my research, bind to receptors on inhibitory neurons that synapse with the dopaminergic neurons of the VTA. The inhibitory neurons become inhibited. Release of inhibition on the dopaminergic neurons therefore increases their release of dopamine (Garland et al., 2013). The NAcc interprets the increase in dopamine as a reward that motivates additional drug use and reinforces associations between drug cues and reward.

Of course, the intended function of opioids is not to hyperactivate the reward pathway. Physicians prescribe them *in spite* of their addictive qualities, not because of them. Rather, the pharmacological benefit of opioids is their analgesic effect. Opioid drugs trigger analgesia when

they bind to opioid receptors in several parts of central nervous system, including the spinal cord, periaqueductal gray, thalamus, hypothalamus, and cerebral cortex, which are all involved in pain signaling and perception (Garland et al., 2013). Binding of an opioid to an opioid receptor on one of these neurons initiates a cascade of reactions that results in a decrease in Ca^{2+} entering the cell and an increase in K^+ leaving the cell (Pathan and Williams, 2012). The neuron therefore becomes hyperpolarized, and its activity decreases. It is through the decreased neurotransmission of signals through these pain-signaling pathways that opioids cause analgesia. However, since opioid receptors are also found on the neurons of the reward pathway, it is not possible for a patient to receive the desired analgesic effects without the risk of developing addiction.

How Drugs of Abuse Affect Cognition Centers

Stimulation of reward pathway alone is not enough to explain all features of addiction, which include not only reward during drug use but also craving, compulsivity, and withdrawal in periods between use. These other features arise because exposure to addictive drugs affects the connections between the reward pathway and several brain structures involved in cognition and other processes. For example, the poor judgment associated with compulsivity (i.e., obsessions and repetitive behaviors, even when dangerous or damaging) is partly attributable to alterations in the glutamate signals that connect the PFC to the NAcc (Kalivas and Volkow, 2005). Further, as dependence develops, drugs change brain stress circuitry: This includes connections between the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the pontine nuclei (Le Moal and Koob, 2007). Acute withdrawal triggers feed-forward stimulation of the stress circuit, causing discomfort (negative reinforcement) that motivates drug-seeking and drug-taking. The striatum–pallidum–thalamic loop translates this motivation to action (Kelley, 2004).

Chronic drug use can affect learning and working memory. After all, forming drug–stimulus associations (i.e., consolidating the salient cues related to a drug) is a learning process, and cue-induced drug seeking (i.e., retrieving and acting on drug cues) is evidence that learning has

occurred. Previous studies have linked several brain regions to the learning that occurs from drug use; these include the striatum (movement toward drug cues), amygdala (emotional processing, such as avoidance of withdrawal symptoms), orbitofrontal cortex (decision making; part of the PFC), hippocampus (memory consolidation and retrieval), thalamus (relay of stimuli to other brain regions), and insula (self-awareness) (Gould, 2010). Overall, changes in these regions affect cognition and behavioral output.

Synaptic plasticity partly explains the widespread changes to the brain that occur as a result of addictive drug use. Drugs affect two forms of synaptic plasticity: In long-term potentiation neural connections are strengthened, whereas in long-term depression neural connections become weaker. The overall change to neural activity due to drug use depends on whether the drug affects long-term potentiation or depression, as well as which neurons are targeted. For example, cocaine has been shown to induce long-term potentiation in the VTA (Argilli et al., 2008). A single dose of cocaine in a drug-naïve rat brain strengthens the connections between glutamergic neurons terminating in the VTA and dopaminergic neurons projecting from the VTA. As a result of these strengthened neural connections, more dopamine is released in response to a subsequent drug dose. Similar to cocaine, opioids also upregulate neural activity, but opioids induce synaptic plasticity by a different mechanism. Exposure to opioids weakens the long-term potentiation of GABA neurons that terminate in the VTA (Nugent et al., 2007). Recall that GABA neurons are inhibitory, so the result of weakening connections between GABA neurons and the VTA's dopamine-producing neurons is that the VTA neurons become less inhibited. Again, these changes cause the reward pathway to respond more strongly to subsequent drug use. As a result of consistently strong responses to drugs, projections between the reward pathway and brain regions involved in memory and decision-making (such as the hippocampus and the PFC) also become stronger. Drug-stimulus associations develop, making future drug use more likely.

Drug-induced synaptic plasticity may be especially relevant in adolescents. Even in the absence of drugs, the adolescent brain is in a period of change: Significant remodeling occurs in the PFC and limbic regions during adolescence. One type of remodeling, called synaptic pruning, reduces the abundance of neural connections. This process is hypothesized to reshape the brain to fit environmental needs (Rakic et al., 1994). For example, synaptic pruning in the hippocampus—which is responsible for memory consolidation and retrieval—and in the PFC help to fine-tune declarative memories and ultimately an individual’s sense of self (Eichenbaum, 2000).

Perhaps due to the reshaping that naturally occurs during adolescence, drug use during this developmental period impacts the brain more profoundly than does drug use during adulthood (Lisdahl et al., 2013). For example, adolescent rat brains are more significantly affected by alcohol consumption than are adult rat brains, especially in regions associated with decision making and memory (Markweise, 1998). Human studies corroborate the results of animal research: Alcohol use in human adolescents is associated with poor performance on cognitive tasks and alterations in brain structure (Spear, 2015). Adolescents who engage in binge drinking have lower white matter “integrity” (that is, the ability of white matter to transmit neural signals) in the corpus callosum, internal capsule, corona radiata, and other white matter tracts (McQueeney et al., 2009). Adolescent drinking also affects gray matter macrostructure, but the direction of the effect depends on gender: in adolescent female drinkers, thickness of the PFC and cingulate cortex increases compared to non-drinkers, whereas in males, cortex thickness decreases (Squeglia et al., 2012).

Use of other drugs during adolescence has also been shown to affect long-term cognitive function. Adolescent smokers face impairments in working memory, as well as cognitive performance in divided attention tasks, even after smoking cessation (Jacobsen et al., 2005).

Administration and then cessation of ecstasy (3,4-Methylenedioxymethamphetamine; MDMA) in adolescent rats leads to a decrease in object recognition memory and an increased likelihood of

engaging in risky behaviors (Piper and Meyer, 2004). Cannabis also impairs working memory, as well as social interactions, in rats several weeks after cessation of drug use (O'Shae et al., 2006). The effects of cannabinoids are significant in not only adolescents but also in perinatal and adult rats.

Current literature has a dearth of information about how opioids affect the adolescent brain. A study done with patients who are exclusively over age 18 and receiving opioids for palliative care showed significant anterograde and retrograde memory impairment (Kamboj, 2005). Another experiment using adult human subjects showed decreases in working memory, as participants on morphine had impaired abilities to recite numbers in reverse order (Friswell et al., 2008). However, both of these experiments used adult participants and investigated the acute effects of opioids on cognition. More research is needed to understand opioids' long-term cognitive effects, as well as how these drugs affect adolescents. After all, teen opioid abuse has risen in recent years as opioids (prescribed almost exclusively to adults) have become more easily available in the home (Sheridan et al., 2016). In 2016, 40% of 12th graders living in the United States said that opioids, such as Vicodin and OxyContin, were "fairly easy" or "very easy" to get (Johnston et al., 2016). Therefore, ignoring opioids' effects on adolescents simply because physicians do not prescribe them to adolescents is a folly.

The adolescent brain is more likely than the adult brain to become sensitized to opioids, presumably because of the adolescent brain's higher potential for synaptic plasticity. In a mouse model, opioids enhance locomotor activity, which is a proxy for how the VTA dopaminergic response to opioids affects behavior in mice; further, repeated administration of the same opioid dose on consecutive days causes an increasing locomotor response (Koek, 2014). The locomotor-sensitizing effects of opioids are not only more pronounced in adolescent mice but are also longer-lasting: Enhanced locomotor responses to opioids extend beyond drug extinction into adulthood, whereas sensitization in adults is no longer present after 5 weeks. These results

suggest that relapse is more common for adolescents than for adults and highlight the need to learn more about how opioids trigger addiction and affect cognition and behavior in adolescents.

The Periaqueductal Gray Is Implicated in Opioid Withdrawal

Opioids are addictive in two ways. First, they positively reinforce psychological dependence through activation of the reward pathway. Hyperstimulation of VTA and NAcc causes downstream consequences, such as strengthening connections in the hippocampus, striatum, and PFC; these changes teach the subject to recognize and pursue drug cues (Wise, 1988; Feltenstein and See, 2008). Second, opioids dampen the stress and pain associated with withdrawal (Wise, 1988; Nestler, 2004). The periaqueductal gray (PAG) is associated with this second phase of opioid addiction.

Typically, when opioids bind to target receptors on a GABAergic neuron, cell activity decreases; as a result, less GABA is released to post-synaptic neurons, which become disinhibited (Gysling and Wang, 1983). As the post-synaptic neurons in the opioid response pathway (such as those in the VTA and PAG) become more active, they release dopamine or serotonin, providing opioids' hedonic effect (Gysling and Wang, 1983). However, during opioid withdrawal, the opposite process occurs: GABA release from the same neurons increases above baseline levels, triggering inhibition of downstream neurons. These changes are mediated by increases in cyclic AMP in GABAergic neurons upon opioid withdrawal (Bonci and Williams, 1997).

Withdrawal from opioids causes feelings of dysphoria, likely due to increased inhibition of neurons that release serotonin and dopamine (Nestler, 2004). Upon cessation of opioid use, changes in the GABAergic neurons that innervate the PAG cause the cells to release abundant GABA, thereby inhibiting serotonin-releasing neurons in the PAG and the adjacent dorsal raphe nucleus (Jolas et al., 2000). (Note that the dorsal raphe nucleus and PAG also contain *dopaminergic* neurons, which may also be inhibited during withdrawal, but this has not yet been

tested.) Opioid withdrawal also causes changes in the VTA, which is immediately ventral to the PAG. The firing rate of dopaminergic cells in the VTA decreases substantially due to increased GABA tone during opioid withdrawal (Bonci and Williams, 1997). Therefore, following opioid cessation, the resulting drop in available dopamine and serotonin in the brain causes a state of unease or dissatisfaction with life.

As described above, repeated stimulation of a neural circuit can strengthen existing neural connections (long-term potentiation), and repeated inhibition of a neural circuit may weaken connections (long-term depression). These changes are due to increases in neurotransmitter release from presynaptic cells or upregulation of receptors in postsynaptic cells. At a cellular level, hyperstimulation of a neural pathway may also lead to formation of new dendritic spines (see Fu et al., 2012, for a description of how clusters of dendritic spines form during learning).

At an even broader scale, the neuroanatomical changes that occur from repeated stimulation or depression of neural circuits can be summarized as volumetric or morphometric changes in brain structure. The brain of an individual who has been addicted to opioids and then has gone through withdrawal may have regional structure changes compared to an opioid naïve brain. In our experiment, we expect the PAG of opioid users to have a different shape from the PAG of opioid naïve rats because the PAG's dorsal raphe nuclei and the neighboring VTA are stimulated during opioid use and inhibited during opioid withdrawal; these drug-induced changes in neural activity likely translate to morphometric changes to the PAG and the structures that flank it.

Understanding how these large-scale changes to brain structure correspond to the behavioral and emotional changes that occur during opioid addiction and withdrawal may be especially valuable to physicians who may need to diagnose drug-induced brain damage at a glance.

Drug-Induced Cognitive Changes May Be Reflected in the Corpus Callosum

The corpus callosum is a primary marker for cognition integrity (Luders et al., 2007). For example, a recent study relating cognition and corpus callosum anatomy found that adult subjects with a thicker callosum (at the midsagittal section of the splenium) were correlated with higher intelligence quotient (IQ) test scores (Westerhausen et al., 2018). Strauss, Wada, and Hunter (1994) describe a similar result: In people with epilepsy, those with a greater cross-sectional area of the posterior corpus callosum perform better on cognitive tasks. A greater callosal area is associated with more commissural fibers (Aboitiz et al., 1992). In other words, the connection between brain cortices is stronger when the corpus callosum has a larger cross-sectional area; this strong connection may explain why many studies link high callosal area to high intelligence. However, this intuitive trend is reversed in children and adolescents. Multiple studies have shown that *smaller* splenial thickness (at midsagittal plane) is associated with greater intelligence in children and adolescents, perhaps as a consequence of more efficient neural networks forming during development (Luders et al., 2011; Hutchinson et al., 2009). Regardless of the direction of the correlation, callosal area and thickness are related to intelligence. (However, see Westerhausen et al., 2018, for a discussion of how a small corpus callosum likely only affects cognitive tasks that require time-sensitive choices and not *intelligence*, per se.)

Drug-induced developmental abnormalities affect the corpus callosum anatomy. For example, fetal alcohol syndrome may cause full or partial agenesis of the corpus callosum, as has been shown via magnetic resonance imaging (MRI) of children that were prenatally exposed to alcohol (Riley et al., 1995). Bookstein et al. (2001) used MRIs of adult subjects to reveal differences in the *shape* of the corpus callosum as a result of fetal alcohol syndrome. (I built on the morphometric methods outlined in Bookstein et al., 2001, for the brain region analyses in my experiment. I will elaborate on these methods in the next section.)

As outlined in the section above titled “How Drugs Affect Cognitive Centers,” drugs of addiction alter both the structure and function of brain regions that confer cognitive processes. Since opioids affect cognitive areas of the brain that use the corpus callosum as an information highway, and since the corpus callosum is an anatomical marker of cognition integrity, I predict that opioids will affect the structure of the corpus callosum. Similar to the PAG analysis, the callosal morphometric data will provide additional evidence for how drugs can damage the brain.

CHAPTER II

REFINING THE USE OF MORPHOMETRY IN NEUROANATOMICAL EXPERIMENTS

Introduction: What Is Morphometry?

Analyzing shape variation using geometric morphometrics (morphometry) is a relatively new approach to neuroscience; it can provide insights into how brain structures change through development, aging, and disease (Whitwell, 2009). Morphometry offers advantages to the more traditional approach of *volumetry*—studying differences in volume. First, analyzing shape provides more information than analyzing volume: Suppose, for example, that disease causes the genu of the corpus callosum to shrink but causes the splenium of the corpus callosum to expand. A volumetric analysis of the corpus callosum might suggest no significant change in volume had occurred, whereas a morphometric analysis would reveal a significant change in shape, which may be clinically important. Second, volumetric analyses often carry the assumption that an enlarged brain structure has greater processing capacity, but this correlation is not always appropriate (Chollet et al., 2014; also, see chapter 1 regarding the inverse relationship between splenial thickness and intelligence in adolescents). In contrast, morphometry suggests that any change in shape from control is abnormal, thereby reducing potential bias in neuroanatomical experiments.

Prior to performing a shape analyses, researchers may scale, translate, and rotate the coordinates of each shape as part of data standardization. In my analysis, for example, subtle variations in the

size, position, and orientation of a specimen when cross section images were captured would affect how the subsequent statistical analysis detected variations in specimen shape. Therefore, using a process called Procrustes superimposition, the digital registration of each specimen is standardized, reducing the “noise” in the data (figure 2.1). In addition, Procrustes superimposition strengthens a neuroanatomical analysis because the natural variation that occurs in brain size (due to specimen age or body mass) does not affect data dispersion. In other words, removing size, position, and orientation makes it easier for an investigator to detect statistically significant changes in regional brain anatomy *due to the independent variable* (e.g., disease progression).

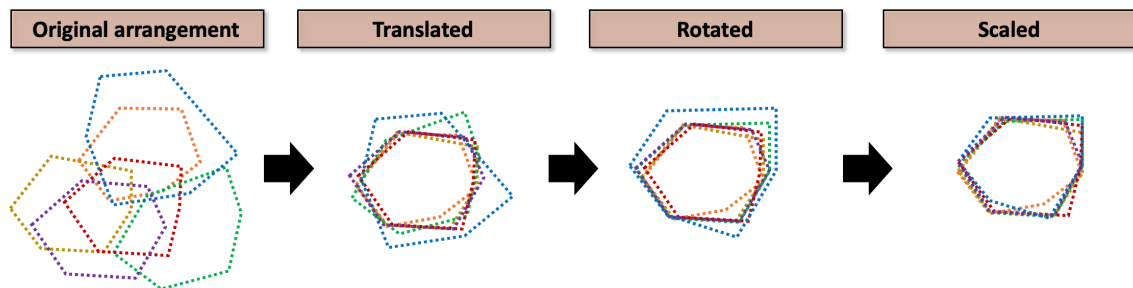


Figure 2.1. *Procrustes superimposition.* Translating, rotating, and scaling specimen images allows the subsequent statistical analysis to detect differences that are only based on shape.

Despite the advantages of morphometry over volumetry, the former has only recently been applied to neuroanatomy, and best practices are still being refined. For example, the shape of a structure can be quantified in multiple ways, either using landmark-based or landmark-free techniques. In morphometrics, a “landmark” is an anatomical point that can be reliably placed for all specimens in an investigation (Bookstein et al., 2001). Landmark-based morphometrics can be used for either conspecific anatomical studies or for comparing homologous landmarks among multiple species, giving it an evolutionary framework (Webster and Sheets, 2010). Statistical analysis determines if shifts in landmarks are evidence of significant differences in shape among specimens. However, use of landmarks is not possible for all morphometric questions. If the landmarks cannot be reliably placed due to either investigator inexperience or poor anatomical cues, then the analysis will overestimate inter-specimen variation in shape simply due to

investigator error. Landmark-free morphometric techniques do not necessarily have these drawbacks. To perform a morphometric analysis without landmarks, anatomists may use computer vision systems (as well as geographic information systems borrowed from landscape analyses) to digitize the topography of a structure (see Mangin et al., 2004, for a computerized analysis of cortical folding patterns). Other landmark-free methods use outline-based analyses to evaluate the contour data of a specimen (Chaiphongpachara, 2018).

My investigation of the shape of the corpus callosum and the periaqueductal gray used an outline analysis. More specifically, I used an elliptical Fourier analysis to compare brain structures among specimens in my experiment. This approach approximates the outline of a structure by iteratively bending an ellipse; with each bend the ellipse more closely matches the shape of the specimen (see figure 2.10c for a visual of this process). The result of elliptical Fourier analysis is a sum of sine and cosine waves that together form a *continuous* perimeter on a specimen, in contrast to the discrete landmarks that are used in other morphological approaches. Elliptical Fourier analysis is therefore useful for two-dimensional shape comparisons when a structure lacks homologous landmarks (Haines and Crampton, 2000), such as the periaqueductal gray. In contrast, a previous experiment established landmarks and semi-landmarks for a parasagittal corpus callosum cross section (Bookstein et al., 2001), but I chose to use an elliptical Fourier analysis of the corpus callosum for two reasons: First, I wanted to use consistent methods for both brain regions of interest, and second, a full outline provides more information than a defined number of landmarks (Crampton, 1995). The average number of points for a corpus callosum outline generated by elliptical Fourier analysis in my investigation was 1038. This vastly exceeds the 40-point corpus callosum outline used in Bookstein et al., 2001. Elliptical Fourier analysis is therefore an improvement on the Bookstein et al. (2001) geometric morphometric technique: It provides a more consistent and complete outline compared to a landmark-based method.

An Introduction to DiceCT

Even before deciding how to determine the shape of a brain region, an investigator must develop a protocol for staining and visualizing specimens. These methods also vary depending on the goals of an investigation. My experiment used an imaging technique called diceCT (diffusible iodine-based contrast-enhanced computed tomography), which offers outstanding opportunities for analyzing small neuroanatomical structures without requiring dissection. DiceCT employs an iodine stain to selectively increase the radiodensity of some soft tissues, allowing for exceptional contrast between structures that were until recently difficult to visualize with X-ray technologies. On the other hand, iodine staining causes challenges for morphometric analyses because of the propensity for the iodine (a salt solution) to cause soft tissues to shrink (Vickerton et al., 2013). To reduce shrinkage in iodine, Wong et al. (2013) proposed that tissues should first be stabilized with hydrogel—a solution of crosslinking acrylamides that solidifies when heated. The drawback of this technique is that infiltrating specimens with hydrogel adds substantial time and additional expenses to an experiment.

Specific Aims

The goal of my first experiment is to test if hydrogel stabilization is routinely necessary step to obtain satisfactory results from diceCT. This investigation adds to the growing discussion of diceCT “best practices” by comparing the efficacy of the hydrogel tissue stabilization protocol against specimens that do not receive hydrogel treatment. In particular, we tested the hypothesis that hydrogel reinforcement protects a brain specimen from significant volumetric and morphological changes that occur during iodine staining. Results that support this hypothesis would include differences in regional brain size and shape between hydrogel-treated and -untreated specimens. On the other hand, if we find no difference in regional brain shape between the two groups, then hydrogel reinforcement may not be worth the time and materials cost for all morphometric analyses.

Methods: The Process of Morphometric Analysis

Using diceCT for a morphometric experiment requires several steps. First, an investigator must obtain and fix the specimens to be analyzed. After fixation, a specimen may be reinforced with hydrogel. The specimens must then be stained in Lugol's iodine prior to being microCT scanned, and, finally, processed using imaging software. Statistical analyses test for changes in specimen shape. Each of these steps is summarized in figure 2.2 and described in detail in the sections below.

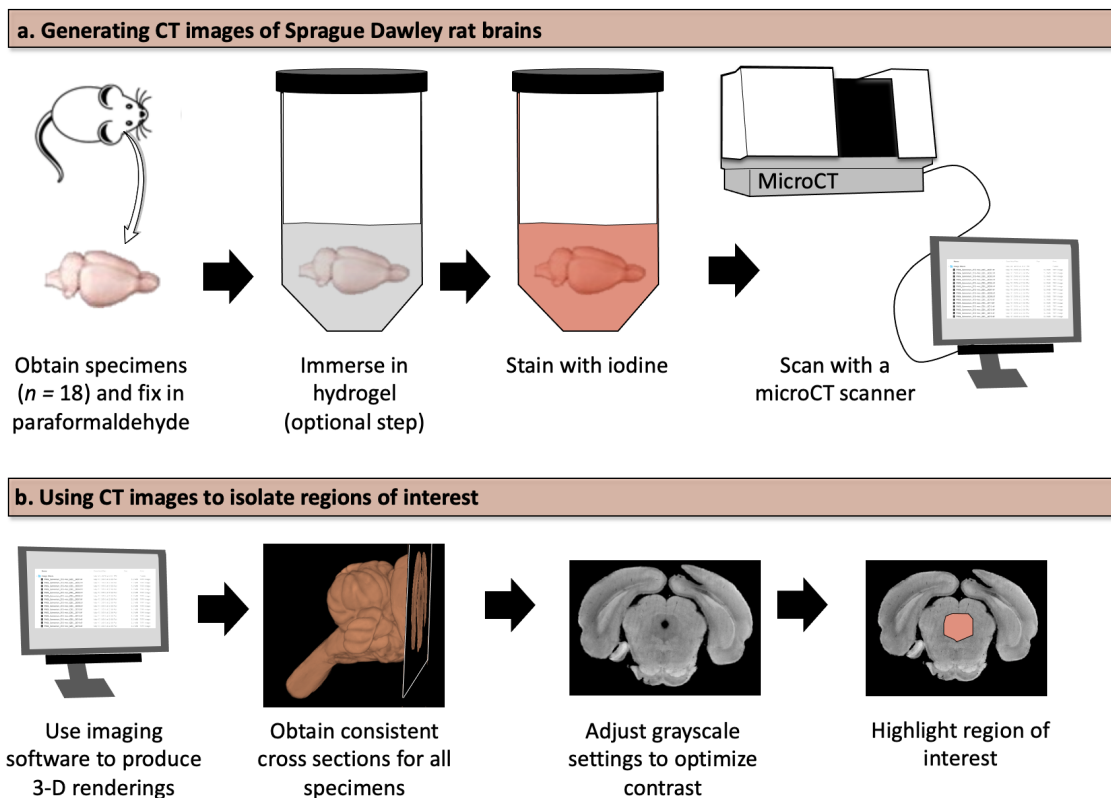


Figure 2.2. *Morphometric methods: A summary.* (a) In this experiment, I obtained brains from adolescent Sprague Dawley rats and scanned them using microCT. (b) I then processed these scans using imaging software.

Obtain Specimens

This experiment uses the postmortem brains of 18 adolescent Sprague Dawley rats. The rats were originally purchased from Charles River Laboratories (Indianapolis, USA). Prior to our use in a hydrogel experiment, the rats were used for drug-exposure experiments: Fifteen were exposed to

morphine, and then eight of the morphine-exposed rats were also given a nociceptin receptor (NOP) agonist. The resulting treatment groups for drug-exposure experiments were therefore opioid-naïve ($n = 3$), NOP-morphine ($n = 8$), and morphine-only ($n = 7$). See chapter 3 for more details about drug exposures. All 18 rats were sacrificed on postnatal day 43 by transcardial perfusion with cold saline solution (0.9% NaCl) followed by cold 4% paraformaldehyde. Each brain was immediately extracted after sacrifice.

Procedures were conducted according to the National Institutes of Health guidelines and were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee (IACUC protocol: 2021-2014). All reasonable efforts were made to minimize discomfort and the number of animals used in this study.

Fixation

Proper preservation—or fixation—of specimen tissues is an important part of any morphological experiment. Potential fixatives include phosphate buffered formalin solutions, Bouin solution, Dent's solution, or ethanol (Gignac et al., 2016). Fixation or storage of a specimen in ethanol is not advised prior to iodine staining since ethanol can disrupt the lipid content of the tissues. As will be explained in the next section, lipids are especially important for neuroanatomical imaging.

The rat brains in this experiment were fixed in 4% paraformaldehyde solution for 24 hours immediately after sacrifice. They were then moved to a 30% sucrose solution until either hydrogel reinforcement or iodine staining commenced; no brain was in a sucrose solution for more than 72 hours.

Hydrogel Reinforcement

An optional intermediate step between fixation and staining is to reinforce a specimen with hydrogel. Hydrogel reinforcement required about 2 weeks for this experiment. The function of hydrogel is to minimize changes that occur to a specimen during the staining process. The

hypertonic iodine solution that is used for diceCT causes shrinkage of soft tissues, such as skeletal muscle, cardiac muscle, and the brain; high concentrations of iodine or long staining durations cause substantial shrinkage (Vickerton et al., 2013). Hydrogel reinforcement is reported to reduce the shrinking effect of iodine staining (see the STABILITY method, coined by Wong et al., 2013).

The potential benefit of hydrogel reinforcement is that a specimen can tolerate higher concentrations of iodine or longer staining durations, thereby improving X-ray contrast and image quality. However, my research has noted six potential drawbacks to using hydrogel: (1) the time investment of adding the hydrogel to the specimen; (2) the time required to remove excess hydrogel that adheres to the outside of the specimen, which interferes with X-ray penetration; (3) the potential damage to external structures when excess hydrogel is removed; (4) the “porcupine artifacts” in CT scans of hydrogel-treated specimens, due to the scattering of X-rays off hydrogel-filled blood vessels (figure 2.3); (5) the materials cost, which totaled over \$2000 for the 18 specimens used in this experiment; and (6) the inability to use the specimens for further investigations (tissue-bound hydrogel cannot be removed and interferes with other staining techniques).

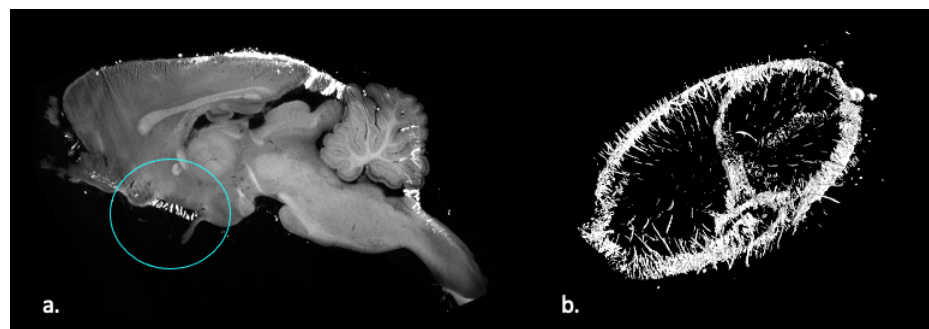


Figure 2.3. *Porcupine artifacts.* (a) Dense areas of hydrogel on the surface of this parasagittal brain slice cause backscattering of X-rays, leading to white areas in the output. (b) Hydrogel-filled blood vessels create projections from this specimen, artifacts of the X-ray scattering.

To weigh the tradeoffs between these potential benefits and drawbacks, we set up an experiment to objectively measure the protective effect of hydrogel against shrinkage and deformation of neural tissues (for experimental design, see figure 2.2 above and table 2.1 below). Out of the 18 rat brains in our experiment, 7 were reinforced with hydrogel and 11 did not receive hydrogel treatment. Then, as described below, all brains were stained and scanned, and then visualized using imaging software. Finally, the shape and size of two brain regions—the periaqueductal gray and the corpus callosum—were analyzed for the hydrogel-treated and -untreated groups. Analysis of these two brain regions should inform us if hydrogel reinforcement has a differential effect on white matter and gray matter.

Step	Description
Obtain and fix specimens	Obtain 18 adolescent rat brains and fix them in 4% paraformaldehyde
Hydrogel reinforcement	Stabilize some rat brains with hydrogel ($n = 7$). Skip this step for remaining brains ($n = 11$).
Staining	Allow all brains to soak in iodine solution.
Scanning	Place each specimen in a microCT scanner, generating an image stack of virtual brain slices.
Visualization	Use software to process the image stacks and isolate cross sections of the PAG and corpus callosum.
Analysis	Use elliptical Fourier analysis in R to determine if treated and untreated brains have regional differences in size and shape.

We modified previously published hydrogel reinforcement methods (Wong et al., 2013; Carlisle et al., 2017). To make the hydrogel solution, we mixed 4% paraformaldehyde (the fixative), a combination of acrylamide and bis-acrylamide (the crosslinking agents of the hydrogel solution), VA-044 (a polymerization initiator), and saponin (a “soapy” glycoside that helps the hydrogel spread through the specimen). The solution is kept on ice to prevent cross linking.

Brains were then immersed in the hydrogel solution for two weeks within a 4°C fridge. The long immersion time allowed the hydrogel solution to become evenly distributed throughout each specimen. After two weeks, the hydrogel solution with immersed specimens was removed from the fridge and a layer of oil was added to the surface of the solution (to prevent air from contacting the gel). The containers were then immediately placed in a 37°C water bath for 3 hours, during which the hydrogel polymerized.

Prior to iodine staining, excess hydrogel must be removed by hand from the surface of the specimen. For small rat brains, this step was challenging, as delicate surface structures such as cranial nerves and olfactory bulbs sometimes peeled off along with the hydrogel.

Staining

The X-ray imaging that is used in standard computed tomography (CT) does not effectively image soft tissues, such as the brain. Staining agents increase the contrast between certain types of soft tissues. For example, diceCT's most popular contrast-enhancing stain—Lugol's iodine (I₂KI)—binds more strongly to myelinated (lipid-rich) nervous tissues than to unmyelinated (lipid-poor) tissues (summarized in Gignac and Kley, 2014). Iodine staining therefore increases the radiodensity of white matter more than it increases the radiodensity of gray matter.

Consequently, during a CT scan of an iodine-stained brain, radiodense white matter is more likely to absorb X-rays compared to less radiodense gray matter, which allows relatively more X-rays to pass through to the machine's detector. This difference in X-ray absorption allows a researcher to distinguish between axonal tracts and nuclei in CT scan images. In my experiment, iodine staining allowed me to visualize, for example, the white matter of the corpus callosum in contrast to the gray matter of the cingulate gyrus that sits dorsally.

The duration of the staining process depends on several factors. First, iodine solution concentration is inversely related to staining duration, such that a lower iodine concentration will

permeate the tissues more slowly, requiring a longer stain. Second, larger specimens require more time for the iodine stain to penetrate to deep tissues. Third, the use of hydrogel reinforcement slows down iodine uptake into tissues, necessitating a higher concentration of iodine solution, a longer duration of staining, or both.

In this experiment, hydrogel reinforced brains were immersed in a 3% Lugol's solution for 6 weeks. The solution was refreshed after 3 weeks. Note that staining occurred in a 4°C fridge to prevent additional polymerization in hydrogel-treated brains. In contrast, brains that did not receive hydrogel treatment were stained at room temperature with a 1% Lugol's solution for 3 weeks and then a 2% Lugol's solution for 2 additional weeks; the 2% solution was refreshed twice.

Scanning

Soft tissue specimens that are sufficiently stained for high-contrast CT scanning often have higher radiodensity than bony tissue (Gignac et al., 2016). For a CT scanner to produce an image, X-rays must pass through the radiodense specimen to a detector. Gignac and Kley (2018) outlined four best practices for microCT scanning iodine-stained soft tissues: 1) high beam energy, 2) small rotation steps, 3) long exposure time, and 4) image averaging.

- 1) High beam energy. The high beam energy allows the X-rays to penetrate the specimen. The drawback is that only expensive, industrial scale microCT scanners are capable of producing high enough beam energies for any specimen larger than an embryo or small postembryo.
- 2) Small rotation steps. During a scan, the specimen is exposed to X-rays stepwise from 360°, and the investigator decides how many rotation steps the scanner uses. For example, if an investigator only requires low resolution images, then the scanner may

take exposures at every 1° angle (360 scans total). Additional exposures at smaller rotation steps improves the resolution of the image.

- 3) Long exposure time. The amount of time that the detector is receiving X-rays during a single exposure may vary. A longer exposure time collects more X-rays, similar to a long camera exposure collecting additional light in a dark setting.
- 4) Image averaging. The investigator may also elect for the scanner to take multiple exposures from a single angle. The computer then averages the data from these independent collections. Using image averaging produces scans with sharper edges compared to scans in which only one exposure is taken per angle. Sharp edges at transitions in tissue are especially important for distinguishing the shape of a brain structure.

Specimens in this experiment were scanned at two facilities. Eleven were scanned at the American Museum of Natural History's Microscopy and Imaging Facility (New York, New York) on a GE phoenix v|tome|x s240 CT scanner. The remaining seven specimens were scanned on a Nikon 225 XTH at the University of Arkansas (Fayetteville, Arkansas; acquired and maintained by the MicroCT Imaging Consortium for Research and Outreach).

The scan specifications varied for each specimen. A complete list of specifications can be found in appendix A; here I will provide an overview. Beam energy averaged 30.8W (range = 12.1–70.0W). The number of rotation steps (i.e., projections) averaged 2405 with a range of 1800 to 3141. Exposure time averaged 395ms (range= 267–708ms). All scans used multi-frame image averaging (mean=6 frames; range= 4–8 frames).

Processing Images

The output of a CT scan is an image stack—a series of image files that represent sequential cross sections of a specimen. Imaging software compiles an image stack into 3-D renderings, allowing a user to navigate through a specimen, home in on a relevant structure, and outline the structure

based on differences in grayscale values between the structure-of-interest and surrounding structures.

We used Avizo (version 9.5.0, Thermo Fisher Scientific) to process CT scan data. Avizo allowed us to visualize an image stack as a 3-D representation of a rat brain (figure 2.4). We then obtained cross sections of the PAG and the corpus callosum. Finally, we compared the shape of the PAG and corpus callosum in hydrogel-treated and -untreated brains.



Figure 2.4. Avizo visualizes an image stack. (a) The output of a CT scan is an image stack that (b) imaging software, such as Avizo, combines into a 3-D rendering of a specimen.

Using Avizo, I obtained consistent cross sections of the PAG for all specimens. I took a coronal cross section just rostral to the pineal gland (figure 2.5). Care was taken to ensure that the cross section matched plate 92 of Paxinos and Watson's *The Rat Brain in Stereotypic Coordinates, Seventh Edition*. Important landmarks for this cross section include subtle separation of the cerebral hemispheres along the dorsal surface (the pineal stalk may be visible between the hemispheres); defined oculomotor nuclei just ventral to the PAG; and a distinct hippocampus shape, with a swollen dorsal hippocampal commissure tapering into a long and rounded alveus of the hippocampus (see figure 2.5). This cross section of the PAG lies approximately dorsal (but just barely caudal) to the ventral tegmental area.

I also obtained consistent cross sections of the corpus callosum along a parasagittal plane (figure 2.6). Again, Paxinos and Watson's *The Rat Brain in Stereotypic Coordinates, Seventh Edition*

provided a guide to consistency (plate 163). I used the shape of the ventricles to make sure each cross section was an analogous distance from the midsagittal plane. The third ventricle, for example, is separated in this view because the dentate gyrus and the stria medullaris touch (see figure 2.6).

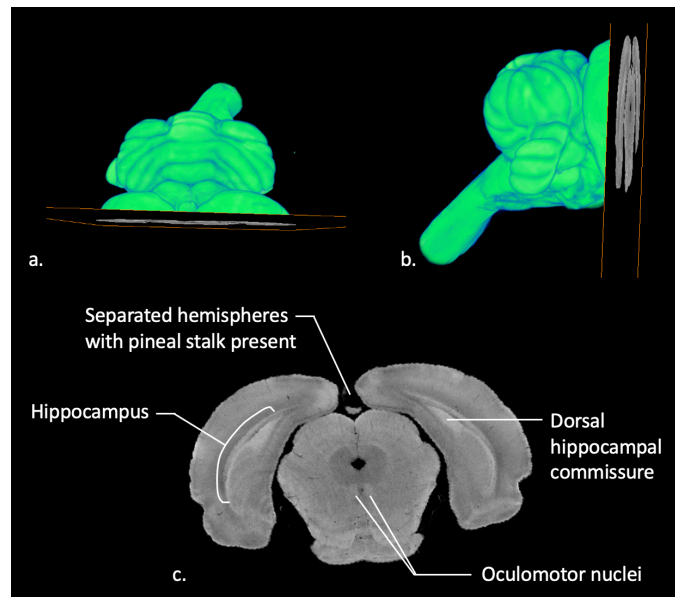


Figure 2.5. *PAG cross section.* All PAG cross sections were taken (a, b) just rostral to the pineal gland. (c) The result is a cross section with the cerebral aqueduct (black circle) in the center. The aqueduct is flanked by the PAG.

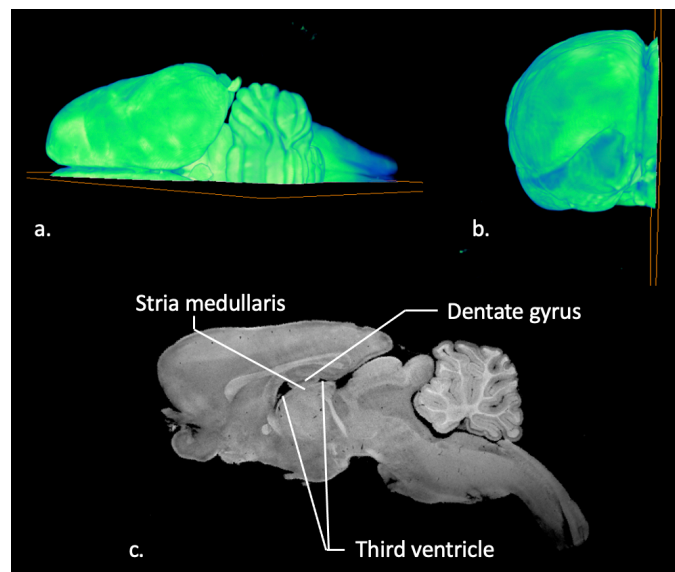


Figure 2.6. *Corpus callosum cross section.* All corpus callosum cross sections were taken (a, b) at a parasagittal plane, just lateral to the midline. (c) The third ventricle was especially useful for obtaining consistent cross sections for all specimens.

Finally, I exported each cross section image and used Microsoft PowerPoint (Mac version 16.48) to outline each PAG and corpus callosum. The result is a colored region representing a brain region within each cross section (figure 2.7).

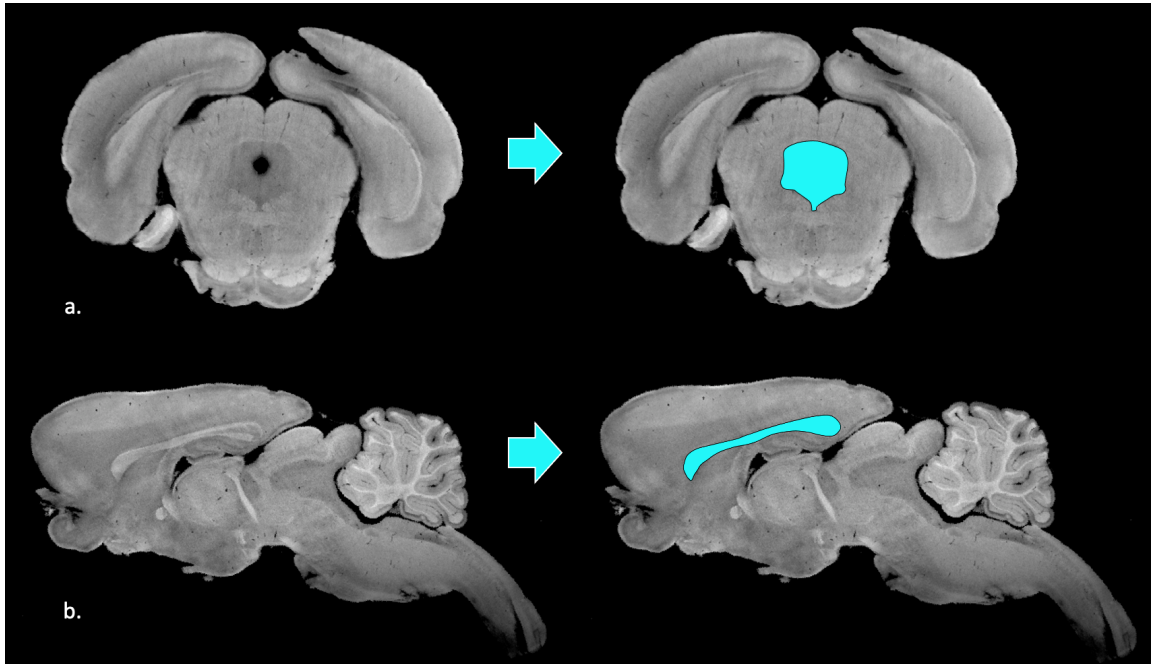


Figure 2.7. *Outlining brain regions.* After obtaining consistent cross sections, I outlined the (a) PAG and (b) corpus callosum for all specimens.

After highlighting the region of interest, I exported a screen capture of the specimen to an analytical program called ImageJ (Fiji version 1.0). Using scale information via the known voxel size for each image provided by Avizo, I calibrated the “area tool” in ImageJ and determined the cross-sectional area of each corpus callosum and PAG (figure 2.8). This last step was used to determine if volumetric changes are occurring in our specimens as a result of hydrogel treatment—for comparison with the potential morphometric changes.

Statistical Analysis

Isolated cross sections of the PAG and corpus callosum revealed variation in these brain structures across specimens (figure 2.9). Determining if shape variation was a result of chance alone or if it was affected by hydrogel treatment required use of statistics.



Figure 2.8. *Calculating cross-sectional area.* The cross-sectional area of each specimen’s corpus callosum and PAG (not shown) was obtained using ImageJ.

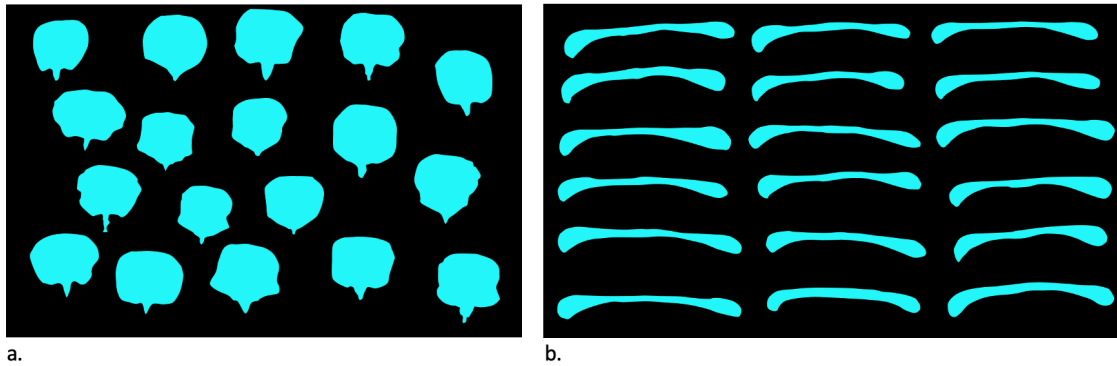


Figure 2.9. *Variation in regional shape.* These collages of (a) PAG and (b) corpus callosum cross sections show variation in the shape of each brain region.

I employed the Momocs package in R to compare the shape of the PAG and corpus callosum in hydrogel-treated brains and non-treated brains. Momocs was designed to provide a toolkit for 2-D morphometrics (Bonhomme et al., 2014). I input the shapes shown in figure 2.9 (as .jpg files) and implemented Procrustes superimposition in Momocs to rotate, scale, and center the outline data (figure 2.10a and 2.10b).

I then used an elliptical Fourier analysis to bend an ellipse into the approximate shape of each brain region (figure 2.10c); this step decomposes the image files into mathematical terms that can be statistically compared. (See this chapter’s introduction for more details on elliptical Fourier analyses, including how this method compares to other morphometric techniques.) Note that the harmonic power (i.e., the amount of shape information contained in the ellipse) increases with each bend of the ellipse. I added harmonics until each brain region reached a harmonic power of 99%, which has become the standard for the field (Crampton, 1995). For the PAG, 99% of the shape information was obtained with 11 harmonics; for the corpus callosum, the same amount of shape information was obtained with 8 harmonics.

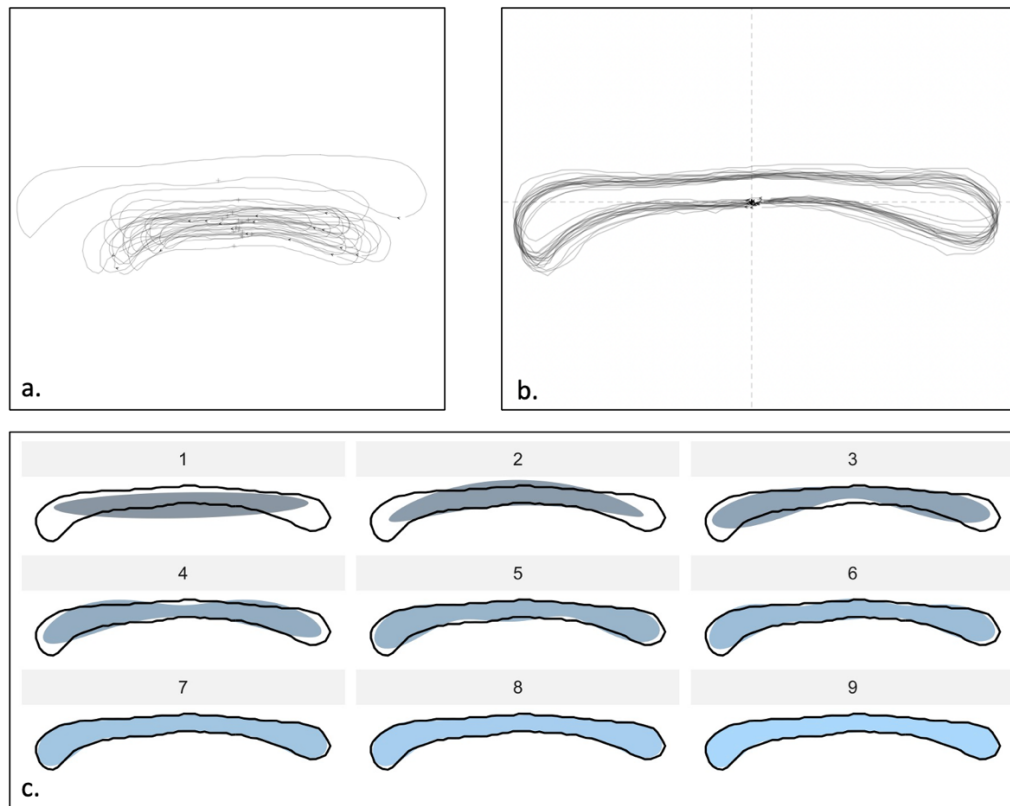


Figure 2.10. *Processing Images in R.* For both the corpus callosum (shown here) and the PAG, (a) I input the cross sections into R and then (b) scaled and centered the images. (c) Elliptical Fourier analysis fitted an ellipse to the approximate shape of each cross section.

Once the elliptical Fourier analysis mathematically approximated the shape of each brain region in each specimen, I employed a principal components analysis (PCA). The PCA separated the outlines based on the major components affecting the shape of each brain region.

Finally, I used MANOVA determine if the treatment groups were significantly different in shape. To avoid including superfluous information in the discriminant analysis, I retained PC axes up until they captured 80% of the total variance in each sample (PAG: 4 axes; corpus callosum: 2 axes). *Post hoc* analysis with a Pillai test revealed the P values between each pair of treatment groups.

As noted above, we also determined whether hydrogel protected a specimen from *volumetric* changes during iodine staining. After obtaining the cross-sectional area of each PAG, we compared treated and untreated brains using a *t*-test. Then we repeated this analysis for the corpus callosum.

Results: Does Hydrogel Treatment Affect Regional Brain Structure in a DiceCT Protocol?

The purpose of hydrogel treatment is to stabilize tissues prior to iodine staining. In our analysis of a gray matter structure (the PAG) and a white matter structure (the corpus callosum), we found no difference in the two-dimensional shape of brain structures that were treated with hydrogel and brain structures that were left untreated.

As shown in figure 2.11, the principal component analysis plot of the PAG reveals extensive overlap between the hydrogel-treated brains (green highlight; “Yes”) and -untreated brains (red highlight; “No”). MANOVA reveals that these two groups were not significantly different ($P=0.52$; $F=0.85$; harmonics=11; four axes retained in PC plot capturing a total variance of 83.0%).

Similar to the analysis of the PAG, the corpus callosum of brains treated with hydrogel was no different in shape from the corpus callosum of untreated brains. The two groups overlap

substantially in the morphospace of the PCA plot (figure 2.12). Again, discriminant analysis finds no difference between the shape of a hydrogel-treated and hydrogel-untreated corpus callosum ($P=0.26$; $F=1.61$; harmonics=8; two axes retained in PC plot capturing a total variance of 81.8%).

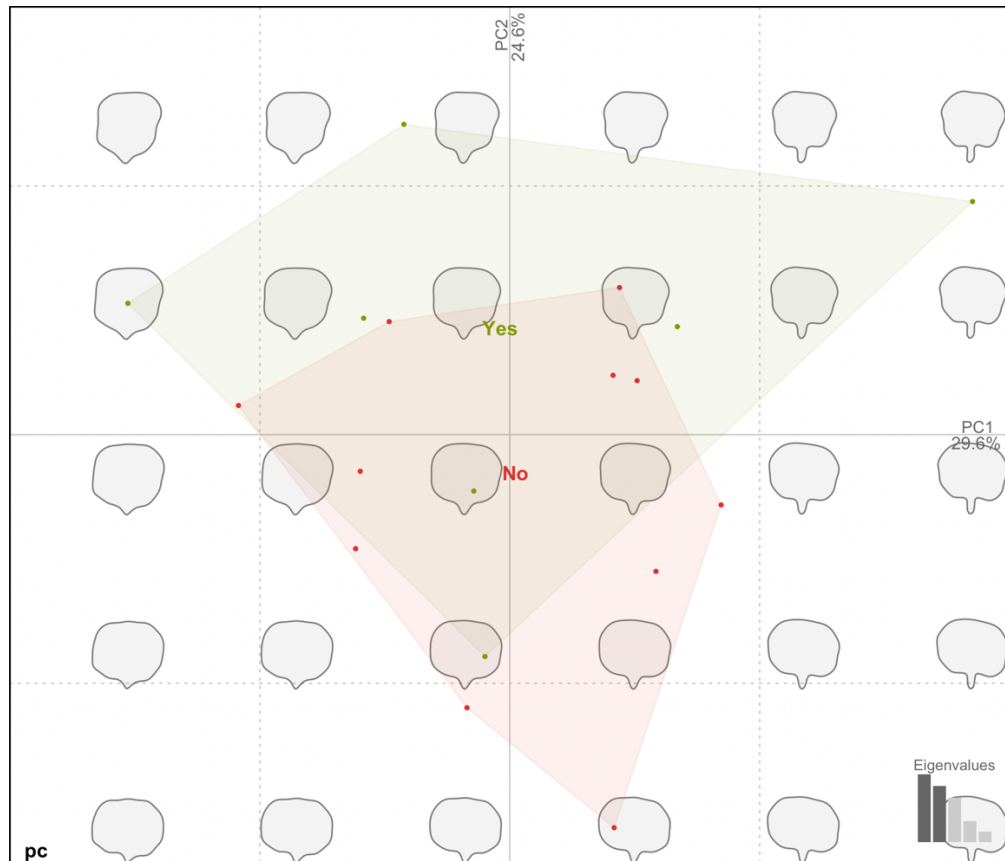


Figure 2.11. Hydrogel treatment does not affect PAG shape. The PAG of hydrogel-treated brains (shown in green and denoted with “Yes”) overlap greatly in shape with the PAG of untreated brains (shown in red and denoted with “No”).

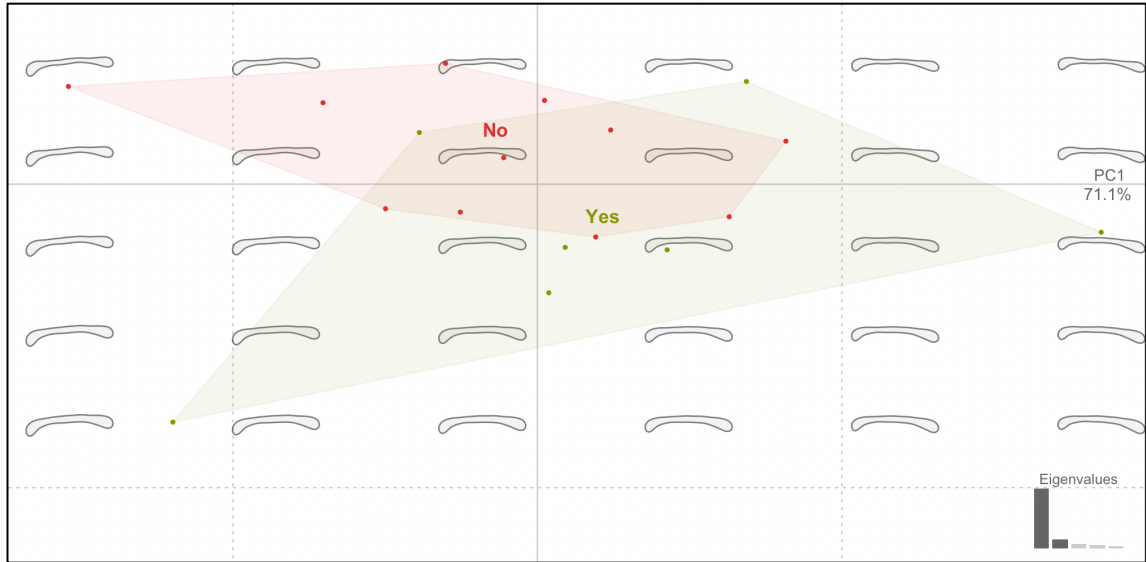


Figure 2.12. Hydrogel treatment does not affect corpus callosum shape. The corpus callosum of hydrogel-treated brains (shown in green and denoted with “Yes”) overlap greatly in shape with the corpus callosum of untreated brains (shown in red and denoted with “No”).

In contrast to *shape* analyses, our investigation did note significant differences in regional brain *size* between hydrogel-treated and -untreated specimens. As shown in figure 2.13a, the average cross-sectional area of the PAG was significantly smaller in brains not treated with hydrogel compared to hydrogel-reinforced specimens ($P < 0.001$; hydrogel-treated cross-sectional area = $3.61 \text{ mm}^2 \pm 0.53 \text{ mm}^2$ (mean \pm standard deviation); no-hydrogel cross-sectional area = $2.48 \text{ mm}^2 \pm 0.21 \text{ mm}^2$). Figure 2.13b shows the same trend for the corpus callosum: Hydrogel treatment led to a significantly larger average corpus callosum area, compared to untreated brains ($P < 0.001$; hydrogel-treated cross-sectional area = $3.04 \text{ mm}^2 \pm 0.13 \text{ mm}^2$; no-hydrogel cross-sectional area = $2.25 \text{ mm}^2 \pm 0.24 \text{ mm}^2$). Note that the average total brain mass, taken after fixation, of both groups (hydrogel-treated and -untreated) was exactly 1.84 g, suggesting that the difference in cross-sectional area is not due to sampling error.

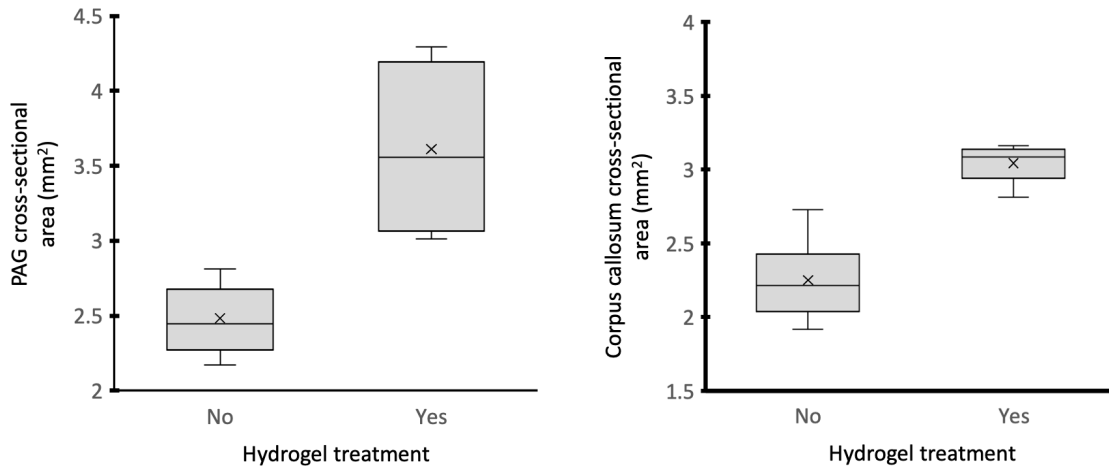


Figure 2.13. *Hydrogel treatment affects cross-sectional area.* Hydrogel-treated specimens had significantly larger cross-sectional areas for (a) the PAG and (b) the corpus callosum. In both box plots, X represents the mean, whiskers represent minimum and maximum values (no outliers), and box borders represent first, second, and third quartiles.

Discussion: Hydrogel Stabilization Is Not Necessary for All Morphometric Experiments

The aim of this experiment was to test the efficacy of hydrogel stabilization; that is, my goal was to learn if hydrogel is necessary to stabilize iodine-stained brains used in morphometric analyses. I anticipated that hydrogel would be protective against volumetric changes that occur during iodine staining, since hydrogel helps to stabilize tissues against the reported shrinkage that occurs in the iodine stain solution (Wong et al., 2013 and Vickerton et al., 2013). On the other hand, I did not anticipate that hydrogel reinforcement would affect the shape of brain structures. My results align with these predictions. Using hydrogel reinforcement prior to iodine staining triggers a significant change in the *size* of brain structures but no significant difference in brain structure *shape* (table 2.2).

Brain Region	Cross-Sectional Area Change?	Shape Change?
PAG	Yes, significantly larger size in hydrogel-treated specimens.	No difference in PAG shape for hydrogel-treated and -untreated brains.
Corpus callosum	Yes, significantly larger size in hydrogel-treated specimens.	No difference in corpus callosum shape for hydrogel-treated and -untreated brains.

As previously mentioned, many published articles suggest that hydrogel treatment protects soft tissues from shrinkage in iodine (see Wong et al., 2013 and Vickerton et al., 2013). Data from our investigation may support these findings. However, an alternative hypothesis is that hydrogel caused the tissues to expand. Park et al. (2018) discusses using polymerized hydrogel and deionized water to expand neural tissues by up to a factor of 8. In this method, polymerization of hydrogel in a high heat and alkaline environment created ionic residues throughout the hydrogel network. The subsequent osmotic influx of water caused the specimen to expand. It is possible that the hydrogel stabilization method that we used in this experiment also causes tissue expansion as the iodine solution fills spaces in the hydrogel network. Most likely, both hypotheses are partially true: Hydrogel treatment is protective against iodine-induced shrinkage *and* causes tissue expansion. To clarify the effect of hydrogel on tissue size, one needs to compare the size of a brain structure (for example) both before and after hydrogel treatment. These measurements can be taken via MRI to avoid introducing iodine as a variable. (As shown in Zhu, 2019, MRI is useful for visualizing hydrogel-infused specimens without use of a contrast agent.)

Regardless of how hydrogel affects the size of brain structures, these size changes do not correspond with significant deformations of shape. The shape of a gray matter region (the PAG) and a white matter region (the corpus callosum) is the same after iodine staining irrespective of hydrogel reinforcement.

Recall that hydrogel reinforcement comes with several drawbacks. These include time, cost, potential specimen damage, imaging artifacts, and interference with other staining techniques. These drawbacks would certainly be worth the cost if hydrogel reinforcement improved the results of a morphometric analysis. After all, it would be challenging to answer questions about how disease, drug addiction, or other variables change regional brain shape if iodine staining caused significant shape deformations prior to CT scanning. However, our results suggest that

hydrogel-free brains accurately represent regional shapes. With notable costs and no obvious advantages, hydrogel reinforcement is not advisable for all morphometric analyses.

Hydrogel treatment is, however, a necessary step for analyses of size. Cross-sectional area may be approximately 25% (white matter) to 33% (gray matter) smaller in iodine-stained specimens that have not been reinforced with hydrogel compared to reinforced specimens. If an investigation has questions about the size of brain regions, then specimens should be reinforced with hydrogel prior to iodine staining. An investigator should be careful to have a well-defined protocol for hydrogel polymerization (i.e., the step in which hydrogel is heated), since a longer duration of polymerization may be directly related to hydrogel-induced tissue expansion (Park et al., 2018).

Hydrogel may also be necessary for visualizing structures without well-defined boundaries. Specimens treated with hydrogel in this experiment had better resolution and greater contrast in CT scan images than specimens that were not treated with hydrogel (figure 2.14). The improved image clarity is likely due to the higher concentration and duration of iodine staining that

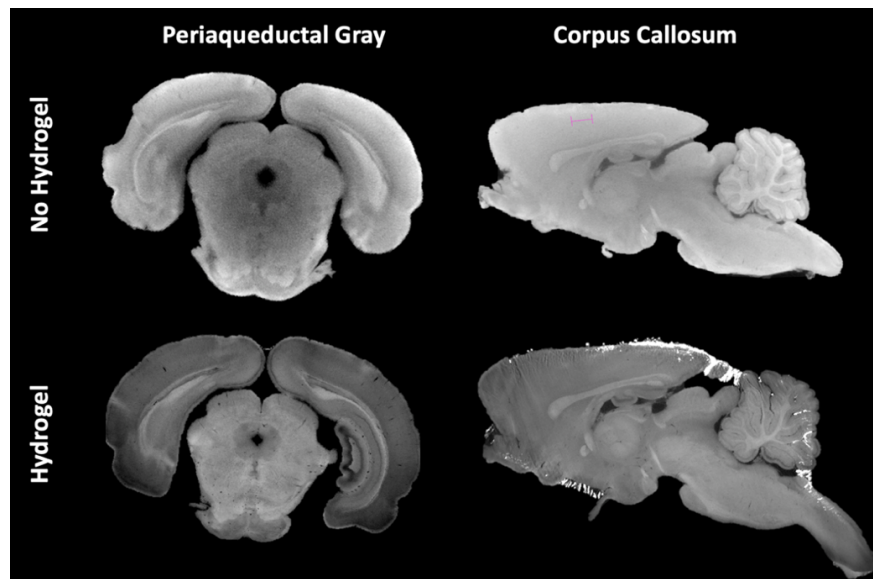


Figure 2.14. *Hydrogel treatment improves image quality. Brains that were treated with hydrogel (bottom) had higher resolution and greater contrast than untreated brains (top).*

hydrogel allows. Fortunately, for both the PAG and the corpus callosum, grayscale differences between these structures and surrounding regions allow for segmentation without hydrogel-enhanced contrast. Other research questions may require the greater contrast that is possible to achieve after hydrogel stabilization.

Given that hydrogel does not affect regional brain shape, we used the same brains in another investigation of the effect of opioid drug exposure. As mentioned above, the rats from which we obtained these brains were exposed to opioids. When hydrogel is used as a cofactor in this follow-up experiment, we see no difference in the results compared to when hydrogel is ignored. This further indicates that hydrogel is not a necessary step in morphometric analyses.

In chapter 3, we will use similar morphometric methods to look more closely at how opioids affect the shape of the PAG and the corpus callosum in the adolescent rat brain.

CHAPTER III

USING MORPHOMETRY TO STUDY HOW OPIOID ADDICTION CHANGES THE ADOLESCENT BRAIN

Introduction: How Has Morphometry Been Used to Study Addiction?

Morphometry has taught scientists and physicians much about how drug addiction changes brain structure (reviewed in Fowler et al., 2007, and Suckling and Nestor, 2017). Most revealing are structural MRI data from individuals who have been chronically exposed to drugs. For example, using structural MRI, Liu et al. (1998) compared the brains of people with chronic polysubstance abuse to matched controls and found that addiction is associated with smaller prefrontal lobes. A smaller prefrontal lobe among drug abusers is likely due to a reduction in white matter (Schlaepfer et al., 2006). However, drugs also affect gray matter. Stimulant drugs such as methamphetamine have been shown to reduce gray matter density in the right middle frontal cortex; gray matter began to recover after 6 months of abstinence (Kim et al., 2006). Structural MRI also reveals other brain regions that drugs affect: Stimulants cause the basal ganglia to enlarge (Jacobsen et al., 2001; Jernigan et al., 2005) and trigger severe gray matter reductions in the cingulate cortex and the hippocampus (Thompson et al., 2004). Overall, these imaging studies reveal that addictive drugs repaint the landscape of the brain.

Understanding how drugs affect the brain can be informative for assessment and monitoring of drug-addicted patients. For example, assessing the degree of gray matter changes to the basal ganglia and cingulate cortex may help to determine a drug addict's motivation to move toward

drug stimuli: Paulus et al. (2005) showed that functional MRI of the prefrontal lobe in methamphetamine addicted individuals accurately predicts whether a person is likely to relapse; that is, low prefrontal lobe activity predicted relapse with 90% accuracy. These results suggest that studying gross anatomical changes to the brain as a result of drug addiction and drug recovery treatment can lead to better patient outcomes.

Relatively few investigations have shown how opioids affect brain region morphometry. Using brain imaging on opioid-addicted and control patients (who were also not addicted to any other drugs), Upadhyay et al. (2010) showed that opioid addiction triggers bilateral volume reductions in the amygdala, and also triggered reductions in white matter connectivity between cortical and subcortical brain regions. These findings may suggest that opioid addicts do not properly process emotions and have impaired impulse control (Upadhyay et al., 2010). Individuals with preexisting prefrontal cortex damage (due to genetics or other factors) are also more likely to have impaired impulse control, making opioid addiction more likely and making its effects more damaging (Kosten and George, 2002).

Previous studies have used Sprague Dawley rats to evaluate opioid-induced changes to brain morphology. For example, Zagon and McLaughlin (1986) showed that exposure to opioid-receptor agonists before weaning triggers significant changes to cerebral width, cerebral area, and cortical area; this same investigation showed that the density of glial cells in the corpus callosum significantly increased in the opioid group compared to control. Researchers have also used morphometric methods on opioid-exposed rat brains to determine that opioids trigger apoptosis of cells in the entorhinal cortex—an area that is responsible for both memory and navigation (Ragab and Mohamed, 2016). Further studies are necessary to determine how opioids affect the morphometry of other brain regions.

Specific Aims

To learn more about how opioids cause anatomical changes in the brain, we compared the postmortem brains of adolescent opioid-exposed Sprague Dawley rats with opioid-naïve rats. We focused on two regions: the periaqueductal gray (PAG) and the corpus callosum. The PAG has been implicated in opioid withdrawal (Bonci and Williams, 1997) and is involved in opioid-induced analgesia (Garland et al., 2013). The corpus callosum is a primary marker of cognition integrity (Luders et al., 2007), and drug addiction has been shown to affect many cognition centers (summarized in Gould, 2010).

We hypothesized that exposure to opioid drugs would significantly affect the shape of both the PAG and the corpus callosum. Chronic opioid use decreases the activity of dopaminergic (DA) neurons (Kosten and George, 2002), perhaps causing anatomical changes to the DA centers of the PAG. Likewise, specific white matter pathways within the corpus callosum may be more likely to be affected by opioids than others (see chapter 1 for a detailed look at how drugs of addiction affect cognitive centers that use the corpus callosum to transmit information); these local changes in white matter activity may be reflected in altered morphometry of the corpus callosum. As described below, I used high-resolution brain imaging to test for shape changes in these regions.

Methods: Employing DiceCT to Learn about Opioid-Induced Changes in Brain Structure

To learn whether exposure to opioids affects brain anatomy, I used an imaging technique called diceCT (diffusible iodine-based contrast-enhanced computed tomography). The details of diceCT are outlined in chapter 2 of this thesis. Here, I apply those methods to obtain and analyze brain scans of adolescent rats—with the goal of determining if opioid exposure affects the gross anatomy of the aforementioned brain regions.

Specimens and Drug Exposure

This experiment uses the postmortem brains of the same 18 Sprague Dawley rats that were described in chapter 2 (i.e., the same brains used to determine the effects of hydrogel reinforcement were also used to test the effect of drug treatment; recall that hydrogel did not affect regional brain shape). All rats had free access to food and water in cages with 1–2 other subjects of the same sex.

To test the effects of opioid exposure on the brain, some adolescent rats were exposed to morphine, whereas others were opioid-naïve. For all subjects, experiments began on postnatal day 33 and ended on postnatal day 43. In this pilot study, the experimental design was somewhat complicated but is summarized here, and the resulting treatment groups are described in table 3.1. All drug-exposed rats received two subcutaneous injections of 5 mg/kg morphine (m8777, Sigma Aldrich, St Louis, MO, USA) on four non-consecutive days; these same rats received saline injections on days between morphine treatments. Drug-naïve rats received injections of saline on all days during this same period. Behavioral tests using a conditioned place preference protocol suggest that morphine-conditioned rats prefer the compartment where they receive morphine; saline-conditioned rats show no compartment preference. These data are evidence that morphine affects memory and approach behaviors in rats. After developing drug preference, all rats then went through extinction, in which they lost preference for a behavioral chamber.

Following extinction were reinstatement trials during which subjects were again exposed to morphine (2.5 mg/kg for this trial) or saline. Prior to each reinstatement trial, all rats (in both drug-exposed groups and the opioid-naïve group; see table 3.1) received injections of either a nociceptin receptor (NOP) agonist (either AT-200, AT-202, or AT-312 at 3mg/kg), vehicle (5% dimethyl sulfoxide; DMSO), or saline. The purpose of the NOP agonist in the behavioral experiment was to determine if binding a related chemical to the opioid receptor system could reduce reinstatement of morphine preference. (NOP agonists were found to be 50–66% effective.)

For our neuroanatomical experiment, these subjects with various drug exposures were divided into three behaviorally relevant treatment groups—morphine, NOP-morphine, and opioid-naïve—summarized in table 3.1.

As described in chapter 2, all 18 rats were sacrificed on postnatal day 43 by transcardial perfusion with cold saline solution (0.9% NaCl) followed by cold 4% paraformaldehyde. Each brain was immediately extracted after sacrifice.

Procedures were conducted according to the National Institutes of Health guidelines and were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee (IACUC protocol: 2021-2014). All reasonable efforts were made to minimize discomfort and the number of animals used in this study.

Table 3.1. Treatment Groups for Opioids Experiment		
Treatment Group	Description	Sample Size
Morphine	Subjects previously exposed to morphine received either a saline injection or a DMSO injection prior to morphine reinstatement.	$n = 7$
NOP–morphine	Subjects previously exposed to morphine received an injection of a nociceptin receptor (NOP) agonist prior to morphine reinstatement.	$n = 8$
Opioid-naïve	Subjects that received only saline in the conditioning trials received an injection of a nociceptin receptor (NOP) agonist prior to the reinstatement trial, in which they again received only saline.	$n = 3$

Fixation

The rat brains in this experiment were fixed in 4% paraformaldehyde solution for 24 hours immediately after sacrifice. They were then moved to a 30% sucrose solution until either

hydrogel reinforcement or iodine staining commenced; no brain was in a sucrose solution for more than 72 hours.

Hydrogel Reinforcement

The function, trade-offs, and procedure of hydrogel reinforcement are described in detail in chapter 2. Out of the 18 rat brains in our experiment, 7 were reinforced with hydrogel and 11 did not receive hydrogel treatment. Reinforcement with hydrogel does not significantly affect regional brain shape in either structure of interest (see chapter 2).

Staining

Hydrogel reinforced brains were immersed in a 3% Lugol's solution for 6 weeks in a 4°C fridge. The solution was refreshed after 3 weeks. Brains that did not receive hydrogel treatment were stained at room temperature in a 1% Lugol's solution for 3 weeks and then a 2% Lugol's solution for 2 additional weeks; the 2% solution was refreshed twice.

Scanning

Specimens were scanned on either a GE phoenix v|tome|x s240 (American Museum of Natural History's Microscopy and Imaging Facility; New York, New York) or a Nikon 225 XTH (University of Arkansas; Fayetteville, Arkansas). High beam energy (average: 30.8W) ensured that X-rays fully penetrated the specimens. Resolution was optimized with a high number of angles for X-ray projections (average: 2405), relatively long exposure time (average: 395ms), and multi-frame averaging of X-ray detection (average: 6 frames per projection angle). For more details about these specifications, see chapter 2 and appendix A.

Visualizing Specimens with Avizo

We used Avizo (version 9.5.0, Thermo Fisher Scientific) to visualize CT-generated image stacks. As described in chapter 2, we obtained two-dimensional, cross-sectional images of the PAG (just dorsal to the ventral tegmental area) and the corpus callosum (parasagittal). After outlining the

region of interest using Microsoft PowerPoint (version 16.48 for Mac), we compared the shape of the PAG and corpus callosum in opioid-exposed brains and opioid-naïve brains using an elliptical Fourier analysis in R. The opioid-exposed group was further divided into rats that received nociceptor agonists prior to morphine exposure (the “NOP–morphine” group; see table 3.1) and rats that received only saline or DMSO prior to morphine exposure (the “Morphine” group).

Note that, upon visualization, we noticed that one opioid-naïve specimen was damaged.

Specimen 3 had an indentation in the right hemisphere of the cerebrum that was most likely a result of packing the specimen too tight prior to transport to the CT scanner. However, the damage did not affect the PAG, which is in the midbrain. In addition, the damage did not appear to affect the corpus callosum; to be sure, the parasagittal cross section of the corpus callosum was taken from left of the midline.

A cross section of each specimen’s PAG and corpus callosum is shown in figure 3.1. Color coding allows for quick visualization of treatment groups.

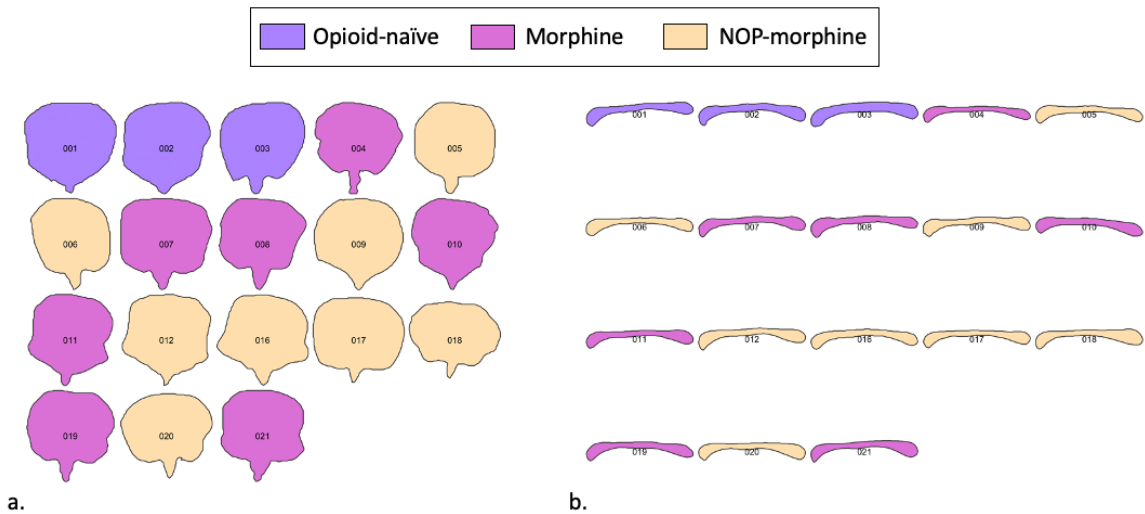


Figure 3.1. *Treatment Groups.* Specimens were divided into three groups—opioid-naïve, morphine, and NOP-morphine—for both (a) the PAG analysis and (b) the corpus callosum analysis.

Statistical Analysis

I employed an elliptical Fourier analysis using the Momocs package in R to compare the shape of the PAG and corpus callosum among the three treatment groups (Bonhomme et al., 2014). I input black-and-white outline jpg files of the PAG and corpus callosum cross sections and then scaled and centered the outlines (figure 3.2a and 3.2b). An essential step to getting accurate shape readouts was to then align the “start points” (i.e., the point at which the program begins to redraw the specimen from the jpg image); see the aligned carats at the top of the PAG drawings in figure 3.2b. I then used an elliptical Fourier transform on each specimen to form a continuous perimeter composed of sine and cosine waves (figure 3.2c). For the PAG, 11 harmonics (i.e., sine and cosine waves) captured 99% of the shape information, which is a standard for the field (Crampton, 1995). For the corpus callosum, 8 harmonics captured the same amount of shape variation. Finally, I used a PCA plot and a MANOVA test to determine if the shape of the PAG or corpus callosum is significantly affected by opioid treatment. For MANOVA, principal components were retained until they captured at least 80% of the total variance in shape. This resulted in retaining four PC axes for the PAG and two PC axes for the corpus callosum. Finally, a Pillai test was used for post hoc analysis.

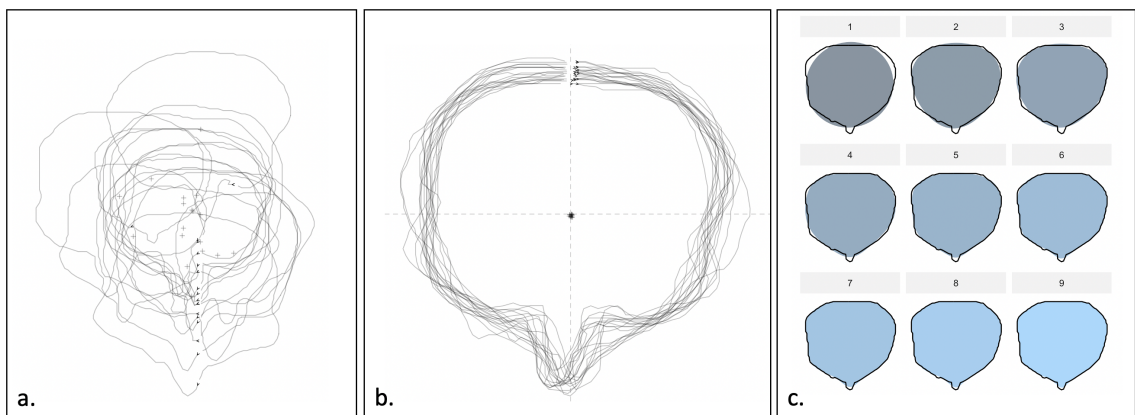


Figure 3.2. *Processing the PAG.* (a) I input the cross sections into R and then (b) scaled and centered the images. (c) Fidelity of the PAG shape (black outline) represented by increasing the number of harmonics (blue hulls), as found during elliptical Fourier analysis. Note that increasing the number of harmonics better represents shape complexity.

Results: Does Opioid Exposure Affect the Structure of the PAG and Corpus Callosum?

I investigated the effect of opioid exposure on the adolescent rat brain, with focus on two regions: the PAG (a gray matter structure) and the corpus callosum (a white matter structure). My analysis revealed a significant change in the shape of both the PAG and the corpus callosum as a result of opioid exposure.

Principal component analysis provides a visual representation of the shape differences between treatment groups. Figure 3.3 shows the effect of opioids on the PAG. Notice the slight overlap between the morphine group and the NOP-morphine group, as well as the separation between the opioid-naïve group and the two morphine groups. MANOVA reveals a significant difference exists between these groups ($P=0.01$; $F=2.51$; harmonics=11; four axes retained in PC plot capturing a total variance of 83%). Hydrogel treatment was used as a covariate in this analysis. A post hoc test reveals that all treatment groups are significantly different from one another (Pillai test; opioid-naïve vs. morphine: $P = 0.018$; opioid-naïve vs. NOP-morphine: $P = 0.006$; NOP-morphine vs. morphine: $P = 0.006$).

The shape of the corpus callosum also appears to change due to drug treatment. The three opioid-naïve specimens occupy a distinct morphospace compared to the “morphine” group and the “NOP-morphine” group (figure 3.4). Discriminant analysis finds a significant difference between the shape of these groups ($P=0.039$; $F=2.49$; harmonics=8; two axes retained in PC plot capturing a total variance of 81.8%). Again, hydrogel treatment was used as a covariate. *Post hoc* analysis reveals that the opioid-naïve group is significantly different from the NOP-morphine group ($P = 0.021$), and the difference between the opioid-naïve group and the morphine group is marginally significant ($P = 0.065$). The morphine group and the NOP-morphine group are not statistically different ($P = 0.99$).

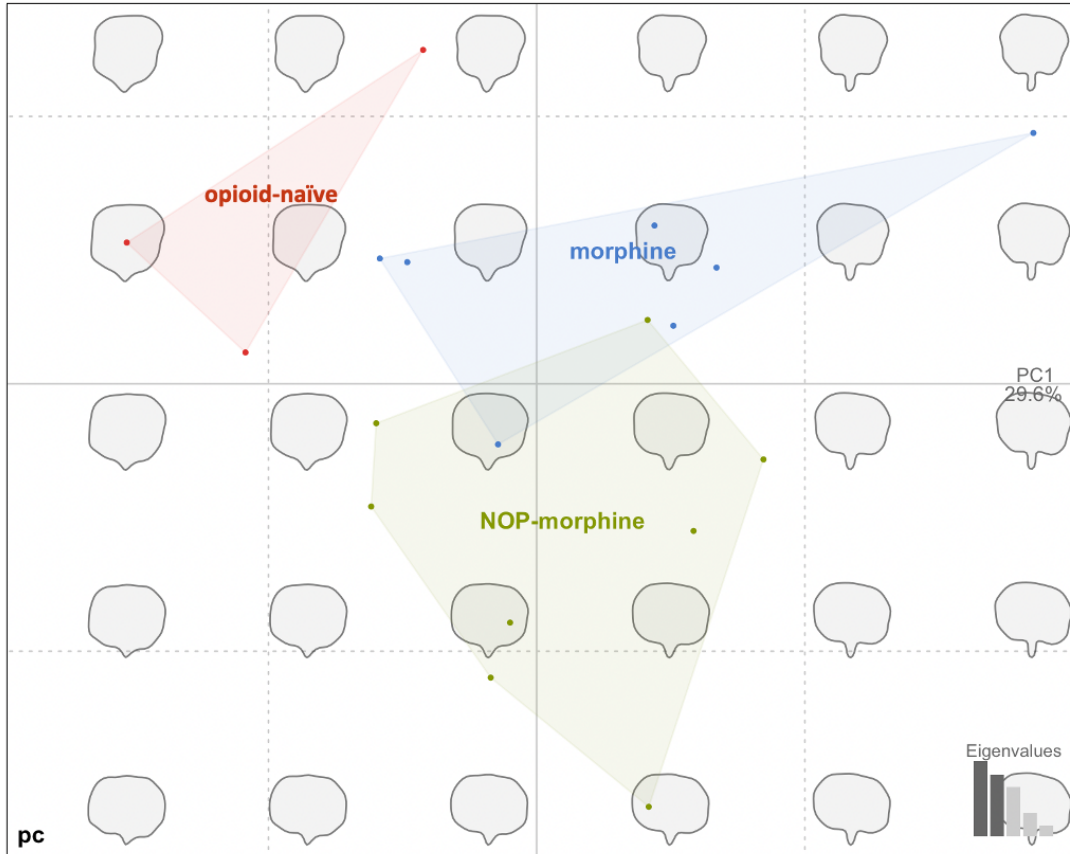


Figure 3.3. Opioid treatment affects PAG shape. The shape of the PAG in the “morphine” group (blue) overlaps with the “NOP-morphine” group (green), but the “opioid-naïve” group (red) occupies a separate morphospace.

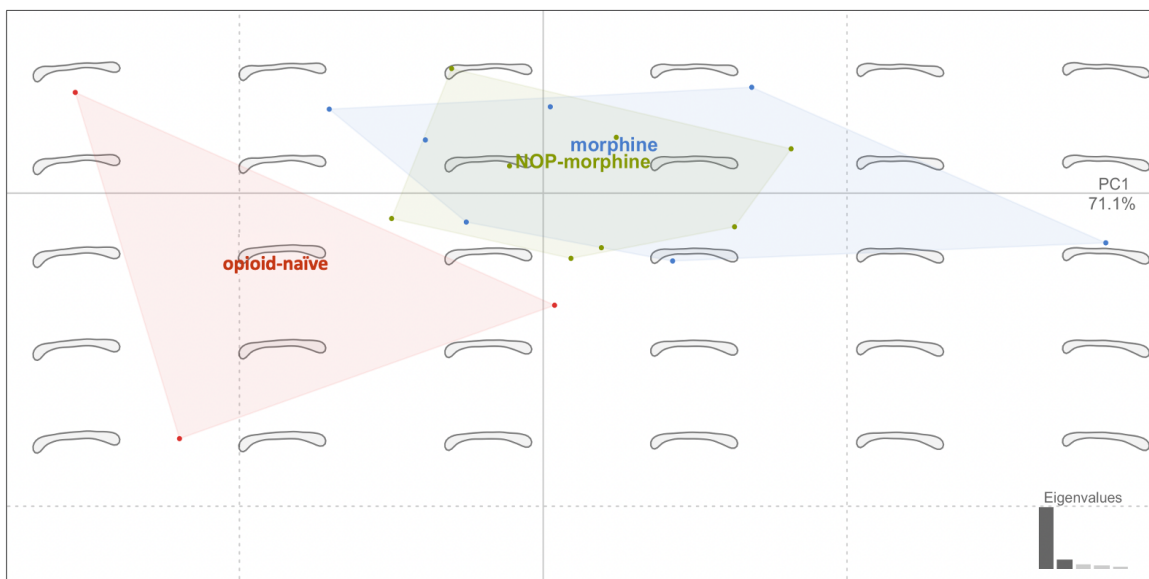


Figure 3.4. Opioid treatment affects corpus callosum shape. The corpus callosum of morphine brains (blue) and NOP-morphine brains (green) are distinct in shape compared to opioid-naïve brains (red).

Looking again at figure 3.4, notice the extensive overlap between the “morphine” group and the “NOP-morphine” group. As a follow-up analysis, I combined these groups into a single “morphine-treated” group to capture all specimens that were exposed to morphine (disregarding the potential effect of the NOP agonist). MANOVA of the shape difference between the “morphine-treated” specimens and opioid-naïve was significant ($P=0.006$; $F=4.05$; harmonics=8; two axes retained in PC plot capturing a total variance of 82.5%).

Discussion: Opioids Likely Affect Gross Brain Anatomy

This was among the first experiments to analyze how opioids affect the gross anatomy of the brain, and, to my knowledge, it is the first to use adolescents as subjects. My results suggest that four injections of opioids during adolescence in a rat model triggers neuroanatomical changes that are detectable via CT scan.

Morphine exposure significantly affected the shape of the PAG. These results are not surprising, considering the role of the PAG in opioid dependence, in dopamine production, and in processing pain. The PAG’s most ventral extent—the ventral subregion of the dorsal raphe nucleus (vDRN)—appears to be a contributing factor to the shape difference among drug treatment groups (figure 3.5). In particular, the vDRN is narrower and longer in morphine-exposed rats compared to opioid-naïve rats. The vDRN primarily houses serotonergic efferent neurons that are activated by rewarding stimuli; these neurons innervate the orbitofrontal cortex and enhance coping behaviors (mice in a forced swim test are more likely to try to escape; Ren et al., 2018).

In addition, dopaminergic neurons project from the vDRN and synapse in the nucleus accumbens (Michelsen et al., 2008). It is therefore possible that the opioid-induced growth of the vDRN primes the brain for more effective serotonin-induced coping and dopamine-induced reward with each morphine administration; these anatomical changes may partly explain why opioid sensitization is long-lasting in adolescents (Koek, 2014).

Morphine also affects the shape of the lateral wings of the dorsal raphe nucleus (lwDRN); these regions are greatly reduced after morphine exposure, leading to the convexity seen in the lateral regions of the PAG's ventral surface in figure 3.5. The lwDRN has serotonergic cells that project to subcortical structures, such as the superior colliculus, trigeminal nuclei, and sensory processing areas of the thalamus (Vasudeva et al., 2011). The lwDRN also has populations of cells that produce nitric oxide (NO), which is hypothesized to regulate stress responses: Blocking NO release from this region induces anxiety behaviors, such as reduced movement in an elevated plus maze (Spiacci Jr. et al., 2008). Opioid-exposure atrophies the lwDRN, and therefore may limit NO release from this region; the resulting heightened stress response may also drive negative reinforcement that occurs during opioid withdrawal. Future studies are needed to clarify if and how anatomical changes to the dorsal raphe nuclei affect neurotransmitter production and release. However, based on these preliminary data, it is possible that exposure to opioids causes anatomical changes that make subsequent opioid use more likely.

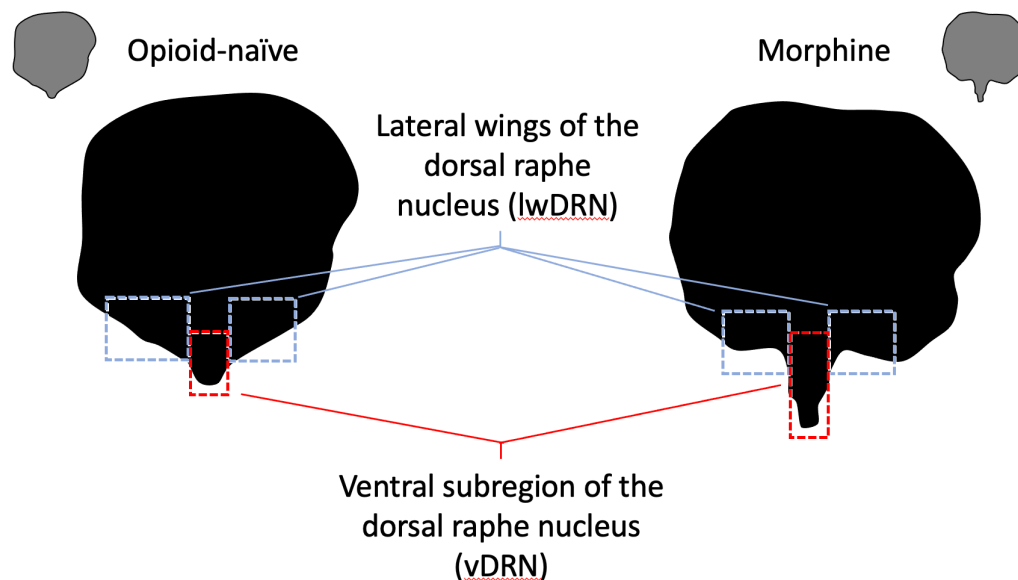


Figure 3.5. *Notable changes in PAG anatomy.* Opioid exposure appears to have affected the size and shape of the ventral subregion of the dorsal raphe nucleus (vDRN, outlined in red) and the lateral wings of the dorsal raphe nucleus (lwDRN, outlined in blue). Shown here are specimen 002 (opioid-naïve) and specimen 019 (morphine). The gray insets in the top corners show the outlines of these specimens without labels and leader lines. Notice the longer and narrower vDRN, as well as the concave lwDRN, in the morphine brain compared to the opioid-naïve brain.

The shape of the corpus callosum was also significantly different in rats that received morphine injections compared to opioid-naïve rats that only received saline injections in this experiment. In particular, the genu and body of the corpus callosum appears to have been affected (see figure 3.6 for corpus callosum anatomy). A close look at the morphospace shown in the PC plot (see figure 3.4) reveals that the primary axis ranges from a relatively wide corpus callosum body (on the left) to a relatively narrow body (on the right). Along the same axis, the genu of the corpus callosum ranges from relatively large to relatively small. Opioid-naïve specimens are closer to the left side of the PC plot (where the corpus callosum body is wider and the genu is larger) compared to specimens that were exposed to morphine. On the other hand, the size and shape of the splenium appears nearly unchanged across the primary PC axis. These results suggest that morphine exposure differentially affects regions of the corpus callosum.

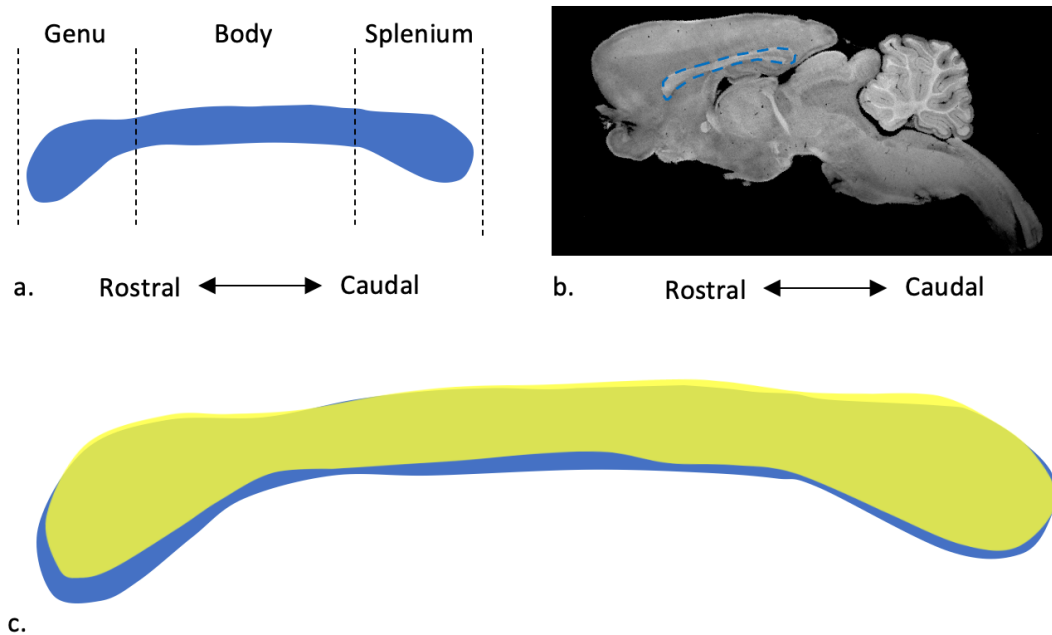


Figure 3.6. *Notable changes in corpus callosum anatomy.* Opioid exposure appears to have affected the genu and body of the corpus callosum but not the splenium, based on analysis of outlines in the PC plot morphospace. These outlines were originally generated on isolated cross sections of adolescent rat brains. (a) Anatomical regions of the corpus callosum; (b) corpus callosum in context (outlined with hashed blue line); (c) an opioid-exposed corpus callosum (*yellow*) superimposed on an opioid-naïve corpus callosum (*blue*) showing the major shifts in shape are in the genu and the body.

Previous studies have shown that opioid exposure in human adults causes retrograde memory impairment (Kamboj, 2005), as well as decreases in working memory (Friswell et al., 2008). These memory limitations could be due to changes in the structure of the corpus callosum, such as reductions in the abundance or connections between commissural fibers. After all, a greater callosal area is associated with more commissural fibers (Aboitiz et al., 1992) and better performance on cognitive tasks (Strauss et al., 1994). While the present study did not directly measure callosal area, it did reveal changes in the shape of the corpus callosum after opioid exposure that suggest impaired functionality. Further studies are required to determine if the gross anatomical changes in the corpus callosum lead to measurable cognitive impairments in the adolescent rat model. For example, opioid-exposed rats could be challenged with Y-maze tests, olfactory or social discrimination, or object recognition. The results of these tests could then be aligned with the degree of corpus callosum distortion.

One limitation of our experiment was the small sample size. To overcome this limitation, we attempted to add more opioid-naïve specimens to the experiment. Unfortunately, the COVID-19 pandemic interfered with scanning these specimens. They therefore remained in a diluted iodine stain for six weeks beyond the typical protocol. The extended staining period resulted in abnormal CT scanning results; in particular, it ruined the contrast enhancement between white and gray matter that is the cornerstone of diceCT. It is possible that iodine diffused extensively into the interstitial spaces of the gray matter. Regardless of the exact cause, gray matter appeared brighter than normal on CT images, making it impossible to accurately outline the corpus callosum and PAG. We attempted to re-stain the specimens following the original protocol, but the gray matter remained too bright on subsequent CT images. While this mistake was mostly outside of our control, it underscores the importance of honing the protocol for fixation, staining, storage, and scanning of specimens before beginning a largescale experiment—as small errors can render the resulting scans unusable.

Other limitations of this experiment include the narrow duration of opioid exposure, which occurred over only four treatments, and the short time frame between opioid exposure and animal sacrifice. More research is needed to understand the effects of chronic opioid exposure on adolescents, as well as the permanence of these effects. These future directions are important because chronic opioid exposure is a reality for some adolescents. While efforts have been made in recent years to curb the rise in opioid prescriptions (Dowell et al., 2016), evidence suggests that opioids are “easy” for many adolescents to obtain (Sheridan et al., 2016; Johnston et al., 2017). A 2020 study by Nguyen et al. showed that the prescription of opioids to an adolescent greatly increases (six-fold) the chance of opioid overdose, and the prescription of opioids to a family member is also strongly associated with overdose risk (two-fold increase). Misuse of opioids during childhood and adolescence is also strongly associated with heroin abuse later in life (Cerdá et al., 2015). These data argue for improvements in patient and family counseling about the risks associated with opioid use, including the potential for developing addiction and the long-term consequences of opioid exposure on the brain; however, the latter is not well enough understood. It is therefore imperative that additional research is conducted into how opioids cause lasting changes in brain anatomy and function. It is especially important to advance these studies in adolescents, who are not only more likely to engage in risky behaviors such as drug abuse but also who are more likely to become sensitized to opioids than are adults (Koek, 2014).

The opioid epidemic has no simple cure. It will require medical interventions, such as opioid agonists for people with opioid use disorders, as well as alternatives for treating chronic pain. It will also require improvements in education so that physicians and family members can more easily prevent and detect opioid addiction. Of course, a key element to both the medical and social interventions of the opioid epidemic is a solid foundation in research.

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APPENDICES

Scanning specifications for each specimen can be found in table A on the following page. Scan dates ranged from early 2018 to early 2019. Scan facilities included both the American Museum of Natural History's (AMNH) Microscopy and Imaging Facility (New York, New York) and the University of Arkansas MicroCT Imaging Consortium for Research and Outreach (MICRO) facility in Fayetteville, Arkansas. Scanners included a GE phoenix v|tome|x s240 CT scanner and a Nikon 225 XTH. Beam energy is measured in watts (W), voxel size in micrometers (μm), and exposure time in milliseconds (ms). These and other column headers shown in table A are described in detail in the *Scanning* section of chapter 2.

Specimen: treatments	Scan date	Facility	Scanner	Beam energy	Voxel size	Exposure time	Image averaging	Rotation steps
001: no, con	1/29/18	AMNH	GE	15.6 W	22.0 μm	333 ms	6x	1800
002: hyd, con	12/17/18	MICRO	Nikon	70.0 W	15.6 μm	267 ms	8x	2500
003: hyd, con	12/18/18	MICRO	Nikon	70.0 W	16.7 μm	267 ms	8x	2500
004: hyd, mor	3/19/19	MICRO	Nikon	14.0 W	16.1 μm	708 ms	8x	3141
005: no, nop	1/31/18	AMNH	GE	12.1 W	21.5 μm	333 ms	5x	1800
006: no, nop	1/31/18	AMNH	GE	12.1 W	21.5 μm	333 ms	5x	1800
007: hyd, mor	12/18/18	MICRO	Nikon	70.0 W	14.1 μm	267 ms	8x	2500
008: no, mor	1/31/18	AMNH	GE	14.4 W	24.3 μm	333 ms	6x	1800
009: no, nop	1/31/18	AMNH	GE	14.4 W	24.3 μm	333 ms	6x	1800
010: hyd, mor	12/18/18	MICRO	Nikon	51.2 W	13.9 μm	267 ms	8x	2500
011: no, mor	5/17/18	MICRO	Nikon	13.9 W	14.4 μm	708 ms	4x	3141
012: no, nop	5/18/18	MICRO	Nikon	13.9 W	20.4 μm	708 ms	4x	3141
016: hyd, nop	12/18/18	MICRO	Nikon	65.8 W	15.2 μm	267 ms	8x	2500
017: no, nop	1/31/18	AMNH	GE	13.2 W	20.1 μm	333 ms	6x	1800
018: no, nop	1/31/18	AMNH	GE	13.2 W	20.1 μm	333 ms	6x	1800
019: hyd, mor	12/19/18	MICRO	Nikon	63.5 W	14.0 μm	267 ms	8x	2500
020: no, nop	5/17/18	MICRO	Nikon	14.1 W	12.1 μm	354 ms	4x	3141
021: no, mor	5/18/18	MICRO	Nikon	13.9 W	17.2 μm	708 ms	4x	3141

Table A.1. Scanning parameters for all specimens. Abbreviations: no=no hydrogel; hyd=hydrogel; con=control group; mor=morphine group; nop=NOP–morphine group.

VITA

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