Structure and Evolution of Dragon Brains

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Declaration

This thesis is structured as a series of manuscripts that have been or will be submitted for publication in scientific journals. I was the primary contributor on all chapters presented in this thesis. My supervisor, Prof. J. Scott Keogh, and my co-supervisor, Assoc. Prof. Martin J. Whiting, provided input on all aspects of this thesis. The remaining major contributions by others to this thesis are as follows: Dr. Jeremy F.P. Ullmann helped plan, analyze, and edit the manuscripts for Chapters 3, 4 and 5. Dr. Andrew L. Janke created the model brains from the original MRI scans and helped edit the methods sections in Chapters 3, 4 and 5 pertaining to model creation and MRI image manipulation. Dr. Timothy Stait-Gardner designed the MRI scanning protocol used in this thesis, performed the MRI scanning, and helped edit the methods sections in Chapters 3, 4 and 5 pertaining to MRI scanning. Yanurita Dwihapsari conducted the MRI scanning with Timothy (it is a two-person job). Prof. William S. Price provided access to the MRI scanner. Marta Vidal-Garcia provided input with data analysis, data visualization, and editing for Chapter 5. Further contributors are listed in the Contributions section. No part of this thesis has been submitted for any previous degree.

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Abstract

This thesis is about the evolution of brain structure in lizards, with a particular focus on the agamid lizards of the genus *Ctenophorus*. Achieving this required advancing the methods with which to study lizard brains. Therefore, this thesis is split about equally between developing the tools to study lizard brain structure and the evolution of brain structure in *Ctenophorus*. Below I present short summaries of each chapter. Full abstracts are presented with each chapter.

Section 1: Development of the framework and tools necessary for the study of the dragon brain

Chapter 1
Among vertebrates, reptiles have lagged far behind birds, mammals, fishes and amphibians in neurobiological research. Nonetheless, in the past twenty years there have been significant advances in our understanding of the neurobiology of reptiles, particularly among squamates (lizards and snakes). The first chapter of this thesis presents a broad literature review of lizard brain research. All peer-reviewed publications since the last major review in 1998 are summarized to give a complete overview of the state of the published literature on squamate neurobiology. I use this overview to highlight what is unique about squamate brains and identify gaps that remain in our understanding of these systems. Finally, I provide a framework for future studies that includes exciting new and unanswered questions about squamate brain evolution, structure and function.

Chapter 2
Perfusion is the most common technique for preserving brains for neuroscience research. Standard perfusion techniques were developed primarily for application in mammals, which are traditional neuroscience research models. A perfusion method has never been published for lizards and following mammalian perfusion protocols for lizards results in failed perfusions. In this chapter, I present a modified perfusion protocol suitable for lizards.

Chapter 3
In this chapter we present a magnetic resonance-based atlas for the brain of an agamid lizard, the tawny dragon (*Ctenophorus decresii*). We use literature sources as well as
histological sections to identify and delineate, in three dimensions, the cell regions and fiber tracts visible in this model. This atlas has acted as a guide for measuring and analyzing brains in the subsequent chapters, and as a template with which to automate brain measurements across many individuals from multiple species.

Section 2: Evolutionary patterns in dragon brain structure

Chapter 4

Two models have been proposed to explain the patterns observed in evolutionary changes in brain morphology: the concerted model and the mosaic model of brain evolution. It is now well understood that both models are relevant in explaining brain evolution but the relative influence of each mode on brain structure varies between vertebrate groups. It remains unclear what factors favour concerted or mosaic brain evolution. In this chapter, we found evidence for both mosaic and concerted brain evolution in dragon lizards. Brains showed a pattern of concerted brain evolution with respect to the morphological characters. In contrast, they showed a pattern of mosaic brain evolution with respect to ecological and life history characters.

Chapter 5

The role of sexual selection in altering brain organisation and structure over evolutionary time is poorly understood. In this chapter we compare the brains of species under strong and weak sexual selection. Males belonging to species that experience strong sexual selection had a larger medial preoptic nucleus and a smaller ventromedial hypothalamic nucleus. Conversely, females did not show any obvious variation in these brain regions. The medial preoptic nucleus controls male reproductive behaviour while the ventromedial hypothalamic nucleus controls female reproductive behaviour and is also involved in male aggression. Therefore, the primary brain nuclei underlying reproductive behavior evolve in a mosaic fashion in dragons, differently between males and females, likely in response to the strength of sexual selection.

Collectively, these findings describe in detail the structure of an agamid brain and some of the ways in which that structure has changed through evolution. In doing so, these results have highlighted both how labile the brain can be in response to evolution, and how conserved brain structure is in general. Lizards, and reptiles in general, are the most understudied vertebrate group in neuroscience, but there is huge potential for discovery in this field.
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Section 1

Development of the framework and tools necessary for the study of the dragon brain
Chapter 1

Structure and function of the lizard and snake brain: recent advances and shifting perspectives
Abstract

Vertebrate animals are remarkably diverse and this is also reflected in their complex brain anatomy. Among vertebrates, reptiles have lagged far behind birds, mammals, fishes and amphibians in neurobiological research. Nonetheless, in the past twenty years there have been significant advances in our understanding of the neurobiology of reptiles, particularly among squamates (lizards and snakes). As the neurobiological research into squamates grows, it is increasingly apparent that they hold great potential for exciting discoveries in neuroscience, particularly in evolutionary neuroscience and the links between brain, behaviour and cognition. Here, we begin by synthesising all the original research published on squamate neuroscience in the past twenty years, particularly since the last major review (1). We synthesize the information for each brain region encompassing cellular structure, neurochemistry, connectivity, function, and any other pertinent information about that region including new advances. Where we have found conflicting viewpoints in the literature, we attempt to offer resolution. We also address the ambiguities present in reptilian neuroscience terminology and suggest a new protocol to provide consistency and to bring reptilian neuroscience in line with avian and mammalian systems. Our over-arching aim is to create a single resource on squamate brain neurobiology for any researcher interested in knowing the current state of the published literature. We also highlight what is unique about squamate brains and identify gaps that remain in our understanding of these systems. Finally, we provide a framework for future studies that includes exciting new and unanswered questions about squamate brain evolution, structure and function. We suggest that squamate reptiles are a valuable model system for advancing our understanding of the evolution of the brain because of their evolutionary history, relationship to mammals and birds, and their amazing diversity of lifestyles, social systems and reproductive tactics, all of which bring influence to bear on the evolution of the brain.
Abbreviations

NMDA: N-methyl-D aspartate

CRMP: Collapsin response mediator protein

PSA-NCAM: Polysialylated-neural cell adhesion molecule

GABA: Gamma-Aminobutyric acid

PCNA: Proliferating Cell Nuclear Antigen
1 Introduction

Neuroscience is one of the largest and most lucrative areas of modern biological research, yet, among vertebrates, the vast majority of neurobiological studies have focused on just four model organisms: mice, rats, monkeys, and humans. Furthermore, the number of neuroscientists working with these model systems greatly exceeds the number working with all other vertebrates combined (2-4). This intense research on four model systems promotes rapid advancement of our understanding of normal brain function and the neurobiological disorders that disrupt it. As a result, the vast majority of neuroscience research is devoted to two things: basic neural function and the neuropathology of neurological disorders. Both these areas of research are immensely broad and important, and they drive rapid technological and methodological advances.

At the same time, there are numerous avenues of research that cannot be examined using such a restricted set of model species. By embracing novel systems we have gained insights into brain function that would otherwise have been difficult, if not impossible, to achieve. For example, echolocation in bats and asymmetric ears in owls provide unique models with which to discern the functional organization of auditory processing areas in the cortex, thalamus and brainstem (5, 6). More recently, voles have emerged as an ideal system in which the neural basis of mating systems can be examined (7). In particular, the neural underpinnings of monogamy, an area of interest to human biology, is a focus of this research. Tree shrews are a useful animal model in two disparate fields: the neurological effects of stress and the organization of visual circuitry (8, 9). Despite this expansion, however, there is still very limited and narrow phylogenetic coverage, and therefore phylogenetic bias, within the organisms used in neuroscience. In order to understand fundamental brain function and evolution, we must cast a wider net. This work has started. Neuroscience, in addition to focussing on a wider range of mammal species, is increasingly focussing on birds. Vocalization and vocal learning are studied extensively using songbirds (10). And the domestic chicken is emerging as a useful model organism in developmental neuroscience (11-13). Quantifying variation in brain structure and function across many species spread across the entire animal phylogeny is an important avenue of research for understanding the basic principles of brain evolution and function.
From an evolutionary perspective, birds can be viewed as an extremely species-rich clade of reptiles. The brains of ‘traditional’ reptiles (lizards, snakes, turtles, crocodiles and rhyncocephalians) are poorly understood even though they are a highly species-rich group. In 1978, Paul MacLean introduced an edited volume on the behaviour and neurobiology of lizards by saying “those who are familiar with lizards realize that there are more reasons for conducting research on these animals than there are investigators prepared to do the necessary work” (14). Unfortunately, this statement may still be true, although there have been some important advances in the interim. By increasing our focus on reptiles, we have an opportunity to close this gap and improve our overall understanding of vertebrate brain structure and evolution. Understanding the reptile brain will not only enable us to better understand the link between birds and mammals from a neurobiological perspective, but there is great potential for advancing neuroscience theory in general. For example, unlike in eutherian mammals, the squamate brain is naturally “split,” in that there is no corpus callosum, and therefore communication between the left and right hemispheres is limited (15). This is highly advantageous in neuroscience research because it allows for the unilateral manipulation of one hemisphere while leaving the other as an unmanipulated control (16). That the two hemispheres communicate very little with each other also provides insight into the natural laterality of brain function. For example, both aggressive and predatory behaviours are lateralized in the squamate brain (17-22). Although eutherian mammals are the only vertebrate group that has a corpus callosum, all the standard neuroscience models belong to this group. Reptiles, and in particular squamates, present the simplest, most convenient system in which to use this natural “split” as a control.

Squamate reptiles comprise all lizards and snakes and together they represent the vast majority (96.4%) of the world’s extant reptiles. Of the neurobiological research published to date, most has focussed on squamates. Turtles and crocodiles, the other two main extant groups of reptiles, are comparatively species-poor groups that are usually large, long-lived and difficult to manipulate, making them less desirable as model organisms. In contrast, squamates are a diverse group of almost 10 000 species, making them about as species-rich as birds and more so than mammals (23). They are ubiquitous, easy to manipulate, and have comparatively stereotyped behaviours that make them easy to study. Most are also small, have short life cycles, and many species are easy to breed. The squamates vary incredibly in terms of their ecology, morphology, and behaviour. These areas of focus for biologists provide a unique opportunity to link...
with neurobiological research. For example, there is increasing interest in cognition in squamate reptiles (24). The variation in cognitive abilities displayed amongst closely related species provides a unique and repeatable opportunity to study the evolution of cognition (24). Furthermore, recent advances in resolving squamate phylogenetic relationships provides an important foundation for comparative cognition and neuroscience studies.

The last major review of the reptilian brain was published as a book chapter in 1998 (1). In it, Donkelaar synthesized and summarized over 100 years of advances in reptile neuroscience, up until 1995. His review provides an overview of all the major brain divisions. In particular, his syntheses of the rhombencephalon and cerebellum largely represent the current state of knowledge, because only limited research has since been conducted on these regions. Donkelaar’s discussion of the rhombencephalon is particularly thorough, describing in detail the connection patterns of its cranial nerves, somatomotor nuclei and sensory and motor relay stations. The cerebellum in reptiles, as in all animals, is a region of motor coordination and control. In squamates, in contrast to other vertebrates, the cerebellum is everted, either lying over the optic tecta or standing vertically, 90° to the rostral-caudal axis (Figure 1). Squamates vary significantly across species in cerebellum size and shape, along with the size and shape of most of the brain, including the olfactory bulbs, telencephalon, and optic tecta (Figure 1). Some of this variation is straightforward, such as the reduction in cerebellum size in snakes and limbless lizards (1), however much of it remains poorly understood.
Figure 1. Variation in overall brain structure within the squamates is immense.

Brains are illustrated from the top (left) and side (right). Species depicted are: (A) Tuatara, *Sphenodon punctatus*, (B) Black-and-white Tegu, *Tupinambis merianae*, (C) Green Anole, *Anolis carolinensis*, (D) Water Monitor, *Varanus salvator*, (E) Reticulated Python, *Python reticulatus*, and (F) Grass Snake, *Natrix natrix*. Brains are standardized in size to midline telencephalic volume to emphasize relative variation in brain region size and shape.
In the intervening years since Donkelaar’s 1998 review, there have been major advances in both our knowledge of reptilian nervous systems and the methods used to study them. Rapid advances in methodology and technology driven by medical necessity have enabled research that was unavailable twenty years ago. This has encouraged the use of a variety of reptile species as model organisms with which to study brain evolution and the links between cognition, behaviour, ecology and neurobiology (Figure 2). Interestingly, studies of lizard cognition have experienced a recent surge. Lizards have been demonstrated to show behavioural flexibility (25), are capable of relatively rapid spatial learning (26), and the first demonstration of social learning in a lizard was recently published (27). In order to make appropriate links between cognition and brain structure and organisation, we need a good understanding of our state-of-knowledge of the squamate brain and need to bridge the gap between what we know about the squamate brain and that of other vertebrates.

Here, we have summarized all the research published on the structure and morphology of squamate brains since the last major review by Donkelaar (1998). We have attempted to synthesize the information for each brain region encompassing cellular structure, neurochemistry, connectivity, function, and anything else known about that region. Where we have found contradictions and synonyms in the literature, we have outlined these in detail and attempted to offer resolutions. However, this is not a region-by-region survey. Only those brain regions that have had significant advances since 1995 are covered (Table 1). There are numerous nuclei in the squamate brain that are small and very poorly known. Many of these regions have been given scant, if any, attention in the literature since Donkelaar (1). We have omitted from our review nuclei where, by and large, the current state of knowledge is presented in Donkelaar (1). Our goal here is to create a single resource that will provide a starting point in understanding the current state of squamate neurobiological research in general and for any particular region of interest. We end by summarising what is unique about squamate brains and by highlighting gaps that remain in our knowledge. Specifically, we provide a framework for future studies that includes new and unanswered questions about squamate brain evolution, structure and function and we link this to squamate behaviour and cognition.
Figure 2. Neuroscience papers published on squamate reptiles since 1995. These papers study the neurobiology of a broad cross-section of the squamate phylogeny, laying the foundation for diverse research in evolutionary neuroscience using a comparative approach. Squamate Families used for neuroscience research are indicated with a solid square (n). Phylogeny modified from Pyron et al. (23).
Table 1. Brain regions covered in this review, with proposed standardized abbreviations (section 6.1.2.2).

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2 Telencephalon

2.1 Cortex

The squamate cortex is made up of four discrete areas. All four are relatively consistent in structure, with three distinct layers. The superficial plexiform layer is the most dorsal and medial, consisting of many axon terminals and dendrites with relatively few neuronal cell bodies. The cell layer sits just below the plexiform layer, with many tightly packed neurons. The inner plexiform layer sits between the cell layer and the lateral ventricles, and is similar in structure to the superficial plexiform layer, with many axonal and dendritic processes and relatively few neurons. A scaffold of glial fibres supports this structure. Radial glial cells in the ependymal layer of the lateral ventricle send their projections radially through the three layers of the cortex, providing structural support to the neurons (28, 29).

2.1.1 Medial Cortex

Cytoarchitecture and Cellular Neurochemistry

The cell layer of the medial cortex contains the vast majority of its projection neurons. Projection neurons are generally pyramidal, round or fusiform neurons (30). Between four and eight types of projection neurons have been identified in the lizard cortex (31-35). These neurons are excitatory (glutamatergic) and have long projection axons. All medial cortex projection neurons send their axons ventrally to the inner plexiform layer. There, they bifurcate and enter the alveus, a layer of fibres running horizontally along the boundary between the inner plexiform layer and the ependymal layer of the lateral ventricle (30). Projection neurons extend their apical and basal dendritic branches into one or both plexiform layers (30). Some projection neurons weakly express the calcium-binding protein calbindin (36). The cellular layer also expresses the kinase regulatory subunit R1a, NMDA receptors, dopamine receptors (rostrally), neuropeptide Y and aromatase (37-41).

Interspersed among the projection neurons of the cell layer as well as throughout the two plexiform layers are small inhibitory neurons. Thirteen types of inhibitory interneuron have been identified by morphology, and their distribution throughout the
different layers of the medial cortex is heterogeneous (42-44). Calcium binding proteins are expressed much more intensely in interneurons than in projection neurons (36). Parvalbumin-expressing interneurons seem to be present in the medial cortex of Podarcis but not Psammomromus (43). Some interneurons express neurotransmitters nitric oxide, neuropeptide Y or somatostatin (45-47). Some proteins, such as nitric oxide synthase and parvalbumin, are distributed homogeneously throughout the medial cortex, suggesting that they are expressed by multiple morphotypes of interneuron (44, 46). Others are highly localized, for example calretinin is expressed mostly in the outermost third of the superficial plexiform layer (43). By contrast, somatostatin is mostly expressed in the inner plexiform layer (45). Both plexiform layers may weakly express receptors for the vitamin D₃ (48). The distribution of acetylcholine expression throughout the three layers of the medial cortex appears to vary substantially among species (49).

Connectivity and Fibrous Neurochemistry

The medial cortex receives input to both the superficial and inner plexiform layers. The densest source of input is the fibre plexus that occupies the outer third of the superficial plexiform layer. The lateral cortex projects to this plexus caudally, while the dorsolateral thalamic nucleus projects more rostrally (50). Projections from the dorsolateral thalamic nucleus are bilateral (49). The dorsomedial cortex, nucleus of the diagonal band, mammillary nuclei and supramammillary nucleus also project to the medial cortex (51, 52). At least some of these dendrites, caudally, express dopamine receptors (53). The medial cortex receives nitric oxidergic input into the inner third of the superficial plexiform layer (46). Scattered orexin inputs and additional projections from the lateral cortex are received in the inner plexiform layer (50, 54). Two rows of fibres, one on either side of the cell layer, contain androgen receptors (55, 56). In this case, aromatase expression in the medial cortex may act as a testosterone antagonist, converting testosterone into estrogen, for which the medial cortex does not express receptors (57). Projections expressing acetylcholine terminate in the superficial and inner plexiform layers (58). The medial cortex also receives some neuropeptide Y, orexin and cholecystokinin innervation (54, 59, 60).

The most prominent medial cortex projections are to the dorsomedial and dorsal cortices, as well as to the contralateral medial cortex. The anterior septal nucleus receives bilateral projections from the rostral and vertical regions of the medial cortex,
while the lateral septal nucleus receives only ipsilateral input from the caudal horizontal portion (52). The medial cortex also sends projections to the dorsal part of the dorsal septal nucleus and the dorsolateral septal nucleus (52).

Functional Correlations

The medial cortex is often compared to the mammalian dentate gyrus due to similarities in neurochemistry and connectivity, primarily its massive projections to the dorsomedial cortex, the putative reptilian hippocampal CA3 region (36). However evidence for a functional equivalency between the mammalian dentate gyrus and the squamate medial cortex is scant and contradictory. The medial cortex is larger in an active forager compared to a sit-and-wait predator, suggesting that it is larger in species that have higher navigational demands (61). In a lizard, medial cortex volume was not correlated with territory size, however in a snake where males have larger home ranges than females it was found to be larger in males (62, 63). In a comparison between territorial and non-territorial individuals of the same species, neuronal recruitment is higher in the territorial individuals (64). In captive lizards, neuronal recruitment is also correlated to enclosure size, which was used as an artificial proxy for home range size (64). However, these results are confused by the fact that lizards housed in larger enclosures had significantly higher locomotor activity levels, and another study was unable to replicate this result in another species (64, 65). The medial cortex is larger in translocated rattlesnakes compared to individuals placed back at their point of capture, presumably due to the higher navigational demands placed on the former individuals (66). However, lesioning the medial cortex did not inhibit the acquisition of a maze navigation task, though it did prolong learning (67). In these experiments it was expected that medial cortex lesions would inhibit task acquisition by blocking spatial learning and memory. However, medial cortex lesions appeared to inhibit pliancy in search strategies rather than memory of the task itself. A caveat of this study is that intact controls did not seem to use a spatial navigation strategy to recall the task, and in fact the strategy they used could not be identified. Further studies with species that use well-characterized navigational strategies are warranted to examine the medial cortex’s role in spatial memory.

In another study, a different kind of pliancy was examined. *Thamnophis* has elevated levels of plasma glucocorticoids during the breeding season, and a switch from
reproductive motivation to appetitive motivation once the breeding season ends correlates with a drop-off in plasma glucocorticoid levels. Administration of a glucocorticoid synthesis inhibitor also induces a switch from reproductive to appetitive motivation (68). The reduction in plasma glucocorticoids and behavioural shift correlates with an increase in neuropeptide-Y expression in the medial cortex (68).

There is also limited evidence that the medial cortex is involved in aggressive behaviour. Lizards shown videos of aggressive behaviour had heightened metabolic activity in the medial cortex, and this was correlated with heightened metabolic activity in a range of nuclei dubbed the “limbic aggression network” (69). Stress is often related to aggression, as situations that require an aggressive response are by and large stressful. A low dose of corticosterone increases serotonin activity in the medial cortex, however a high dose does not (70). Restraint stress reduces serotonin and noradrenaline levels in the medial cortex (71). After an aggressive interaction between two male conspecifics, the concentration of dopamine in the medial cortex is reduced (72).

**Neurogenesis and Neural Repair**

Neurogenesis is constitutive in the squamate medial cortex, even in adulthood (29, 73). In some species, the medial cortex has the highest constitutive neurogenesis rate of any telencephalic region, and may be even higher in adults than in juveniles (73-75). Neurons are generated from radial glial cells, and related cells called tanycytes, which act as neuronal stem cells in the ependymal layer of the lateral ventricle (28, 73, 75, 76). From there, they migrate along a scaffold of radial glial cells to the cell layer and the plexiform layers (73, 75, 77). Therefore, all neurons are likely to be the same when they are generated in the ependymal layer, and specialize once they are incorporated into the cortical layers (78). There may even be further cell division of neurons after reaching the cell layer (77). Newly generated neurons integrate completely into the existing neuronal network (73). Cells in the medial cortex express PCNA, CRMP-4, PSA-NCAM, Tbr-1 and doublecortin, proteins known to be markers of neurogenesis (79-81). The number of neurons in the medial cortex increases as much as five-fold over the normal lifespan of the lizard (29).

In lizards that live in temperate climates, adult neurogenesis rates are at their highest in spring and summer, and are virtually absent in winter (29, 82). Low temperature inhibits
neuronal migration from the ependymal layer, but whether low temperature also affects the rate of constitutive neurogenesis may be species-dependent (83, 84). In contrast, short photoperiods decrease the rate at which neurons are generated from radial cells in the ependymal layer but increase the rate of neuronal migration from the ependymal layer to the medial cortex (84). Therefore, the shortening of the photoperiod as summer transitions to fall may prepare the medial cortex for the oncoming temperature reduction by moving new neurons to the cortex and preventing the creation of more neurons before neuronal migration is halted. Neurogenesis may also be inhibited by testosterone, but only in the summer (65). Furthermore, temperature during incubation also affects the development of the medial cortex. Lizards incubated at warmer temperatures have a higher density of neurons, while lizards incubated at cooler temperatures have larger neurons.

Chemical lesion of the medial cortex induces dramatic increases in the rate of neurogenesis in the ependymal layer (52, 73, 77, 85). Cellular debris is transported along the radial glia scaffold to the ventricular zone, where radial glia cells phagocytize the debris (77, 86). Microglia are not involved in the initial stages of lesion repair and disappear from the lesioned area (87). However, somatostatinergic interneurons are resistant to chemical lesion and somatostatin expression is upregulated post-lesion (77, 84). This subgroup of interneurons may be involved in the control of reactive neurogenesis during the early stages of repair (77). As the repair progresses, microglia reinvade the damaged area and may be involved in downregulating neurogenesis later in the reparation process (77, 87). Microglia are also involved in the final stages of debris removal through phagocytosis (87). Though the time it takes to repair the medial cortex varies among species, reparation is complete and no evidence of the lesion remains (29, 73, 74, 77, 88). Following lesion, neurogenesis in the ventricular zone is upregulated even under winter temperatures, but migration to the cortex is inhibited and the new neurons accumulate in the ventricular zone (84).

2.1.2 Dorsal Cortex

Cytoarchitecture and Cellular Neurochemistry

The basic structure of the dorsal cortex is similar to that of the medial cortex, with glutamatergic projection neurons localized to the middle cell layer and GABAergic...
interneurons scattered through all three layers. Like the medial cortex, projection neurons express small amounts of calbindin, while interneurons express calbindin, calretinin, and parvalbumin (36, 43). In contrast to the medial cortex, interneurons in the dorsal cortex co-express both calretinin and parvalbumin (43). These neurons are found in the deep plexiform layer and are more abundant caudally and medially (43).

Between four and ten morphotypes of neuron have been identified in the dorsal cortex, depending on species (32-35, 89). Cells that are likely projection neurons express androgen receptors on their dendritic arbours in both plexiform layers (55, 90). In contrast to the medial cortex, the dorsal cortex expresses estrogen and progesterone receptors and only weakly expresses kinase regulatory subunit R1α (41, 57, 91). It also expresses acetylcholine in all three layers (58). The inner plexiform layer interneurons express somatostatin, corticotropin-releasing hormone, and, weakly, vitamin D3 receptors (45, 48, 92, 93). The dorsal cortex cell layer expresses aromatase, 5α reductase, dopamine receptors (rostrally), androgen receptors, progesterone receptors, serotonin and neuropeptide-FF (38, 39, 90, 91, 94-96). While a study in Gecko found significant expression of nitric oxide in the cell layer of the dorsal cortex, a study in Cnemidophorus found none (47, 97). Neuropeptide Y has long been thought to be restricted in the cortex to the medial and dorsomedial cortices, however a recent study found neuropeptide Y expression in the dorsal cortex of a snake (37, 68).

Connectivity and Fibrous Neurochemistry

The dorsal cortex receives diverse sensory input including projections from the anterior olfactory nucleus, anterior dorsal ventricular ridge, spherical nucleus, nucleus of the diagonal band, dorsal lateral geniculate nucleus, dorsolateral thalamic nucleus, mammillary nuclei and supramammillary nucleus (51, 59, 98, 99). Furthermore, it receives input from the contralateral dorsal cortex and the ipsilateral medial and dorsomedial cortices (76, 100). Projections from the lateral cortex to the medial cortex pass through the superficial plexiform layer of the dorsal cortex and some axons synapse in the dorsal cortex (50). The dorsal cortex receives input from terminals expressing orexin, cholecystokinin, galanin, dopamine, serotonin, noradrenalin and acetylcholine (45, 54, 58, 59, 101).
The dorsal cortex projects to many regions involved in motor integration and motivation. These include the medial cortex, dorsomedial cortex, septum, preoptic area, diagonal band of Broca, ventral anterior and dorsolateral amygdaloid nuclei, dorsal striatum, accumbens nucleus, olfactostriatum, periventricular hypothalamus, dorsal hypothalamic area, lateral hypothalamic area and ventromedial hypothalamus (52, 99, 102). The dorsal cortex also sends projections to sensory regions such as the main and accessory olfactory bulbs, lateral cortex, and lateral anterior dorsoventricular ridge (99).

The dorsal cortex projects heavily to the lateral, medial and dorsal septal nuclei (52, 102). The inferior septal nucleus receives less prominent projections, but seems to receive projections in a topographically organized fashion (52). Uniquely, the dorsal cortex sends both glutamatergic projections from the cell layer and GABAergic projections from the inner plexiform layer to the septum (52, 102).

Functional Correlations

Like medial cortex lesions, dorsal cortex lesions do not abolish learning of a maze task, but rather increase the time it takes to learn the task (67). However, lizards with dorsal cortex lesions learn the maze task faster than those with medial cortex lesions, and do not show the same pliancy issues present in lizards with medial cortex lesions (67). Also like the medial cortex, the dorsal cortex is larger in active-foraging lizards compared to sit-and-wait predators (61). The dorsal cortex is larger in territorial male lizards than in non-territorial males (62). However, it is smaller in lizards housed in large enclosures compared to lizards housed in small enclosures (65) and translocation did not affect the volume of the dorsal cortex as it did the medial cortex (66). Although the function of the dorsal cortex has been a source of debate and controversy, it is not generally believed to be involved in memory and navigation.

The dorsal cortex is believed to be an area of sensorimotor integration and processing, by and large due to its extensive and varied afferent and efferent connections (44). However, some functional evidence exists. For example, stimulation of the retina with light flashes evokes electrical activity in the contralateral dorsal cortex (103). The dorsal cortex also appears to play a role in the mitigation of aggressive behaviour. Serotonergic projections to the dorsal cortex are thought to be involved in mediating aggressive responses, as increased serotonergic activity in the dorsal cortex correlates
with aggressiveness (101). Serotonergic activity is also positively correlated with stress (104). In addition to serotonergic input, the dorsal cortex receives dopaminergic and noradrenergic input (101). After an aggressive interaction, noradrenergic activity is higher in more aggressive individuals, while dopaminergic activity is higher in less aggressive individuals (101).

**Neurogenesis and Neural Repair**

Neurogenesis in the dorsal cortex is similar in almost all regards to neurogenesis in the medial cortex (section 2.1.1), however doublecortin is not expressed (79). As in the medial cortex, neurogenesis in the dorsal cortex is constitutive and occurs throughout the lifespan (73). One key difference is that in the dorsal cortex, under normal conditions, only interneurons are generated and they remain in the inner plexiform layer (73, 75). No new neurons are transported to the cell or superficial plexiform layers. However, after lesioning, all three cortical layers are repaired with new neurons, including new projection neurons (105).

**2.1.3 Dorsomedial Cortex**

**Cytoarchitecture and Cellular Neurochemistry**

The dorsomedial cortex has long been considered homologous to the mammalian CA3 region of the hippocampus due to many similarities including the expression of NMDA receptors on projection neurons (40, 44). Like other cortical regions, projection neurons are glutamatergic, larger than in other cortical regions, located in the cell layer and send their axons to the alveus, while interneurons are GABAergic and have short axons that remain in the dorsomedial cortex (36). In various species, between five and ten different types of neurons have been identified (32-35, 106). Neurons in the cell layer express progesterone receptors, aromatase, NMDA receptors, and the R1α regulatory kinase subunit (39-41, 91, 107). As with the dorsal cortex, calretinin-expressing interneurons are restricted to the inner plexiform layer, while parvalbumin-expressing interneurons are found throughout all three layers (43, 44). Interneurons in the inner plexiform and cell layers express somatostatin and neuropeptide Y while interneurons in all three layers express nitric oxide and acetylcholine (37, 45, 46, 58, 92). Nitric oxide is expressed in *Psammodromus* but not in *Gecko* (46, 97).
Connectivity and Fibrous Neurochemistry

The dorsomedial cortex receives its strongest input in the form of glutamatergic projections to both plexiform layers from the medial and dorsal cortices (44, 73). The lateral cortex projects to the fibre plexus in the superficial plexiform layer (50). It also receives projections expressing nitric oxide to the superficial and inner plexiform layers, calcitonin-gene-related peptide to the inner plexiform layer, and cholecystokinin to the cell layer (46, 59, 108). Dopaminergic, noradrenergic, and serotonergic projections are received from brainstem nuclei (101). The dorsomedial cortex has limited output. It projects bilaterally to the dorsal and medial cortices, and to the contralateral dorsomedial cortex (52).

Functional Correlations

In lizards, the dorsomedial cortex is most strongly associated with the establishment and retention of social structure (109). Prior to an aggressive interaction that will determine which of two male lizards will be socially dominant and which will be socially subordinate, there is no difference in serotonergic activity in the dorsomedial cortex between the two lizards (110). During the conflict, serotonergic activity is increased in both male lizards (111, 112). After the conflict, both serotonergic and noradrenergic activity are elevated in the lizard who lost the conflict, and is now the subordinate male, but not the lizard who won the conflict and is the new dominant male (101, 113-115). Once the two individuals have already established their dominance hierarchy, baseline serotonergic activity decreases in the dominant male. Lizards in established hierarchies do not display aggressive behaviours towards each other, and the reduction in serotonin may be more involved in stress relief than in aggression (109). Lizards in established dominant-subordinate social pairs show enhanced NMDA receptor expression (40). Systemic corticosterone injection enhances NMDA expression, as does stressful social interaction (40). Increased stress, for example from physical restraint, also increases serotonergic activity, as does corticosterone administration systemically or directly to the dorsomedial cortex (112, 116). Conversely, injection of GABA into the dorsomedial cortex inhibits serotonin release (112).
Dopaminergic activity in the dorsomedial cortex may also be associated with social interaction in lizards. Following a conflict to establish a dominance hierarchy between two unfamiliar individuals, dopaminergic activity is suppressed in the loser, who becomes the subordinate individual (101). Once the social hierarchy is established, baseline dopamine levels in the dorsomedial cortex decrease as compared to unestablished male lizards, and this effect is more pronounced in dominant male lizards compared to subordinate male lizards (116). In an interaction between a dominant lizard and a subordinate lizard with an already well-established dominance hierarchy, the dominant lizard shows an decrease in dopaminergic activity and the subordinate lizard shows an increase in dopaminergic activity (117). A lizard’s social position also influences its dopaminergic response to stress. Following restraint stress, dopaminergic activity decreases in subordinate but not dominant lizards (116).

Neurogenesis and Neural Repair

Neurogenesis is constitutive in the dorsomedial cortex as in other cortical regions (29, 73). There is less neurogenesis in the dorsomedial cortex compared to other cortical regions, and there does not seem to be any seasonal variation in neural proliferation (82). Effects of incubation temperature on neuronal development are also the same as in the medial cortex (section 2.1.1).

Repair post-lesion does not seem to be complete as in other cortical regions. After lesion new neurons seem unable to migrate to the cell layer, instead forming an abnormal cell plate in the inner plexiform layer and there differentiating into projection neurons (88). The absence of CRMP-4 from the dorsomedial cortex and the absence of PSA-NCAM outside the inner plexiform layer may be involved (76, 81). Whether these neurons are still capable of reforming normal connectivity is unknown.

2.1.4 Lateral Cortex

Cytoarchitecture and Cellular Neurochemistry

The lateral cortex is composed of two parts. In the dorsal region projection neurons are larger and loosely arranged, and in the ventral region they are smaller and more densely packed (50). Three types of projection neurons and one interneuron have been
identified, the least diversity of neuronal morphotype in the cortex (33-35, 118). Projection neurons in the lateral cortex stain weakly for calbindin, and this is the only cortical region with projection neurons that express calretinin (36, 43). Interneurons also express calbindin and calretinin (36, 43). Aromatase, 5-α reductase, dopamine receptors, serotonin receptors, androgen and estrogen are expressed in the cell layer (39, 53, 57, 90, 94, 95). Corticotropin releasing hormone is expressed by the interneurons of the inner plexiform layer (93). Both plexiform layers contain nitric oxide expressing cells, which are localized close to the cell layer (97). The lateral cortex weakly expresses the following proteins: vitamin D3 receptors and neuropeptide-FF in the cell layer; and acetylcholine and protein kinase regulatory subunit R1α in all three layers (41, 48, 58, 96). Neuropeptide Y has long been thought to be restricted in the cortex to the medial and dorsomedia cortexes, however a recent study found neuropeptide Y expression in the lateral cortex of a snake (37, 68).

**Connectivity and Fibrous Neurochemistry**

The lateral cortex is the only cortical recipient of bilateral projections from the main olfactory bulbs (119). These terminate in the fibre plexus that forms the outer part of the superficial plexiform layer (50). In contrast, the spherical nucleus projects to the inner plexiform and cell layers (100). Although there is no overlap between the olfactory and spherical fibres, pyramidal neurons in the lateral cortex cell layer extend dendrites to both plexiform layers and could potentially form synapses with both projections (100). The lateral cortex also receives projections from the anterior olfactory nucleus, diagonal band of Broca, amygdala, dorsolateral thalamic nucleus, paraventricular nucleus of the hypothalamus, and triangular area (50, 98). Projections from the brain stem innervate the lateral cortex with serotonin and norepinephrine, while dopaminergic projections reach only the ventral region (50). The lateral cortex also receives scattered orexin and calcitonin gene-related peptide terminals to the inner plexiform and cell layers (54, 108). Nitric oxide and neuropeptide-FF fibres run in both plexiform layers (96).

The lateral cortex projections are specific to the dorsal or ventral regions. The dorsal region projects mainly to both plexiform layers of the caudal medial cortex (50). Other regions that receive projections from the dorsal region include the contralateral lateral cortex, dorsal cortex, dorsomedial cortex, striatoamygdaloid transition area, accumbens nucleus, olfactostriatum, and the granular layer of the olfactory bulb (50). The ventral
region projects bilaterally to the external & dorsolateral amygdaloid nuclei and the anterior olfactory nucleus; ipsilaterally to the medial amygdala; and contralaterally to the lateral cortex (50). Outside of the telencephalon, the ventral region of the lateral cortex sends weak projections to the dorsal hypothalamic area (120).

**Functional Correlations**

The lateral cortex is considered to be the main olfactory region of the squamate telencephalon due to the massive and unique projections it receives from the main olfactory bulbs (36, 50). As the lateral cortex also receives projections from the spherical nucleus, it may represent a region where vomeronasal information and olfactory information are integrated (100, 121). It is less pronounced in a sit-and-wait predator than an active forager (61). There is an increase in neuropeptide-Y expression at the end of the breeding season, when snakes go from reproducing to feeding (68). This increase can be induced by blocking glucocorticoid synthesis (68).

**Neurogenesis and Neural Repair**

Neurogenesis is constitutive in adults and proceeds essentially as per the medial cortex, including seasonal variation in proliferation and migration (section 2.1.1). More new neurons seem to be generated in males than females (122).

**2.2 Olfactory Bulb and Olfactory Peduncle**

Though the olfactory bulb extends well in front of the forebrain along a stalk known as the olfactory tract, it is derived from the ventral pallium during development and is part of the telencephalon. The olfactory bulb is divided into two regions: the main olfactory bulb anteriorly and the accessory olfactory bulb posteriorly. The olfactory bulb generally consists of six architectonic layers. From dorsal to ventral they are the layer of the olfactory nerve fibres, the glomerular layer, the outer plexiform layer, the mitral layer, the inner plexiform layer, and the granular layer (75). A second granular layer, called the external granular layer, may be present below the glomerular layer. It is often absent or indistinguishable from the glomerular layer. However, one study found no laminar organization in the main olfactory bulb and only two layers to the accessory
olfactory bulb: a dorsal plexiform layer and a ventral cellular layer, suggesting that cytoarchitectural organization in this nucleus may vary by species (85).

In the mammalian literature, the olfactory bulbs together with the olfactory peduncle are traditionally referred to as the rhinencephalon. We have never encountered this term in the squamate neurobiological literature and will avoid using it here.

2.2.1 Main Olfactory Bulb

Cellular Neurochemistry

The inner granular and internal plexiform layers contain light-staining aromatase expressing cells, while the mitral and external plexiform layers contain dark-staining aromatase expressing cells (39). Dopamine receptors are expressed by cells in the mitral layer, and to a lesser extent in the external plexiform, glomerular, and internal plexiform layers, while tyrosine hydroxylase is mainly expressed in the glomerular layer (53). A few cells expressing nitric oxide are scattered around the periphery of the olfactory bulb (97). The olfactory bulb shows the highest aromatase activity anywhere in the squamate brain and conversion of testosterone to estrogen is greater in the spring compared to the fall (123). Tbr-1 and doublecortin are expressed, but not co-expressed in the same neuronal cell bodies (79).

Connectivity and Fibrous Neurochemistry

The main olfactory bulb receives its principle projections from the olfactory sensory neurons of the nostril and it is the first relay for all olfactory information entering the brain (124). In addition, the retrobulbar formation, diagonal band of Broca and the ventral lateral cortex send projections back to the main olfactory bulb (119). Cholinergic projections to the olfactory bulb originate from the nucleus of the diagonal band and the substantia innominata (102). Fibres co-expressing tyrosine hydroxylase and neuropeptide-FF are found in the internal granular layer (53, 96). Scattered orexinergic and thyrotropin-releasing hormone terminals also project to the internal granular (54, 125). In contrast, fibres expressing gonadotropic-releasing hormone are diffused through all layers except the internal granular layer (22).
The olfactory bulb sends massive bilateral projections to the superficial plexiform layer of the lateral cortex, and additionally projects to the anterior olfactory nucleus, external and ventral anterior amygdalar nuclei, olfactory tubercle, and the diagonal band of Broca (102).

**Neurogenesis**

The olfactory bulb is the site of perhaps the most intense recruitment of neurons some species of squamates, though in others recruitment is stronger in the medial cortex (29, 73, 75, 78). Neurogenesis does not occur in the olfactory bulb (73). Instead, neurons are generated along the ependymal layer of the lateral ventricle in the rostral forebrain (126). They then undergo the only long-distance migration of neurons known in vertebrates (73). These neurons travel along a highly restricted migratory route known as the rostral migratory stream from the rostral forebrain through the olfactory tract to the olfactory bulb (73, 126). There, they are integrated mostly into the granular cell layer (73, 75). Some new neurons are integrated into the mitral and glomerular layers (75). Neurogenesis in the ependymal layer of the lateral ventricle is greater in the spring and summer compared to the fall and winter (82, 83). Transport along the restricted migratory route slows in the summer and neurons reach the olfactory bulb primarily in the other three seasons (78). Males seem to have a higher rate of neuronal recruitment, mirroring the lateral cortex to which the main olfactory bulb has its most significant projections (122).

**2.2.2 Accessory Olfactory Bulb**

**Cytoarchitecture and Cellular Neurochemistry**

Rostrally, the accessory olfactory bulb usually consists of the six layers of the olfactory bulb, however caudally only three layers remain: the external plexiform, mitral, and internal granular layers (39, 75). Aromatase is expressed prominently in the external plexiform and mitral layers, and less prominently in the internal granular layer (39). Dopamine receptors are expressed mostly by cells in the mitral layer, but are also found in the external plexiform, glomerular, and internal plexiform layers, while tyrosine hydroxylase is expressed by the glomerular layer (53). Some neurons also express nitric
oxide synthase (127). As in the main olfactory bulb, Tbr-1 and doublecortin are expressed, but not co-expressed in the same neuronal cell bodies (79).

Connectivity and Fibrous Neurochemistry

The accessory olfactory bulb receives projections from vomeronasal chemoreceptor neurons in the vomeronasal organ (Jackobson’s organ) and the tongue (124). Its primary projections are to the hilus of the ipsilateral spherical nucleus (119). It also projects to the medial and accessory olfactory amygdaloid nuclei as well as to the olfactory tubercle (119) 1997). Although the accessory olfactory bulb projects ipsilaterally to the spherical nucleus, reciprocal projections from the spherical nucleus to the accessory olfactory nucleus are bilateral (119). They terminate bilaterally on the granular layer and ipsilaterally on the internal plexiform layer (119). The accessory olfactory bulb also receives projections from the bed nucleus of the terminal groove (102, 128). Fibres expressing tyrosine hydroxylase and neuropeptide-FF project to the internal granular layer and fibres expressing gonadotropin releasing hormone project to all layers except the internal granular layer (22, 53, 96).

Functional Correlations

The accessory olfactory bulb is the first recipient of vomeronasal information from the tongue and vomeronasal organ (129). These detect chemicals that have a higher molecular weight than the olfactory system, including pheromones. As such, the accessory olfactory bulb likely plays a role in reproduction, as it does in other vertebrates (1). The accessory olfactory bulb is sexually dimorphic, being larger in males than females (122).

Neurogenesis

The accessory olfactory bulb recruits neurons in the same way as the main olfactory bulb (section 2.2.1). Neurons travel from the proliferation zone of the lateral ventricles along the olfactory peduncle and are deposited primarily in the granular layer (126).
2.2.3 Anterior Olfactory Nucleus

The anterior olfactory nucleus is situated at the base of the olfactory tract in the rostral telencephalon in a region referred to as the retrobulbar formation. Anterior olfactory neurons express androgen receptors, dopamine receptors, nitric oxide, Tbr-1 and neuropeptide-FF (53, 79, 96, 130). It receives ipsilateral projections from the main olfactory bulb and bilateral projections from the lateral cortex, and projects back to the lateral cortex as well as to dorsal cortex (50). Projections to the anterior olfactory nucleus express dopamine receptors, calcitonin gene-related peptide and neuropeptide-FF (53, 96).

The anterior olfactory nucleus sits at the rostral extent of the lateral ventricles and is the site of neurogenesis for the neurons that will be transported to the olfactory bulb (78). As with elsewhere along the lateral ventricle, new neurons are generated from radial glial cells in the ependymal layer and then migrate to their destinations (82). Proliferation varies by season and peaks in the spring and summer (82). New neurons are also incorporated into the cell layer of the anterior olfactory nucleus (73, 78).

2.3 Dorsal Ventricular Ridge

2.3.1 Anterior Dorsal Ventricular Ridge

Cytoarchitecture and Cellular Neurochemistry

The anterior dorsal ventricular ridge is a pallium-derived structure unique to reptiles (including avian reptiles) (24, 51). It is made up of three concentric zones: a periventricular zone with scarce cells, an intermediate zone of cell clusters, and central cell-poor zone (131-133). In addition, the anterior dorsal ventricular ridge can be divided medially to laterally into three cytoarchitectonic regions. In some species, the medial may not have a clear laminar organization, or may have a narrow periventricular zone and an intermediate zone cell clusters populated with small cells (131-134). The intermediate region has been described as having a wide or narrow periventricular zone, a wide intermediate zone of small cell clusters that have either small, medium or large cell bodies, and a central cell-poor zone (131, 133, 134). The lateral region has been described as having a wide or narrow periventricular zone, a zone of very big cell
clusters, and a narrow deep zone (131, 133, 134). These conflicting descriptions are not due to interspecific variation as they mostly come from studies on *Psammodromus algirius*.

The intermediate and lateral regions are rich in calbindin-expressing neurons in their cell-cluster and deep zones (133). Unlike in the cortical regions, these cells are not GABAergic interneurons, and are likely projection neurons (133). Other neurons, expressing parvalbumin, lie in the deep zone on the border with the striatum and project their axons to the intermediate region (133). A different study found neurons that co-express nitric oxide synthase and parvalbumin; it is unclear whether these represent the same population of neurons (46, 47, 97, 135). Nitric oxide expressing neurons are most abundant in the intermediate region and in the deep zone (97). Other neurons express estrogen, progesterone, serotonin & dopamine receptors, aromatase, acetylcholine, vitamin D3 receptors, 5-α reductase, somatostatin, the R1α regulatory kinase subunit, and neuropeptide-Y (37-39, 41, 45, 48, 53, 57, 58, 91, 94, 95, 136).

GABAergic interneurons exist throughout the anterior dorsal ventricular ridge, though they are preferentially found in the lateral region (133). There are three populations of GABAergic neurons: those that co-express parvalbumin, those that co-express calretinin, and those in which a calcium-binding protein was not detected (133). GABAergic neurons in the periventricular zone of the lateral region express calretinin. These neurons have a single long dendrite which projects to the lateral and dorsal cortices (133). There are a few scattered calretinin-expressing cells throughout the rest of the anterior dorsal ventricular ridge (133). Interneurons expressing parvalbumin are preferentially distributed laterally and in the deep zone (133).

**Connectivity and Fibrous Neurochemistry**

The anterior dorsal ventricular ridge receives projections from the major sensory regions of the brain: The rotund nucleus (visual), the medial nucleus of the thalamus (auditory), and the posterior medial and the posteroentral thalamic nuclei (somatosensory) (132-134, 137). Though the rotund nucleus receives bilateral visual input, the visual region of the anterior dorsal ventricular ridge seems to receive visual information purely from the contralateral eye (137). The globus pallidus sends acetylcholinergic projections to the anterior dorsal ventricular ridge, and serotonergic,
noradrenergic and dopaminergic projections come from the brainstem, including the ventral tegmental area (102, 131). The anterior dorsal ventricular ridge has reciprocal connections with the ventromedial hypothalamus (51, 128). Fibres expressing tyrosine hydroxylase and nitric oxide form dense plexuses in the periventricular and cell cluster zones, particularly around dopamine receptor expressing neurons (53). Other fibres express cholecystokinin, neuropeptide-Y, somatostatin, and neuropeptide-FF (37, 45, 92, 96, 138). Fibres expressing calcitonin gene-related peptide innervate the lateral region, mainly in the periventricular zone (108).

Although the three sensory modalities are largely isolated in the anterior dorsal ventricular ridge, there is some evidence for sensory integration in this region (132). However, the anterior dorsal ventricular ridge is also thought to project multiple sensory modalities caudally to other regions of sensory integration, including to the striatum and the posterior dorsal ventricular ridge (51, 102, 131).

**Functional Correlations**

The lateral visual region receives topographic visual input into two zones that likely represent the cell-cluster and deep zones, but projections to the putative periventricular zone are not topographic (137). In both topographic zones, the upper visual field is represented anteriorly, and the lower visual field posteriorly (137). The putative cell cluster zone receptive fields, which have an inhibitory surround, are responsive to flashes of light but prefer moving light stimuli. In the putative deep zone, neurons respond to the onset and offset of light, but not to movement. These receptive fields are larger and do not have inhibitory surrounds (137). The putative periventricular region has weaker responses to visual stimuli (137). The receptive fields of neurons in the somatosensory region cover most of the contralateral body, but have regions of high and low sensitivity (137).

Metabolic activity, as measured by cytochrome oxidase expression, increases with age and social experience (139). In a species with temperature-dependent sex-determination, males incubated at a ‘feminizing’ temperature have greater metabolic capacities than males incubated at other temperatures and females incubated at the same temperature (140). Females had higher metabolic capacities, as high as males, when incubated at a
‘masculinizing’ incubation temperature (140). Castration reduces metabolic activity and testosterone implants rescue it in both males and females (141).

Furthermore, metabolic capacity is elevated in male lizards in both the central visual region and the lateral somatosensory region after repeated viewing of an individual displaying aggressive behaviours (69). Castration reduces metabolic activity and testosterone implants rescue it in both males and females (141). The anterior dorsal ventricular ridge was described as part of the “functionally connected” “sensory aggression network” which includes both the central and lateral regions as well as the rotund nucleus (69). The anterior dorsal ventricular ridge varies in volume, relatively to the rest of the pallium, across reptiles (24). This variation seems to be positively correlated with complexity in novel behaviour and social cognition (24).

**Neurogenesis**

Like all brain regions bordering the lateral ventricle, the anterior dorsal ventricular ridge shows neurogenesis in adults (29, 82, 85, 87, 88, 122). Neurons in the periventricular zone co-express PSA-NCAM, Tbr-1 and doublecortin (76, 79). Neurogenesis is constitutive and is similar to neurogenesis in the medial cortex, including seasonal variation in cell proliferation (82, 83). However, neuronal recruitment may be limited to the periventricular zone (75). The rate of neurogenesis in the anterior dorsal ventricular ridge, relative to neuronal density, is the highest in the telencephalon (75).

**2.3.2 A Note on the Rhynchocephalian Anterior Dorsal Ventricular Ridge**

In *Sphenodon*, the sister clade to squamates (Figure 2), the anterior dorsal ventricular ridge has a trilaminar organization like the cortex. The intermediate cell layer resembles the cortical cell layer and is continuous with the lateral cortex, and the dorsal cortex rostrally (142). Apical dendrites from cell layer neurons project ventrally and radially to a plexiform layer (142). This plexiform layer is continuous with the superficial plexiform layer of the cortex, but as the anterior dorsal ventricular ridge is ventral to the lateral ventricle, this ‘superficial’ plexiform layer is ventral to the cell layer (142). Basal dendrites project dorsally to the plexiform layer that is continuous with the cortical inner plexiform layer. Both plexiform layers contain scattered fusiform cells, which are presumably inhibitory interneurons similar to in the cortex (142).
Compared to squamates, the sensory-modality specific regions of the Rhynchocephalian anterior dorsal ventricular ridge are greatly reduced. Rostrolaterally there is a small visual region, and caudomedially there is a small auditory region (142).

There has been a tendency to categorize squamates as having either a rhynchocephalian-like, or “type I” anterior dorsal ventricular ridge cell pattern, or a “type II” anterior dorsal ventricular ridge cell pattern, with cells more broadly distributed throughout the ridge as described in section 2.3.1. Type I squamates are said to be gekkoids and lacertids while type II squamates are snakes, scincomorphs, anguimorphs and iguanoids (24, 143). However, a true systematic analysis encompassing a wide array of species from both putative types has yet to be conducted. For clarity, the studies used to form the summary of the anterior dorsal ventricular ridge in section 2.3.1 are from a mix of both types. There were no conflicting findings between the two types.

2.3.3 Posterior Dorsal Ventricular Ridge

Projections from the three sensory regions of the anterior dorsal ventricular ridge converge on a region caudal to it, the posterior dorsal ventricular ridge or amygdaloid complex. This suggests that the posterior dorsal ventricular ridge is an area of multimodal sensory processing (132-134) and could be involved in sexual behaviour, aggression, and stress response. The posterior dorsal ventricular ridge is larger and expresses more arginine vasotocin in males than females, and is larger in the breeding season (144, 145). Males who display more sexual behaviour have larger cells (146). Males housed with females have a higher metabolic capacity than males housed in isolation (147). Testosterone treatment also increases arginine vasotocin expression (148). Females have a higher density of aromatase-expressing cells than males (149).

When a male is presented with a social threat, dopamine and serotonin levels increase immediately (150). However, when the environment is disturbed, representing a non-social threat, only serotonin levels increase, and they do not increase as much as with a social threat (150). After an aggressive social encounter serotonin levels decrease (69). The amygdaloid complex forms part of the “functionally” connected “limbic aggression network” (69). Following a restraint stress (where the animal is manually restrained), there is increased noradrenergic activity in the amygdaloid complex (116). Serotonergic
activity is also increased in more aggressive individuals, whereas dopaminergic activity is increased in less aggressive individuals (116).

Here, we consider the terms “posterior dorsal ventricular ridge” and “amygdaloid complex” synonymous, however some authors do not (24). Generally, it appears that the term “posterior dorsal ventricular ridge,” when not applied to the entire amygdaloid complex, is used to denote a large, homogenous, sparsely populated region vaguely dorsomedial to the identifiable amygdaloid nuclei, bounded dorsally and medially by the lateral ventricle. This region is clearly amygdalar and can usually be thought of as “all the amygdalar tissue that is not obviously part of a definable cell cluster.” However, this is a relatively new definition of “posterior dorsal ventricular ridge” starting with Lanuza & Halpern (119) (not referred to in their text, but see table 1).

Prior to 1998, “posterior dorsal ventricular ridge” sometimes referred to the lateral amygdala. The region currently referred to as the posterior dorsal ventricular ridge was referred to as the caudal part of the anterior dorsal ventricular ridge or the centromedial dorsal ventricular ridge (51, 119). Some authors still use the term posterior dorsal ventricular ridge to refer to the lateral amygdala (39), and others use it to refer to the ventromedial amygdala (151). Care should be taken with results associate with the “posterior dorsal ventricular ridge”. If possible, check figures to see where their putative posterior dorsal ventricular ridge is located, otherwise examine whether sources cited for nomenclature date from before or after 1998.

Furthermore, identification of the various nuclei within the amygdaloid complex is confused by the broad array of species used to study the basic cytoarchitecture of the amygdaloid complex. Authors frequently coin new terms to describe the nuclei they identify instead of attempting to reconcile their findings with previously identified nuclei (Table 1). In a variety of species, with extremely variable life histories, it is expected that the same nuclei will be of slightly different shape and architecture across species. However, effort should be made to maintain terminology across species for nuclei that are ontogenetically the same. Here we have used the terminology that is most common, and identified synonyms where they have been identified in the literature. Two terms frequently used are “rostral amygdala” and “ventral amygdala”. The rostral amygdala consists of the external amygdala and the ventral anterior amygdala (121). The ventral amygdala consists of the ventral anterior and the ventromedial amygdala
The rarely used term “interstitial amygdala” refers to a subregion of the medial amygdala that may or may not be distinct (128).

2.3.3.1 Ventromedial Amygdala

This amygdaloid nucleus, one of the few not thought to be involved in sensory processing, projects to the ventromedial septal nucleus, preoptic area, ventromedial hypothalamus and the lateral hypothalamic area (52, 128, 152). It receives projections from the posterior medial nucleus (134). All ventromedial amygdaloid neurons appear to express aromatase (38, 39). Cell density is greater in the non-breeding season compared to the breeding season (153). The density of these aromatase-expressing cells is greater in females than in males, echoing the amygdaloid complex as a whole (154). Neurons also express 5\(\alpha\) reductase, androgen & estrogen receptors, and somatostatin (45, 57, 95, 155-157). Calcitonin gene-related peptide fibres innervate this nucleus (108).

Lesions of the ventromedial amygdala inhibit male sexual behaviour (157-159). Neuronal cell body size is larger in the breeding season than the non-breeding season, and the volume is greater in males than females (95, 154, 157, 160). Testosterone increases cell body size in gonadectomised males, and this effect is amplified in the breeding season (157). However, testosterone decreases neuronal activity (146). Estrogen implants upregulate dopamine in ovariectomized females (161). The average size of the cell bodies in the breeding season correlates with the rate of dewlap extensions in males (146, 157). Males have larger neuronal cell bodies than females (151). However, cell density is greater in females than males, and cell density increases outside of the breeding season (154, 160). Expression of 5\(\alpha\) reductase and estrogen receptors are also greater in females compared to males (57, 155).

After an aggressive interaction, lizards show a decrease in serotonin in the ventromedial amygdala and females show an increase in norepinephrine (162).

2.3.3.2 External Amygdala

The external amygdala consists of an outer cell-poor shell and a core of small, tightly-packed cells (119). It receives projections from the main olfactory bulb, the lateral
cortex, preoptic area, and bilateral projections from the spherical nucleus (119, 121, 134). It projects to the accumbens nucleus, olfactostriatum, and hypothalamus (60, 120). It expresses dopamine, androgen & progesterone receptors, aromatase, acetylcholine, neuropeptide-FF, and nitric oxide (38, 39, 53, 58, 90, 91, 96, 163). Androgen receptor expression was detected in *Cnemidophorus* but not in *Sceloporus*, this may be due to confusion as to where the boundary lies between the external and ventromedial amygdaloid nuclei (55).

Metabolic activity increases with age in male lizards, and decreases with castration (139, 141, 163). Testosterone administration increases metabolic capacity in gonadectomised lizards (163). In a species with temperature-dependent sex determination, in gonadectomised lizards there is more metabolic activity in females that come from a temperature that produces ‘masculinized’ females (163). At a temperature that produces normal females and ‘feminized’ males, metabolic capacity is higher in males than females (163). Sexually experienced females show more metabolic activity than naïve females (139, 163).

### 2.3.3.3 Ventral Anterior Amygdala

This nucleus is a sheet of small-to-medium sized cell bodies, all of which appear to express aromatase (119, 128). It receives projections from the main olfactory bulb, the spherical nucleus (bilateral), the dorsal cortex and the medial posterior thalamic nucleus and projects to the preoptic area, dorsomedial thalamic nucleus, dorsolateral thalamic nucleus, the ventromedial hypothalamus and the lateral hypothalamic area (98). The ventral anterior amygdala is innervated by fibres expressing calcitonin gene-related peptide (108). It projects to the lateral hypothalamic area and the lateral tubermammillary area (152).

### 2.3.3.4 Medial Amygdala

The medial amygdala is a nucleus of medium-sized cells that has a cell-sparse core surrounded by a cell-dense shell (119, 128). Cell bodies express progesterone receptors, acetylcholine, NMDA receptors, aromatase, thyrotropin-releasing hormone and nitric oxide (38, 47, 58, 97, 125). Medial amygdalar neurons may weakly express tyrosine hydroxylase, however this was only reported recently despite numerous other studies on
tyrosine hydroxylase expression in the lizard brain (164). Possible explanations include species differences and the difficulty inherent in detecting weak expression. Administration of testosterone decreases NMDA receptor expression (165).

The medial amygdala is divided into three divisions. The rostral division receives input from the accessory olfactory bulb (120). The dorsal division receives input from the spherical nucleus (120). The ventral division receives input from the lateral cortex (120). Additional projections come from the stria tum, anterior dorsal ventricular ridge, dorsal, medial and lateral cortex, lateral amygdala, contralateral medial amygdala, dorsolateral amygdala, spherical nucleus, dorsomedial thalamic nucleus, medial thalamic nucleus, rotund nucleus, preoptic area, posterior hypothalamic nucleus, lateral posterior hypothalamic nucleus, dorsomedial hypothalamic nucleus, and ventromedial hypothalamic nucleus (98, 166). It projects to the olfactory tubercle, retrochiasmatic area, ventromedial hypothalamus core, the lateral posterior hypothalamic nucleus, the lateral hypothalamic area, torus semicircularis, (128, 152, 166, 167). It is innervated by calcitonin gene-related peptide and orexin fibres (54). Projections to the ventromedial hypothalamus are nitric oxidergic (97).

There is some confusion as to where androgen receptors are expressed in the amygdala. Krohmer (39) and Moga (55) did not report any androgen receptors in either the medial or external amygdalae. Rhen (130) reports them in the external amygdala, however Rhen (168) reports them in the medial amygdala. This confusion may be at least in part due to their using the same acronym, AME, for both nuclei.

More aggressive (dominant) males have less baseline serotonergic activity than less aggressive (subordinate) males (110). During an aggressive interaction between two males noradrenergic activity decreases in the more aggressive individual and serotonergic and noradrenergic activity increase in the less aggressive individual (101, 114). How dopaminergic activity varies during and aggressive encounter is less clear. One study examining dopamine activity found that dopaminergic activity increases in the subordinate individual, while another analysing dopamine and dopamine metabolite concentrations separately found that they increase in the dominant individual (72, 101). In dominant individuals serotonergic activity peaks after as little as one hour of interaction, but in subordinates individuals the activity is delayed and peaks after three
weeks of cohabitation (111, 115). This peak in the subordinates coincides with a peak in systemic corticosterone, suggesting a role in stress response (111).

In a parthenogenic, all-female species of lizard, progesterone receptor expression is greater in the medial amygdala compared to females of the ancestral sexual species (169).

2.3.3.5 Lateral Amygdala

In *Gekko gecko*, the lateral amygdala consists of a central core of large, multipolar cells surrounded by a loose capsule of large acetylcholinergic cells (128). However, in *Thamnophis sirtalis*, the lateral amygdala consists of loosely packed, small and medium acetylcholinergic cells (119). This nucleus receives projections from the anterior dorsal ventricular ridge, rotund nucleus and posterior medial nucleus (128, 134, 167). It projects to the core of the ventromedial hypothalamus (128, 170). The lateral amygdala receives orexin, calcitonin gene-related peptide and neuropeptide-FF innervation (54, 96, 108, 128). Serotonergic activity increases after an aggressive interaction between males in the more aggressive (dominant) individual, and dopaminergic activity increases in the less aggressive (subordinate) individual (72, 114).

2.3.3.6 Dorsolateral Amygdala

The dorsolateral amygdala is made up of large, sparse, acetylcholinergic neurons that also express PSA-NCAM (76, 134, 171). It receives projections from the dorsal and lateral cortices and the spherical nucleus (119, 120). It sends bilateral cholinergic projections to the striatum, the striato-amygdalar transition area, olfactostriatum and the accumbens nucleus and also projects to the hypothalamus (172, 173). Dopaminergic, cholinergic, calcitonin gene-related peptide and calretinin fibres innervate this nucleus (108, 133, 172, 173).
2.3.3.7 Spherical Nucleus

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The spherical nucleus has a trilaminar structure similar to the cortex (134). Like the cortex, projection neurons occupy the mural, or cell layer and GABAergic interneurons the plexiform layers (172). The outer and inner plexiform layers are called the hilus and marginal layers respectively (172). The mural layer expresses aromatase, somatostatin and progesterone receptors (38, 39, 45, 91, 92, 174). Acetylcholinergic activity occurs only in the medial hilus, while nitric oxide is only expressed laterally (47, 58). Neuropeptide Y was detected in the mural layer of a snake, but not previously in any lizards (37, 68).

Connectivity and Fibrous Neurochemistry

The spherical nucleus hilus is the main recipient of projections from the accessory olfactory bulb and is the main vomeronasal processing center of the brain (51). Other projections come from the nuclei of the accessory olfactory tract, diagonal band, dorsomedial thalamic nucleus, preoptic area and anterior commissure (39, 98).

The spherical nucleus projects topographically from the mural layer to the marginal & mural layers and the submural rim of the hilus of the contralateral spherical nucleus (100). This pattern of innervation is complementary to accessory olfactory innervation (175). Projections are sent to the lateral cortex, rostral dorsal cortex, medial amygdala, external amygdala, ventral anterior amygdala, the nucleus of the accessory olfactory tract and dorsolateral amygdala (100, 120). In snakes and some lizards there is an additional, massive bilateral projection to the olfactostriatum; whether this projection innervates the accumbens nucleus is controversial (60, 173). The spherical nucleus is not thought to project to the ventromedial hypothalamus, as previously reported (100). Its only confirmed hypothalamic output is a meagre bilateral projection to the medial preoptic area, lateral hypothalamic area and the lateral tuberomammillary nucleus (120, 152).
The size of the spherical nucleus varies greatly between species according to the importance of the vomeronasal system (100). In snakes and at least one nocturnal lizard, which use vomeronasal information extensively, the spherical nucleus is an extremely prominent component of the amygdaloid complex (141). However, in highly visual lizard species the spherical nucleus is almost absent (141, 176). Seasonal variation in the spherical nucleus may reflect seasonal variation in specific behaviours, such as reproduction and feeding, that rely on vomeronasal input. Aromatase activity is greater in the spring than the fall (177). Furthermore, aromatase activity is maintained during hibernation at 4°C, this is possibly associated with the reproductive period that follows emergence in the spring (177). Like the other primary chemosensory region in the brain, the lateral cortex (section 2.1.4), neuropeptide Y expression increases at the cessation of the reproductive period, and experimentally when glucocorticoid synthesis is inhibited (68).

The spherical nucleus is sexually dimorphic, with greater metabolic capacity in males than females (163). It is also larger in the breeding season than the non-breeding season (145). Lesions of the spherical nucleus facilitate courtship behaviour and increase circulating androgen levels (178). Metabolic capacity in the spherical nucleus increases with age in adults (139). In a temperature sex-determined species, both males and females incubated at a ‘masculinizing’ temperature have a higher metabolic capacity (140, 163). Castration reduces metabolic capacity in males and testosterone rescues this effect (141, 163).

Neurogenesis

Like other telencephalic regions bordering the lateral ventricle, the spherical nucleus has constitutive neurogenesis (29, 73, 82). Neuronal proliferation occurs in the ependymal layer bordering the ventricle, from where the neurons migrate to the marginal and mural layers (73, 75, 82, 88). Like other neurogenic regions, there are CRMP-4, Tbr-1, doublecortin and PSA-NCAM expressing cells in the mural layer (76, 79, 81). Proliferation rates are highest in the spring and the summer, and are higher in males than females (82, 83, 122).
2.3.3.8 Nucleus of the Accessory Olfactory Tract

Formerly considered part of the medial amygdala, this nucleus consists of an aggregation of cells which projects vomeronasal input from the accessory olfactory bulb (119). It expresses acetylcholine and projects to the spherical nucleus, olfactostriatum, preoptic area and lateral hypothalamic area (152, 167, 173).

2.3.3.9 Striatoamygdaloid Transition Area

This is a nucleus of large, densely-packed, cholinergic neurons that project to the lateral posterior hypothalamic nucleus (167). These neurons also express tyrosine hydroxylase and androgen receptors, potentially only in males (173). The nucleus receives projections from the lateral cortex, deep lateral cortex, anterior dorsal ventricular ridge, dorsolateral amygdala, posterior medial nucleus and posterior medial nucleus and is innervated by dopaminergic, serotonergic, calcitonin gene-related peptide and neuropeptide-Y terminals (108, 128, 167, 170, 172, 173). It projects to the dorsomedial and dorsolateral thalamic nuclei and the lateral posterior hypothalamic nucleus (98, 152, 167). Though this is a cell-dense region, it becomes less dense laterally (173). Acetylcholinesterase and tyrosine hydroxylase expression also weaken laterally, while innervation by calcitonin gene-related peptide is more dense laterally (108, 173).

Tonic immobility, a freezing response to fear-inducing stimuli, is considered the final line of defence in a sequence of anti-predator behaviours (179). The length of time spent immobile is a quantitative proxy used to measure fear response. Fear-increasers increase the time spent immobile, and fear reducers decrease it. Lesions to the striato-amygdaloid transition area reduce the duration of tonic immobility (179).

2.3.3.10 Bed Nucleus of the Terminal Groove

This nucleus receives projections from the deep lateral cortex, the accumbens nucleus and the dorsomedial thalamic nucleus (98, 173). There is some indication that it receives vomeronasal projections from the accessory olfactory bulb as well as weak projections from the septum (180). The bed nucleus of the terminal groove sends cholinergic projections to the ventromedial hypothalamus, and also projects to the accessory olfactory bulb, retrochiasmatic area and lateral posterior nucleus (102) 1997).
Neurons also express androgen, progesterone & estrogen receptors, 5α reductase, arginine vasotocin and vitamin D3 receptors (48, 56, 57, 91, 95). In Anolis, arginine vasotocin expression is higher in males than in females, is higher in the breeding season than the non-breeding season, and is upregulated by testosterone (144). Cells expressing arginine vasotocin were activated by copulation, and the faster males and females engaged in sexual behaviour the more cells were activated (181). Aggressively biting an intruder also activated arginine vasotocin expressing neurons (181). In contrast, in Cnemidophorus there are no sex differences in arginine vasotocin expression, and no effect of testosterone (Hillsman et al., 2006). Androgen receptors may be expressed only in males (55).

2.3.3.11 Deep Lateral Cortex

This cell nucleus was first described by Novejarque et al. (173). It lies lateral to the anterior dorsal ventricular ridge, which would make it a rostral extension of the amygdaloid complex. It projects to the bed nucleus of the terminal groove, the striatoamygdaloid transition area, and the accumbens nucleus (173). Projections to the accumbens nucleus are bilateral with ipsilateral predominance (173).

2.4 Septum

The septum is a part of the limbic system involved in reproductive and social behaviour (180). Aromatase, serotonin receptors and estrogen receptors are expressed over the entire septum, however aromatase expression is more intense rostrally (39, 57, 94, 130, 160, 174). The septum also receives somatostatinergic input over its entire extent (45, 92). More estrogen is produced from testosterone via aromatase activity in the fall than in the spring, and lesions of the septum facilitate courtship behaviour and prolong the breeding season (123, 178). Metabolic activity increases with age (139). Both males and females show a reduction in metabolic activity with gonadectomy and testosterone implants rescue this effect (163). Both males and females have higher metabolic capacities when incubated at a ‘masculinizing’ temperature as opposed to a ‘feminizing’ temperature (163). Females have higher concentrations of norepinephrine than males, and estrogen implants upregulate serotonin in ovariectomized females (161, 162). In males, more aggressive individuals have less serotonergic activity in the septum, and serotonergic activity is reduced after an aggressive encounter with another male (110, 179).
Dopaminergic activity also decreases after an aggressive interaction, but only in the subordinate male (72).

Like all regions bordering the lateral ventricles, the septum has constitutive neurogenesis in adults (73, 82, 85, 122). Proliferation is increased in the spring compared to the other seasons (82, 83).

2.4.1 Anterior Septal Nucleus

The anterior nucleus of the septum primarily consists of regularly distributed, medium sized glutamatergic projection neurons, but also contains GABAergic interneurons (182). It receives bilateral input from the medial cortex (51, 52). Ipsilateral projections come from the anterior part of the dorsolateral thalamic nucleus, the ventral tegmental area, and weakly from the locus coeruleus (52). It projects to the anterior commissure, anterior hypothalamus, nucleus of the diagonal band, nucleus of the medial forebrain bundle lateral hypothalamic area, preoptic area, dorsal hypothalamic area, posterior hypothalamic nucleus, supramammillary nucleus, mammillary nuclei, raphe nuclei, interpeduncular nucleus, laminar area of the torus semicircularis, central grey, and ventral tegmental area, with minor projections to the ventrolateral hypothalamus and the shell of the ventromedial hypothalamus (128, 167, 180). It is innervated by terminals expressing serotonin, tyrosine hydroxylase (but not dopamine receptors), neuropeptide-Y, substance-P, leu-enkephalin, and calcitonin gene-related peptide (53, 60, 108, 182, 183).

2.4.2 Dorsal Septal Nucleus

The neuronal cell bodies of the dorsal septal nucleus are closely associated with the anterior pallial commissure (182). The dorsal part of the nucleus has strong cholinergic and serotonergic expression in the neuropil, while the central part has strong tyrosine hydroxylase and enkephalin innervation as well as enkephalin-expressing cell bodies (182). Since dopaminergic innervation is weak, these tyrosine hydroxylase terminals are likely noradrenergic or adrenergic (182). GABAergic and nitric oxide neurons are spread at low density throughout the nucleus, however GABA innervation is strong throughout (182). This nucleus also receives substance-P, neuropeptide-FF and calcitonin gene-related peptide innervation (96, 108, 182). Both parts receive
projections from the preoptic area while only the dorsal part receives projections from the dorsal and medial cortex (52). The dorsal septal nucleus also receives projections from the dorsomedial thalamic nucleus and the substantia nigra (52, 98). This nucleus sends projections to the posterior hypothalamic area, preoptic area, shell of the ventromedial hypothalamus and the lateral hypothalamus (128, 167, 180).

2.4.3 Dorsolateral Septal Nucleus

The dorsolateral septal nucleus is a thin sheet of cell bodies surrounded by a strongly cholinergic neuropil (182). The nucleus appears weakly glutamatergic (182). The dorsomedial cortex sends bilateral projections, and strong ipsilateral projections project from the mammillary nuclei and the ventral tegmental area, with additional scattered projections from the periventricular hypothalamus (52). The dorsolateral nucleus projects to the nucleus of the medial forebrain bundle, the periventricular hypothalamus, the folded part of the lentiform thalamic nucleus, the laminar region of the torus semicircularis, the central grey, and the dorsal part of the interpeduncular nucleus (180). It is innervated by GABA, serotonin, dopamine, substance-P, and to a lesser extent enkephalin (182).

2.4.4 Lateral Septal Nucleus

The lateral nucleus of the septum is a thin layer of cells on the boundary with the accumbens nucleus that consists of both glutamatergic and GABAergic neurons (182). It receives topographically organized excitatory and inhibitory projections from the dorsal cortex (52). The medial cortex also projects to the lateral septal nucleus (52). Some cells in the dorsolateral and ventromedial aspects of the nucleus express dopamine receptors (53). In the dorsolateral aspect, these cells are innervated by fibres expressing tyrosine hydroxylase (53). Expression of neuropeptide-FF is also restricted to the dorsal aspect (96). This nucleus also expresses aromatase, progesterone, estrogen & androgen receptors, and weakly nitric oxide (38, 91, 130). Expression of androgen receptors is upregulated in late vitellogenic females (168). In a parthenogenic, all female lizard species, estrogen downregulates estrogen receptor expression (169). Metabolic activity increases after viewing a video of aggressive behaviour and the lateral septum is included in the “functionally” connected “limbic aggression network” (69).
The lateral nucleus receives projections from the dorsal cortex as well as from the periventricular hypothalamus (52, 128, 167). The primary projections from the lateral nucleus are to the preoptic area, but it also sends projections to the eminentia thalamus, dorsomedial anterior thalamus, anterior hypothalamus, ventral tegmental area, nucleus of the anterior commissure, posterior hypothalamic area, lateral hypothalamic area, subcommissural organ and lightly to the core of the ventromedial hypothalamus (128, 167, 180). This nucleus is innervated by dopamine, neuropeptide-Y, cholecystokinin, enkephalin, arginine vasotocin, orexin, vasopressin, calcitonin gene-related peptide, corticotropin releasing hormone, gonadotropin releasing hormone, neuropeptide-FF and substance-P (37, 38, 54, 58, 91, 93, 96, 108, 130, 138, 182, 184, 185). Testosterone upregulates the expression of arginine vasotocin in males (144).

2.4.5 Medial Septal Nucleus

The medial nucleus consists of large cells surrounding the anterior pallial commissure (182). Cells are glutamatergic or GABAergic and some also express enkephalin, cholecystokinin, nitric oxide, and dopamine receptors (53, 182). This region may or may not be cholinergic (58, 182). It is the only septal region to express the R1α regulatory kinase subunit (41). Innervating fibres strongly express GABA, and also express dopamine, dopamine & androgen receptors, substance-P, enkephalin, cholecystokinin, orexin, thyrotropin releasing hormone, and neuropeptide-FF (53, 54, 56, 96, 125, 138, 182). Projections come from the dorsal cortex, nucleus of the anterior commissure, dorsomedial thalamic nucleus, periventricular hypothalamus, locus coeruleus, ventral tegmental area, and supramammillary nucleus (52). The medial nucleus then projects to the nucleus of the medial forebrain bundle, anterior hypothalamus, dorsomedial thalamic nucleus, posterior hypothalamic nucleus and mammillary nuclei (180). The medial nucleus increases metabolic activity after viewing a video of aggressive behaviour and is included in the “functionally” connected “limbic aggression network” (69).

2.4.6 Nucleus of the Posterior Pallial Commissure

These cells are arranged radially and are mildly glutamatergic (182). This nucleus receives projections from the ventral aspect of the dorsal cortex, the dorsomedial thalamic nucleus, anterior hypothalamus, periventricular hypothalamus, and
mammillary nuclei (52). Massive projections are sent to the lateral habenula and lesser projections to the nucleus of the medial forebrain bundle, eminentia thalamus, dorsomedial & dorsolateral thalamus, preoptic area, ventromedial hypothalamus and mammillary nuclei (180). It receives massive GABAergic and serotonergic innervation as well as mild dopaminergic, calcitonin gene-related peptide, substance-P and enkephalin innervation (108, 182). The posterior pallial commissure is one of only two brain nuclei that contain fibres expressing rhodopsin (186).

2.4.7 Inferior Septal Nucleus

The inferior septal nucleus is weakly glutamatergic with a densely cholinergic neuropil (182). It expresses beta estrogen receptors and 5α reductase (57, 95). The dorsal cortex projects to it, as do the dorsomedial thalamic nucleus and the anterior hypothalamus (52). The inferior nucleus sends massive projections to the lateral habenula, and also projects to the anterior hypothalamus and the ventromedial hypothalamus (180). It receives mild serotonergic and dense gonadotropin releasing hormone innervation (22, 182).

2.4.8 Ventromedial Septal Nucleus

This compact nucleus contains a low density of GABAergic neurons and a dense cholinergic neuropil (182). It receives massive projections from the anterior hypothalamus, and sends back reciprocal, neuropeptide-Y projections (52, 180). Additional projections come from the bed nucleus of the terminal groove, ventromedial amygdala, dorsolateral thalamic nucleus, preoptic area, anterior hypothalamus, and periventricular hypothalamic nucleus (52). Secondary projections from the ventromedial nucleus reach the preoptic area and the tuberomammillary hypothalamus (180). Ependymal cells on the ventricular wall of the ventromedial septal nucleus express opsin and vasopressin and project dendrites into the third ventricle (187). Fibre terminals innervate the ventromedial nucleus with serotonin and neuropeptide-Y (180).
2.4.9 Ventrolateral Septal Nucleus

The ventrolateral septal nucleus is a compact group of cells, most of which are GABAergic and a small proportion of which are glutamatergic (182). Some neurons also express enkephalin (182). The ventromedial hypothalamus provides the major input to this nucleus, with projections also coming from the ventral aspect of the dorsal cortex (52). Minor reciprocal connections exist between the ventrolateral septal nucleus and the preoptic area and anterior hypothalamus, and projections also go to the lateral hypothalamic area and mammillary nuclei (52, 180). This nucleus receives GABAergic, substance-P and enkephalin innervation (182).

2.5 Basal Ganglia

The nomenclature of the basal ganglia nuclei is particularly complex as different combinations of the same nuclei have different names. The caudate and the putamen together are referred to as the “corpus striatum” or the “dorsal striatum” (113). The putamen and the globus pallidus are together called the “lenticular nucleus” (113). The caudate, putamen and the globus pallidus all together are called the “neostriatum” or simply the “striatum” (113). The accumbens nucleus, the olfactostriatum and the olfactory tubercle are called the “ventral striatum” or “limbic striatum”, those three nuclei plus the ventral pallidum are the “paleostriatum” (113, 173). Both the dorsal and ventral stiata express doublecortin (79). The dorsal striatum expresses vitamin D$_3$ receptors, while the paleostriatum expresses serotonin receptors and aromatase (39, 48, 94).

The functions of the basal ganglia nuclei include aspects of voluntary motor control, habitual learning, and emotions. Lesions of the entire basal ganglia, unsurprisingly, abolish social and territorial displays (188). When two male *Anolis carolinensis* form a stable dominant/subordinate social structure, the dominant male shows increased activity in the dorsolateral aspect of the basal ganglia when displaying dominant behaviours, while subordinate male shows decreased activity during submissive behaviours (189). In the paleostriatum the reverse is true: dominant males showed decreased activity while subordinates showed increased activity (189). Dopamine levels decrease after an aggressive interaction in subordinate individuals and increase in dominant individuals (72).
The paleostriatum in particular is believed to be involved in regulating aggression (111). Lesions of the paleostriatum increase dominant behaviour and serotonergic levels in the ventral striatum are elevated during an antagonistic interaction (112). Note, however, that Summers et al.’s ventral striatum included the medial amygdala. The expression of presynaptic serotonin receptors is upregulated in less aggressive (subordinate) individuals (190). Male *Anolis* lizards with paleostriatal lesions display aggressive territorial behaviour, but are unresponsive to releasers of territorial aggression (113).

### 2.5.1 Olfactory Tubercle

The olfactory tubercle receives projections from the main olfactory bulb, rotund nucleus, and ventral tegmental area and projects to the nucleus of the diagonal band and the unnamed substance (102, 170). It contains neurons expressing dopamine receptors, acetylcholine, nitric oxide, enkephalin and cholecystokinin (53, 58, 97, 136, 138). Fibres innervating the olfactory tubercle express serotonin, tyrosine hydroxylase, nitric oxide, neuropeptide-FF, neuropeptide-Y, and cholecystokinin (60, 96, 97, 138). In the medial portion, cells expressing dopamine receptors are innervated by dense plexuses of tyrosine hydroxylase expressing fibres (53).

### 2.5.2 Accumbens nucleus

**Cytoarchitecture and Cellular Neurochemistry**

The accumbens nucleus contains many GABAergic, cabindin expressing spiny projection neurons that express substance P and/or enkephalin (191). In contrast, aspiny interneurons do not express GABA. Some interneurons are cholinergic, while others express both neuropeptide-Y and somatostatin (191). The accumbens nucleus also expresses androgen, progesterone, estrogen, NMDA, serotonin & dopamine receptors, as well as nitric oxide, 5α reductase, aromatase, acetylcholine, vitamin D3 receptors and gonadotropin-inhibiting hormone (39, 47, 48, 56-58, 90, 91, 94, 95, 165, 192). Expression of nitric oxide, 5α reductase and androgen receptors is particularly weak.
The accumbens nucleus can be divided into rostromedial and caudolateral regions. The rostromedial region contains many cell bodies that express GABA receptors and calbindin as well as dopamine receptors (53, 191). Neuropeptide-Y expressing neurons are rare but distributed laterally (191). Calbindin-expressing cell bodies are more loosely packed in the caudolateral region, with some also expressing GABA receptors (191).

Connectivity and Fibrous Neurochemistry

The rostromedial region has dense plexuses of dopamine, neuropeptide-Y, enkephalin and substance-P innervation (60, 191). The substance-P and enkephalin innervation is from collaterals of accumbal spiney projection neurons (191). The innervation of dopamine and substance-P is weaker in the caudolateral region, as is gonadotropin releasing hormone innervation (22, 191). In contrast, neuropeptide-FF innervation is more dense in the caudolateral region (96). The accumbens nucleus also receives serotonin, norepinephrine, nitric oxide, arginine vasotocin, galanin, orexin, thyrotropin releasing hormone, and calcitonin gene-related peptide innervation (45, 47, 54, 60, 125, 193). Innervation by terminal expressing tyrosine hydroxylase is particularly dense around neurons expressing dopamine receptors. Serotonergic innervation to the accumbens nucleus may be limited to lizards in which the olfactostriatum is reduced or absent (60).

Bilateral, but predominantly ipsilateral, projections from the dorsal cortex innervate the accumbens nucleus, primarily in the rostromedial region (102, 191). The accumbens nucleus also receives input from the ventral lateral cortex, external and dorsolateral amygdaloid nuclei, diagonal band of Broca, dorsomedial thalamus, ventral tegmental area, raphe nuclei and locus coeruleus (194). One study also reports projections from the suprapenduncular nucleus, lateral hypothalamus and periventricular hypothalamus in a snake (194). Projections from the dorsal cortex may be topographic, with the rostral-to-caudal gradient in the dorsal cortex projecting ventromedial-to-dorsolaterally in the accumbens nucleus (60, 191). Projections from the medial cortex are also likely (191, 194).

The most massive projection from the accumbens nucleus is to the ipsilateral ventral pallidum (195). The accumbens nucleus projects bilaterally, with ipsilateral
predominance, to the preoptic area and lateral hypothalamic area (196). Ipsilateral fibres project to the ventrolateral septal nucleus, bed nucleus of the terminal groove, dorsomedial thalamic nucleus, nucleus of the basal optic tract, ventral tegmental area, substantia nigra, retrorubral cell group, peribrachial region, interpeduncular nucleus and superior raphe nucleus (195). The extent of septal innervation may vary between species (52, 60, 195). There may also be a minor projection to the vertical limb of the diagonal band of Broca (195).

**Functional Correlations**

The expression of arginine vasotocin is higher in males than in females, and higher in the breeding season than the non-breeding season (144). Testosterone treatment reduces NMDA receptor and dopamine receptor expression, but increases nitric oxide synthase and arginine vasotocin expression in gonadectomised males (144, 165). After viewing a receptive female, males incubated at a ‘feminizing’ incubation temperature, but not a ‘masculinizing’ incubation temperature show elevated levels of dopamine (197, 198).

Before an aggressive encounter, the male that will lose (the future subordinate) has more serotonergic activity than the male that will win (the future dominant) (110). When presented with a social threat (or another threat) both dopamine and serotonin levels rise (150). Dopamine levels rise more in animals that respond to the threat with a warning behaviour (throat swelling) than those that do not (150).

After an aggressive interaction, serotonin and norepinephrine levels increase in the loser (subordinate male) and dopamine levels increase in the winner (dominant male) (72, 112-115). Some studies have found that serotonergic and noradrenergic activity are also increased in the dominant individual (112, 114). Subsequently, presynaptic serotonin receptors seem to be upregulated in subordinate individuals and dopamine metabolism increases in dominant individuals (190). However, if a dominance hierarchy between the two individuals had been previously established, there is no change in serotonergic activity (117). If the animal is stressed, for example due to physical restraint, serotonergic and dopaminergic activity are both elevated, along with epinephrine and norepinephrine levels (104). The accumbens nucleus is part of the proposed “functionally” connected “limbic aggression network” (69).
2.5.3 Olfactostriatum

The olfactostriatum appears to be a specialized tertiary vomeronasal structure derived from a ventral expansion of the accumbens nucleus shell (60). The main distinction between the accumbens nucleus and the olfactostriatum is the massive chemosensory input into the latter, primarily vomeronasal input from the spherical nucleus (60, 173). Additional input arrives from the nucleus of the accessory olfactory tract, lateral and dorsal cortices, external and dorsolateral amygdaloid nuclei, dorsomedial thalamic nucleus, ventral tegmental area and raphe nuclei (60). Though it does not receive direct projections from the accessory olfactory bulbs, it does project to them (119). Other projections from the olfactostriatum include the lateral cortex, septum, ventral pallidum, bed nucleus of the terminal groove, external, ventral anterior & dorsolateral amygdaloid nuclei, lateral preoptic area, lateral posterior nucleus, ventral tegmental area, substantia nigra, and raphe nuclei (60)b). The olfactostriatum receives strong serotonergic and neuropeptide-Y innervation and weak tyrosine hydroxylase innervation (60).

The olfactostriatum is thought to be primarily a tertiary vomeronasal structure and it is prominent in snakes, which rely heavily on vomeronasal input. Though the olfactostriatum appears to be present, but greatly reduced in size and serotonergic input in lizards, to our knowledge all studies to date use lizard species that are not heavily reliant on vomeronasal input (60). Additional studies on the olfactostriatum in lizards, especially using comparative approaches, are needed.

2.5.4 Ventral Pallidum

The ventral pallidum can be divided into a rostral and a caudal division. The rostral division is cell-poor, cholinergic, and expresses substance-P and a little tyrosine hydroxylase (173). In the caudal division acetylcholine and substance-P expression decrease and tyrosine hydroxylase expression increases (173). This region also expresses dopamine and progesterone receptors and is innervated by calcitonin gene-related peptide terminals (53, 91, 108). The ventral pallidum receives projections from the accumbens nucleus and olfactostriatum and projects to the posterior dorsal ventricular ridge (102, 195, 196).
2.5.5 Dorsal Striatum (Caudate and Putamen)

The dorsal striatum is thought to be involved in stress, aggression and stereotyped behaviours (113). The dorsal aspect of the dorsal striatum is a dense cluster of cells with low expression of acetylcholine, substance P and tyrosine hydroxylase. The ventral aspect, however, is strongly cholinergic, receives dense tyrosine hydroxylase innervation, and patchy substance P innervation (173). Striatal neurons also express dopamine, androgen & progesterone receptors, nitric oxide, substance P, somatostatin and vitamin D3 receptors (48, 53, 56, 91, 92, 135, 173). The lateral aspect of the dorsal striatum receives projections from the rotund nucleus and dorsolateral thalamic nucleus and projects to the globus pallidus, anterior entopeduncular nucleus, suprapeduncular nucleus, ventromedial thalamic nucleus, and reticulated substantia nigra (98, 199). The medial aspect receives projections from the dorsomedial thalamic nucleus, medial thalamic nucleus, posteromedial thalamic nucleus, posterolateral thalamic nucleus, caudomedial thalamic nucleus, posterior nucleus of the lenticular loop, nucleus Z, posterior entopeduncular nucleus and projects to the globus pallidus and the ventromedial thalamic nucleus (98, 199). This region in innervated by fibres expressing thyrotropin-releasing hormone, tyrosine hydroxylase, nitric oxide, calcitonin gene-related peptide, neuropeptide-Y, and cholecystokinin (60, 108, 125, 138, 200).

Aggressive (dominant) males have less serotonergic activity in the dorsal striatum than nonaggressive (subordinate) males (110). Serotonergic activity increases in response to stress (71, 104). Subordinate individuals housed with dominant individuals increase expression of presynaptic serotonin receptors and have increased inhibition of serotonin release (190). Metabolic activity is reduced in females incubated at a ‘masculinizing’ temperature and males incubated at a ‘feminizing’ incubation temperature in a temperature sex-determined species (201). During physical exertion, serotonergic and dopaminergic activity decrease and adrenergic activity increases (71).

Neurogenesis occurs throughout the lifetime in the dorsal striatum, like all regions bordering the lateral ventricle, and varies with season (73, 82, 83, 122). The rate of neurogenesis in the dorsal striatum may be the lowest in the forebrain (75, 85).
2.5.6 Globus Pallidus

The globus pallidus consists of a dense fibre plexus enriched with cell bodies. The globus pallidus expresses acetylcholine, substance P and androgen receptors (90, 173). Its main projections come from putamen, and they are so massive the two nuclei often cannot be distinguished (113). Additional projections may come from the cortex, most likely from the deep lateral cortex, as well as from the entopenduncular nucleus and medial posterior nucleus (170, 173). The globus pallidus projects to the anterior dorsal ventricular ridge (102). This nucleus is innervated by fibres expressing dopamine receptors, thyrotropin-releasing hormone, calcitonin gene related peptide, and tyrosine hydroxylase (53, 108, 125, 173).

2.6 Remaining Nuclei of the Basal Forebrain

2.6.1 Diagonal Band of Broca

The diagonal band of Broca consists of a horizontal and a vertical limb (96). It contains neurons expressing neuropeptide-Y, somatostatin, neuropeptide-FF, nitric oxide, tyrosine hydroxylase, and gonadotropin releasing hormone (22, 37, 45, 96, 135). Nitric oxide and tyrosine hydroxylase expressing neurons are present only in the horizontal limb, which projects to the main olfactory bulb (119). The gonadotropin releasing hormone expressing neurons stain the darkest in the brain and project to the terminal nerve ganglion (22). The diagonal band also projects to the lateral cortex, accumbens nucleus, dorsomedial thalamic nucleus, dorsolateral thalamic nucleus and suprachiasmatic nucleus (22, 50, 98). The diagonal band of Broca is innervated by dense fibres expressing tyrosine hydroxylase, serotonin, vasopressin, orexin, somatostatin, neuropeptide-FF, cholecystokinin, and substance-P (22, 37, 45, 54, 60, 125, 138, 185, 202). Projections, presumably dopaminergic, come from the ventral tegmental area (60).

2.6.2 Bed Nucleus of the Anterior Commissure

This small cell cluster expresses estrogen receptors, acetylcholine and 5 α reductase (57, 58, 95). It sends reciprocal connections to the septum (52, 180). This nucleus receives
additional input from the dorsomedial thalamic nucleus and projects to the spherical nucleus, dorsal hypothalamic area and ventromedial hypothalamic nucleus (128, 167).

2.6.3 Nucleus of the Medial Forebrain Bundle

This nucleus is composed of an outer cell plate and an inner plexiform layer (108, 173). Cell bodies express acetylcholine, androgen receptors and substance-P (90, 173). It receives cholinergic innervation from the septum (52, 180, 182). This nucleus also receives input expressing calcitonin gene-related peptide (108).

2.6.4 Nucleus of the Diagonal Band

The nucleus of the diagonal band is sometimes considered part of the medial pallidum, along with the nucleus of the medial forebrain bundle, but not always (173). It consists of a horizontal and a vertical limb (51). Neurons are cholinergic and express calretinin (43, 173). The nucleus of the diagonal band has reciprocal projections with the septum and projects to the dorsal cortex, main olfactory bulb, spherical nucleus, posterior medial nucleus, dorsal hypothalamic area and lateral hypothalamic area (51, 52, 167). It receives calcitonin gene-related peptide innervation and projections from the olfactory tubercle (102, 108).

3 Diencephalon

3.1 Epithalamus

The epithalamus consists of the epiphysis (not covered in this review), the habenular complex and their associated structures. The habenular complex receives galaninergic, tyrosine hydroxylase, nitric oxide, somatostatin and corticotropic-releasing hormone projections (45, 53, 93). Most studies have found no sexual dimorphism in this region in volume, metabolic activity or number of cells expressing androgen receptors (140, 203). However, one study found that the habenula is smaller in the non-breeding season compared to the breeding season in females but not in males (145). More cells express androgen receptors in males that are more aggressive and show male-specific (sexually dimorphic) colouration compared to males that are less aggressive and show female
colouration (203). There is some evidence of neurogenesis occurring in the epithalamus (204, 205).

3.1.1 Lateral Habenular Nucleus

The lateral habenular nucleus is a moderately cell-poor nucleus with a ventrally located cell-poor subhabenular neuropil (206). A small number of cells express calbindin, and the subhabenular neuropil contains calbindin and calretinin expressing cells and fibres (206). The lateral habenular nucleus receives septal projections from the inferior septal and posterior pallial commissural nuclei and additional projections from the dorsomedial and ventromedial hypothalami (128, 167, 180, 182). It receives orexinergic, neuropeptide-FF and thyrotropin-releasing hormone projections (54, 96, 125).

3.1.2 Medial Habenular Nucleus

The medial habenula is divisible into dorsal and ventral components as well as a cell-poor subhabenular neuropil (206). Dopamine receptors are expressed throughout this nucleus (53). The dorsal component is present only in the left hemisphere; this is the only part of the medial habenular nucleus where cells express calbindin and calretinin, however these calcium binding proteins are also expressed by the subhabenular neuropil (206). The medial habenular nucleus receives septal projections, but these are more sparse than to the lateral habenular nucleus (180). Parvalbumin-expressing projections project to the ventral component, likely from the nucleus of the posterior pallial commissure (206). The dorsal component is unique in the brain in that it receives bilateral projections from the parietal eye. It sends dopaminergic projections to the interpeduncular nucleus (53, 206). The habenula receives bilateral projections from the ventral tegmental area, the likely source of dopamine for these projections (207).

3.1.3 Subcommissural Organ

The cell bodies of the subcommissural organ are elongated, with apical dendrites that contact the cerebrospinal fluid of the third ventricle (208). These cells express Reissner’s fibre, however the quantity produced by the organ and its distribution within the cell bodies appears to vary extensively between species (208, 209). Projections from
the subcommissural organ expressing Reissner’s fibre contact the third ventricle, however whether they also contact blood vessels in the brain appears to be variable between species (208). This region is innervated by fibres expressing serotonin, and when serotonin is blocked by a tyrosine hydroxylase inhibitor, Reissner’s fibre expression is reduced (209). Expression of Reissner’s fibre is enhanced in lordotic animals, and subcommissural organ cells have a higher metabolic capacity in lordotic animals as well (208). Reissner’s fibre expression, along with oxytocin expression, are highest in the summer, while expression of sialic acid is highest in the winter (210, 211). This organ also expresses the hormones oxytocin and vasopressin and secretes them into the third ventricle (211). Furthermore, bradykinin and nitric oxide are expressed (127, 212).

3.2 Thalamus

3.2.1 Lateral Geniculate Nucleus

The lateral geniculate nucleus is the main recipient of visual information in the thalamus (213). It has two parts: dorsal and ventral, both of which are retinorecipient (213). The intergeniculate leaflet (section 3.2.5) separates the dorsal and ventral parts and projects to the ventral part (214). Both parts of the lateral geniculate nucleus consist of a medial cell plate and a lateral cell-poor neuropil, however this may be obscured in the dorsal part in some species (206, 214). The cell plate is made up of large, fusiform projection neurons that, in the dorsal part, are roughly organized into parallel rows (214). Small, round, GABAergic interneurons are scattered throughout the cell plate and the neuropil (214). Some cell bodies express parvalbumin and others either calbindin (in the dorsal part) or calretinin (in the ventral part), while the neuropil expresses calbindin and calretinin (206). Dorsal neurons also express cholecystokinin and neuropeptide-Y (37, 138).

The ventral lateral geniculate nucleus receives visual input bilaterally from both the retina and the optic tectum (180, 184, 214). Projections from the optic tectum innervate both the cell plate and the neuropil, whereas retinal input is centered on the neuropil (214). Projections from the optic tectum to the ventral, but not dorsal, part seem to be organized topographically (215). Projections from the retina to the ventral and pretectal geniculate nuclei are also topographically organized (216). Following damage to the
optic nerves, bilateral retinal innervation is restored first to the ventral part and subsequently to the dorsal part (217).

The dorsal part projects ipsilaterally to the pallial thickening and is the main retinorecipient thalamic structure that projects to the telencephalon (214). The ventral part projects bilaterally to the red nucleus and topographically to the optic tectum (216). This part also projects to the nucleus of the posterior commissure, dorsolateral nucleus, lentiform mesencephalic nucleus, tectal grey area, torus semicircularis, tegmental optic nucleus, and inferior olivary nucleus (98). Fibres expressing neuropeptide-Y and substance P innervate the neuropil (37, 96, 202, 214). The retinorecipient region of the dorsal neuropil is also innervated by GABAergic fibres (214).

3.2.2 Oval Nucleus

The oval nucleus consists of a medial cell plate made up of small cells that express calbindin, calretinin, and neuropeptide-Y and a lateral neuropil with large, scattered cells that express calbindin, neuropeptide-Y, and GABA (37, 206, 214). It also receives neuropeptide-Y and GABAergic innervation (214). Primary input is from the retina (184, 214).

3.2.3 Triangular Area

The triangular area receives visual projections from the optic tectum and olfactory projects from the lateral cortex (50, 180). It is a dense nucleus with cells that express calbindin and neuropeptide-Y and terminals that express calcitonin gene-related peptide and androgen receptors (37, 108, 206).

3.2.4 Rotund Nucleus

Rotund nucleus neurons are excitatory, express calretinin and are surrounded by a neuropil that expresses parvalbumin (206, 218). The rotund nucleus is the main thalamic recipient of bilateral projections from the optic tectum (180, 218). Projections are also received from the subpretectal nucleus, posterior nucleus of the ventral supraoptic commissure, ventromedial nucleus, posteroverentral nucleus, suprapenduncular nucleus, anterior entopenduncular nucleus, suprapenduncular nucleus,
ventral pretectal nucleus and principle sensory trigeminal nucleus (170, 199). It projects to the visual subregion of the anterior dorsal ventricular ridge as well as to the lateral amygdala, olfactory tubercle and globus pallidus (170). After damage to the optic nerve, projections to the rotund nucleus are regenerated but later lost (217).

3.2.5 Perirotundal Belt

Most of the cells of the perirotundal belt are large, round, bipolar or multipolar, and clustered, however there are some additional small, scattered cells (214). These cells express calbindin, calretinin and parvalbumin, however the surrounding neuropil expresses only calbindin (206). Neuropeptide-Y and GABA expressing cells are abundant in the large cell clusters, and some cells also express neuropeptide-FF and substance-P (37, 96, 202, 214). The perirotundal belt receives visual projections from the optic tectum and from the retina as well as projections from the principle sensory and descending trigeminal nuclei (180, 213, 214, 219, 220). It projects to the contralateral perirotundal belt, ventral lateral geniculate nucleus, dorsolateral thalamic nucleus suprachiasmatic nucleus, retrochiasmatic nucleus, and the deep layers of the optic tectum (98, 214).

3.2.6 Dorsomedial Thalamic Nucleus

This region can be divided into dorsal and ventral portions. The ventral portion is also known as the intermediomedial nucleus (206). Both portions strongly express calbindin and calretinin in their cell bodies and neuropil (206). Cell bodies also express progesterone & androgen receptors, aromatase and vasotocin (39, 90, 91). Projections come from the septum, striatum, diagonal band of Broca, accumbens nucleus, straitoamygdaloid area, ventral anterior amgdaloid nucleus, lateral hypothalamic area, lateral posterior hypothalamic nucleus, entopeduncular nucleus, preoptic area, supraoptic nucleus, ventromedial hypothalamus, mammillary nuclei, infundibulum, ventromedial nucleus, lentiform thalamic nucleus, torus semicircularis, optic tectum, ventral tegmental area, intercollicular nucleus, and superior raphe (98, 134, 166, 195). This region receives dense substance-P innervation and neuropeptide-FF innervation as well as cholecystokinin, calcitonin gene-related peptide, neuropeptide-Y, orexin, nitric oxide, thyrotropin releasing hormone and tyrosine hydroxylase (37, 54, 96, 108, 125, 138, 202). The dorsomedial thalamic nucleus projects to the dorsal, medial, inferior and
posterior commissural septal nuclei and to the posterior dorsal ventricular ridge, bed nucleus of the terminal groove, striatum, globus pallidus, dorsomedial retrobulbar formation, accumbens nucleus, medial amygdala, nucleus of the anterior commissure, spherical nucleus and to a lesser extent the olfactostriatum, diagonal band of Broca, and olfactory tubercle (52, 60, 98).

3.2.7 Dorsolateral Thalamic Nucleus

This nucleus is often referred to in lizards as the dorsolateral anterior thalamic nucleus, which is divided into magnocellular and parvocellular parts. Dorsolateral thalamic nucleus is more commonly used to describe the same nucleus in turtles (1). Additionally, some studies refer to a dorsolateral posterior thalamic nucleus, which is part of the same cell region (206).

The dorsolateral thalamic nucleus contains sparse neurons that weakly express calbindin and calretinin, however the posterior region more strongly expresses both proteins (206). They also express aromatase and nitric oxide (38). Androgen receptor expression is restricted to the parvocellular part (90). This cell region receives dense substance-P input as well as calcitonin gene-related peptide, cholecystokinin, thyrotropin releasing hormone, tyrosine hydroxylase, nitric oxide, orexin and neuropeptide-Y input (37, 54, 108, 125, 138, 202).

Auditory input is received from the torus semicircularis and visual input from the optic tectum and retina (50, 184, 214). Retinal stimulation increases electrical activity in this nucleus (103). Somatosensory projections to this region from the spinal cord are arranged topographically (206). Additional input is received from the diagonal band of Broca, striatoamygdaloid area, nucleus of the posterior pallial commissure ventral anterior & ventromedial amygdaloid nuclei, preoptic area, suprachiasmatic nucleus, ventromedial nucleus, ventrolateral nucleus, nucleus of the posterior commissure, mesencephalic lentiform nucleus, tectal grey, lentiform thalamic nucleus, lateral hypothalamic area, mammillary nuclei, lateral geniculate nucleus, perirotundal belt, ventral tegmental area, intercollicular nucleus, superior raphe, locus coeruleus, dorsolateral vestibular nucleus, dorsal column nucleus and sensory trigeminal nucleus (98). The dorsolateral thalamic nucleus then projects to the anterior and ventromedial septal nuclei as well as to the dorsal hypothalamic area, striatum, lateral cortex, dorsal
cortex, and medial cortex (49, 50, 52, 98, 99, 167, 220). Projections to the dorsomedial cortex were detected in *Psammodromus algirus* but not in *Podarcis hispanica* (98, 99).

### 3.2.8 Dorsolateral Caudal Nucleus

Identified by Davila et al. (206) as a distinct nucleus containing cell bodies and neuropil expressing calretinin.

### 3.2.9 Medial Nucleus

Medial nucleus is the term used to denote this nucleus in lizards, while nucleus reuniens is used in turtles and crocodilians. The medial nucleus appears to be a relay for auditory information from the torus semicircularis (173, 221). These cells express progesterone & dopamine receptors, calbindin, calretinin, parvalbumin, and the R1α regulatory kinase subunit (41, 53, 91, 206, 221). It receives projections from the torus semicircularis and projects to the auditory region of the anterior dorsal ventricular ridge (134, 206, 221). It receives input expressing orexin, thyrotropin releasing hormone, calcitonin gene-related peptide, tyrosine hydroxylase, nitric oxide, and neuropeptide-FF (53, 54, 96, 108, 125).

### 3.2.10 Suprapeduncular Nucleus

Suprapenduncular neurons express parvalbumin, GABA, cholecystokinin, nitric oxide, arginine and vasotocin; this nucleus receives projections from the optic tectum and projects to the red nucleus, medial posterior nucleus, nucleus rotundus and dorsal striatum (138, 180, 206, 218). The suprapenduncular nucleus and the ventromedial nucleus are together referred to as the reticular thalamus (180, 206).

### 3.2.11 Entopeduncular Nucleus

This premotor area, which can be divided into anterior and posterior regions, receives projections from the striatum and projects to the globus pallidus, rotund nucleus, dorsomedial thalamic nucleus, optic tectum and red nucleus (170).
3.2.12 Ventral Thalamic Nuclei

Both the ventrolateral and the ventromedial thalamic nuclei receive visual projections from the optic tectum, though the ventrolateral nucleus receives them only to its ventral part (180). The ventrolateral nucleus is also retinorecipient (214). The ventrolateral nucleus expresses both calbindin and parvalbumin, while the ventromedial nucleus expresses only calbindin (206). The ventrolateral nucleus also expresses calcitonin gene-related peptide, while the ventromedial nucleus expresses estrogen receptors and 5α reductase (57, 95, 108). Both nuclei project to the red nucleus (216). The ventromedial nucleus has reciprocal connections with the posterior medial and posterior lateral thalamic nuclei (222). The ventromedial nucleus also projects to the dorsomedial and dorsolateral thalamic nuclei and the dorsal hypothalamic area (98, 167). The ventrolateral thalamic nucleus projects only to the dorsolateral thalamic nucleus (98).

3.2.13 Posterior Thalamic Nuclei

The posterior medial nucleus is one of the main somatosensory relays of the thalamus. Cells express the calcium-binding proteins calbindin & calretinin as well as dopamine receptors, calcitonin gene related peptide and nitric oxide (53, 108, 206). They are innervated by tyrosine hydroxylase expressing fibres (53). Projections come from the principle & descending trigeminal nuclei, superior olive, posteroentral nucleus, suprapeduncular nucleus, lentiform thalamic nucleus, nucleus of the ventral tectothalamic tract, optic tectum, torus semicircularis, profound mesencephalic nucleus, substantia nigra, superior isthmus, nucleus of the lateral lemniscus, ventral striatum and the nucleus of the diagonal band (134). This region projects to the medial, ventromedial, ventral anterior & lateral amygdaloid nuclei, striatoamygdaloid area, dorsal striatum, globus pallidus, anterior dorsal ventricular ridge and the torus semicircularis (134, 170). There are reciprocal connections between the posterior medial thalamic nucleus and the ventromedial thalamic nucleus (222).

Not as much has been written about the remaining posterior nuclei of the thalamus. The lateral posterior nucleus receives projections from the medial amygdala, the bed nucleus of the terminal groove and the olfactostriatum, all regions that receives heavy vomeronasal input, and projects to the motor nucleus that controls tongue movement (60). It also has reciprocal connections with the ventromedial thalamic nucleus (222). In
contrast, the similarly named posterolateral nucleus, a calbindin- and calcitonin gene-related peptide-expressing cell cluster identified in 2000, projects to the posterior dorsal ventricular ridge and the dorsal striatum (108, 206). The posteroventral nucleus, a cluster of parvalbumin-expressing cells, projects to the rotund nucleus, ventromedial hypothalamus and lateral hypothalamic area (128, 167, 206, 218). The posterocentral nucleus relays somatosensory information to the anterior dorsal ventricular ridge (173).

3.3 Hypothalamus (including the preoptic area)

3.3.1 Supraoptic Nucleus

Supraoptic cell bodies express neurophotoreceptors, arginine vasotocin, mesotocin, androgen receptors, nitric oxide, oxytocin, vasopressin, orexin and cystyl aminopeptidase (90, 185, 211, 223-225). However, no orexin was detected in the supraoptic nucleus of a viper (226). The neurophotoreceptors may be in the melanopsin family, which has been detected in the lizard brain (227). This nucleus expresses galanin in cells and fibres only in the active (wet) season, not the inactive (dry) season in a desert lizard (228). Other fibres innervating this nucleus express thyrotropin-releasing hormone and corticotropin releasing hormone, (93, 125). It receives projections from the retina and the septum (180, 214). Projections from the paraventricular nucleus are mesotocinergic, vasotocinergic and serotonergic (192). The caudalmost part of the supraoptic nucleus is sometimes referred to as the retrochiasmatic nucleus (93). It receives projections from the amygdala (152). This region receives calcitonin gene-related peptide input (108). It projects to the ventromedial hypothalamus, dorsomedial thalamic nucleus and the medial eminence (98, 128, 229).

This region is believed to be involved in the homeostatic regulation of water and salt balance. Both hypo- and hyper-osmolality increase the metabolic capacity of the supraoptic nucleus (230). Metabolic capacity is also higher in the morning as compared to the evening (192). Lesions of this and the paraventricular nuclei abolish hypernatraemia-associated thermal depression (224). Administration of arginine vasotocin rescues this effect (224).
The supraoptic nucleus may also be involved in reproductive behaviour. When a male is presented with a mate (female) or competitor (male), activity increases in cells expressing arginine vasotocin (181).

### 3.3.2 Paraventricular Nucleus

This, one of the few magnocellular hypothalamic nuclei, is involved in homeostatic regulation, particularly hydration. It projects to the supraoptic nucleus and the lateral cortex (50, 229). It is the primary brain region expressing vasopressin, thyrotropin releasing hormone and corticotropin releasing hormone (93, 125, 185, 228). In addition, these cells express somatostatin, oxytocin, bradykinin potentiating peptide, arginine vasotocin, mesotocin, and gonadotropin inhibiting hormone (92, 185, 192, 211, 212, 224). However, in a viper no oxytocin was detected (226). Cells express galanin in the wet season but not the dry season in a desert lizard (228). The paraventricular nucleus is more metabolically active in animals that are hyper-osmotic compared to animals that are hypo-osmotic or isosmotic (230). This region receives input expressing galanin, orexin and tyrosine hydroxylase (45, 54, 125). Gonadotropin inhibiting hormone expressing neurons project to the nucleus of the paraventricular organ and the medial eminence, where they synapse onto cerebrospinal fluid-projecting serotonergic neurons (192).

Magnocellular neurons are larger in males than females, and parvocellular neurons are larger in the breeding season compared to the non-breeding season (144). In males, testosterone increases the volumes of both cell types (144). Arginine vasotocin expression is greater in the breeding season, and is also upregulated by testosterone (144). During an aggressive interaction between two male lizards, arginine vasotocin expressing cells are activated (181). When a female is presented with a male, the more she displays to him, and the more they engage in reproductive behaviour, the more arginine vasotocin expressing cells are activated in the male (181).

Lesions of this and the supraoptic nuclei abolish hyponatraemia-associated thermal depression (224). Administration of arginine vasotocin rescues this effect (224).
3.3.3 Periventricular Preoptic Nucleus


In comparing progesterone receptor expression between an ancestral sexually reproducing species and a derived parthenogenic species, Godwin et al. (235) found that mRNA expression was higher in the parthenogenic species, whereas O’Connell et al. (91) found that more cells are immunoreactive in the sexual species. Godwin et al. (235) also found that progesterone receptor expression was greater in males than in females, whereas O’Connell et al. (91) did not. Estrogen receptor expression appears to be greater in the parthenogenic species (169). Estrogen receptor expression is also lower in vitellogenic females compared to postvitellogenic females, whereas the opposite pattern is seen for progesterone receptor density (233, 236). Testosterone increases metabolic capacity and progesterone receptors levels (141, 233). Estrogen increases progesterone receptor expression (233, 237).

In the ancestral sexual species, most males display male reproductive behaviour when injected with testosterone. Some also display reproductive behaviour when injected with progesterone. Gonadectomized males do not display male reproductive behaviour in response to testosterone or progesterone. In those individuals that do not display male sexual behaviour due to gonadectomy or insensitivity to progesterone, nitric oxide expression in the periventricular peroptic nucleus is reduced compared to those that do (47). In the derived parthenogenic species, postovulatory females display male sexual behaviour while preovulatory females do not, and postovulatory females express more nitric oxide than preovulatory females (47). In gonadectomised females and males, testosterone-implanted females express more nitric oxide than sham-implanted females (47, 238).
3.3.4 Dorsal Hypothalamic Area

The dorsal hypothalamic area is usually subdivided into a cell-dense medial nucleus and a relatively cell-poor lateral zone, both parvocellular. ‘Dorsal hypothalamus’ and ‘dorsal hypothalamic nucleus’ generally refer to the dorsomedial hypothalamic nucleus, with the lateral zone may also be referred to as the dorsolateral hypothalamus. This region is sometimes considered as part of the posterior hypothalamic nucleus with which it is closely associated (128).

Dorsomedial cells express cholecystokinin, progesterone & estrogen receptors, nitric oxide, orexin and tyrosine hydroxylase (47, 91, 138, 183, 193, 206, 233). Cells closer to the third ventricle express orexin, while others further from the ventricle express tyrosine hydroxylase (54). Some orexin-expressing cells project dendrites into the cerebrospinal fluid of the third ventricle (54). The neuropil is immunoreactive for calbindin (206). The region receives visual projections from the optic tectum and additional projections from the dorsal cortex, anterior septal nucleus, nucleus of the diagonal band, nucleus of the anterior commissure, ventromedial hypothalamus, mamillary nuclei, posterior hypothalamic nucleus, lateral hypothalamic area, lateral habenula, dorsolateral thalamic nucleus, torus semicircularis, ventral tegmental area, superior raphe, inferior isthmal nucleus, and dorsal tegmental nucleus (128, 180). Galaninergic fibres innervated this nucleus, which projects to the red nucleus (228).

The dorsomedial hypothalamic nucleus has shown some sex-related variation. Estrogen receptor expression is higher in females than in males, and administration of estrogen upregulates estrogen and progesterone receptor expression in females but not in males (183, 233). In contrast, males that are more sexually vigorous have more cells that express tyrosine hydroxylase than sexually sluggish males (193). Females have a higher metabolic capacity than males (163). In a temperature-sex determined species both sexes have higher metabolic capacities when incubated at ‘masculinizing’ temperatures as compared to ‘feminizing’ temperatures (139, 163). Parthenogenic females express more progesterone receptor mRNA than females of a sexual species (169). Gonadectomy reduces metabolic capacity in both sexes, and testosterone increases it (163).
3.3.5 Medial Eminence

Medial eminence neurons are small, round and express galanin and somatostatin (37, 45). This region receives projections expressing cholecystokinin, neuropeptide-Y, galanin, somatostatin, orexin, tyrosine hydroxylase, gonadotropin releasing hormone, calcitonin gene-related peptide and rhodopsin (22, 37, 45, 54, 108, 138, 186, 228). In gravid females, neurons expressing vasopressin project into the cerebrospinal fluid of the third ventricle (185). The supraoptic and paraventricular nuclei send mesotocin and vasotocin projections to the medial eminence, and the paraventricular nucleus sends additional projections expressing corticotropin release hormone (93). The medial eminence also receives oxytocinergic innervation, and gonadotropin-releasing hormone expressing innervations in Eumeces and Sceloporus but not in Anolis (185, 239). In a viper, fibres in the medial eminence are the only fibres in the brain found to express oxytocin (226). Additional projections come from the septum (180).

3.3.6 Nucleus of the Paraventricular Organ

The cells of this nucleus are photoreceptive, expressing neurophotoreceptors and serotonin (186, 192). Other neurons in this nucleus express transducin and cystyl aminopeptidase (192, 223). The paraventricular nucleus projects fibers expressing mesotocin and vasotocin (229). The photoreceptive, serotonergic neurons project to the lateral hypothalamic area where they modulate the activity of neurons that express gonadotropin releasing hormone (192). In addition, both the photoreceptive, serotonergic cells and the transducinergic cells project dendrites into the cerebrospinal fluid of the third ventricle (186, 192). This region is innervated by fibres expressing corticotropin releasing hormone (93).

3.3.7 Suprachiasmatic Nucleus

These neurons express estrogen & progesterone receptors, neuropeptide-Y, parvalbumin, arginine vasotocin and tyrosine hydroxylase (37, 91, 144, 193, 206, 240, 241). This nucleus is most metabolically active in the morning (242). Arginine vasotocin expression is greater in the breeding season than the non-breeding season (144).
Fibres innervating this nucleus express neuropeptide-Y, corticotropin releasing hormone and orexin (37, 54, 93). Fibres expressing gonadotropin releasing hormone project from the diagonal band of Broca (22). Additional input is received in the form of visual projections from the retina and periorotundal belt, and additional input comes from the dorsolateral thalamic nucleus (98, 184, 214). The suprachiasmatic nucleus projects bilaterally to the rotund nucleus and ipsilaterally to the ventromedial hypothalamus and lateral hypothalamic area (128, 167, 218).

The suprachiasmatic nucleus is believed to be the primary circadian pacemaker of the reptilian brain. Bilateral lesions to this region are the only ones to abolish circadian rhythms (241, 243-245). In lizards with bilateral pinealectomies and unilateral lesions of the suprachiasmatic nucleus, daily injections of melatonin or day/night light cycles are sufficient to entrain circadian locomotor rhythms, however they are incapable of restoring behavioural circadian rhythms in animals with bilateral lesions of the suprachiasmatic nucleus (241, 242).

Metabolic activity increases after viewing a video of aggressive displays (69). This nucleus forms part of the author’s ‘functionally’ connected ‘sensory aggression network’ (69).

3.3.8 Preoptic Area

*Cytoarchitecture and Cellular Neurochemistry*

The preoptic area is divided into a cell-dense medial nucleus and a cell-poor lateral zone. The medial nucleus may further be divided into dorsal and ventral portions, with cell body size increasing ventrally (151). Neuron bodies of the medial nucleus express androgen, estrogen & progesterone receptors as well as arginine vasotocin, nitric oxide, neuropeptide-FF, aromatase, 5α-reductase, calcitonin gene-related peptide, tyrosine hydroxylase and gonadotropin releasing hormone (22, 39, 56, 57, 90, 91, 96, 108, 155, 164, 174, 231, 238, 239, 246). Testosterone receptor-expressing cells were detected in only males in *Sceloporus undulatus* (55). 5α-reductase activity is low in the preoptic area and throughout the hypothalamus (247). Nitric oxide may be preferentially expressed in the dorsal portion of the medial preoptic nucleus (97). Gonadotropin-
releasing hormone is expressed in \textit{Thamnophis}, \textit{Eumeces} and \textit{Sceloporus}, but not in \textit{Anolis} (22, 239).

\textit{Connectivity and Fibrous Neurochemistry}

The preoptic area receives projections from the dorsal cortex, ventromedial and ventral anterior amygdaloid nuclei, septum, and accumbens nucleus (52). Innervating fibres express androgen receptors, neuropeptide-Y, orexin, neuropeptide-FF, gonadotropin releasing hormone, calcitonin gene-related peptide, vasopressin, oxytocin and galanin (22, 37, 45, 54, 96, 108, 185). The preoptic area projects to the septum, external amygdala, dorsomedial and dorsolateral thalamic nuclei, ventromedial hypothalamus, lateral hypothalamic area, lateral posterior hypothalamic nucleus and hypoglossal nucleus (98, 128, 166, 167, 180). The preoptic area projects fibres expressing aromatase to the spherical nucleus (39).

\textit{Functional Correlations}

The preoptic area is associated with several functions, including body temperature regulation and hydration (178, 180, 248). As the temperature drops, neuronal firing rate in the preoptic area increases (249). However, the preoptic area is most strongly associated with male sexual behaviour and motivation. Lesions of the preoptic area have consistently abolished male sexual behaviour across multiple squamate species, and testosterone or estrogen implants into the lesioned area restore courtship behaviour (39, 157, 159, 233, 250, 251). Furthermore, the preoptic area may have an inhibitory role in female reproductive behaviour. This has made the preoptic area one of the key regions of interest in many investigations into the links between behaviour, endocrine function, and brain function in reptiles.

Overall volume and cell body size in the preoptic area are greater in the breeding season than in the non-breeding season (145, 153, 160). In \textit{Anolis carolinensis}, \textit{Urosaurus ornatus} and \textit{Cnemidophorus inornatus} overall volume is also greater in males than females both at hatching and during the breeding season, but not during the non-breeding season (95, 145, 160, 240, 252). However, the preoptic area is not sexually dimorphic in \textit{Eublepharis macularius} and \textit{Thamnophis sirtalis} (163, 248, 252). In male \textit{Cnemidophorus inornatus} that are genetically female but have been sex-reversed with
an aromatase inhibitor during development, the preoptic area is female-sized, not male-sized (253).

Aromatase activity in males is greater during the non-breeding season (123). Volume decreases with age and metabolic capacity increases with age (in males) and sexual experience (in females) (139, 197). Metabolic activity is higher in females after they have mated and are unreceptive than while they are still receptive (254). In males, metabolic capacity is higher in those housed with females than in isolation (147). Males express more androgen receptors, aromatase, and arginine vasotocin than females (144, 154, 157, 233). Females express more progesterone and estrogen receptors than males, and expression is increased when they are previtellogenic compared to when they are post vitellogenic (91, 157). Females also have higher noradrenergic activity (162). Though males and females do not differ in dopaminergic activity, injection of a dopamine agonist into the preoptic area facilitates courtship and reproduction (255). Furthermore, tyrosine hydroxylase expressing cells are larger in more sexually vigorous males (200). In females, androgen receptors are upregulated post vitellogenesis and testosterone increases the expression of arginine vasotocin, while in males testosterone upregulates nitric oxide and reduces activity (146, 165, 168).

Gonadectomy decreases the volume and metabolic capacity of the preoptic area, while testosterone rescues these effects and upregulates progesterone receptor expression (141, 163, 246). In contrast, gonadectomy increases androgen receptor expression (in males) and estrogen receptor expression (in both sexes) (233). Testosterone rescues both these effects as well (163, 233). Gonadectomized females implanted with testosterone show male courtship behaviour, and metabolic capacity in the preoptic area increases with the frequency of male courtship displays in these females (163, 238, 248).

In a sexually dimorphic species, males have more cells that express androgen receptors than females. However, in a closely related monomorphic species, there is no difference in androgen receptor expression between males and females (203). In another sexually dimorphic species, overall volume is greater and cell bodies are larger and more dense in males disguising themselves as females, compared to normal males (178, 256). Males from a lizard species that engages in elaborate courtship and territorial behaviours has
reduced androgen receptor expression compared to a species without elaborate courtship and territorial behaviours (257).

In a species with temperature-dependent sex determination, lizards incubated at a ‘masculinizing’ temperature have larger preoptic areas than lizards incubated at a ‘feminizing’ temperature (163). Metabolic capacity is lower in males from the ‘masculinizing’ temperature than the ‘feminizing’ temperature, and the latter more frequently engage in courtship displays (140, 201). Females incubated at the ‘masculinizing’ temperature are more likely to show male courtship behaviour and have a higher metabolic capacity than females incubated at ‘feminizing’ temperatures (163).

In a parthenogenic, all-female species, lesioning the preoptic area or implanting serotonin into the preoptic area both abolish pseudo-male copulatory behaviour while androgen implants into the preoptic area and serotonergic antagonists restore this behaviour in the respective treatments (233, 258, 259). Furthermore, serotonegenic hormones downregulate serotonergic activity in this region (259). The preoptic area in parthenogenic females is the same volume as that of females of the ancestral sexual species, despite the parthenogenic species displaying male-typical copulatory behaviour (233, 252). However, metabolic activity in the preoptic area is greater in parthenogenic females displaying male pseudo-copulatory behaviour than female pseudo-copulatory behaviour (252). Compared to the ancestral sexual species females, parthenogenic females have fewer cells that express tyrosine hydroxylase and progesterone receptors, but they have a greater expression of estrogen and progesterone receptor mRNA (91, 193, 246, 252). Estrogen upregulates estrogen and progesterone receptor expression only in the parthenogenic species (236). Testosterone upregulates nitric oxide expression in parthenogenic females, and presence of another female increases nitric oxide activity (238). Post vitellogenic parthenogenic females, which take on the male pseudocopulatory role, show increased metabolic activity and aromatase expression in the medial preoptic area compared to previtellogenic females, which perform the female pseudocopulatory role (38, 246). Postvitellogenic females also have lower serotonin levels than previtellogenic females (258).

The preoptic area may also be involved in aggressive behaviour. Metabolic activity increases after viewing aggressive behaviour, and this region is part of Yang’s ‘functionally’ connected ‘limbic aggression network’ (69). In Anolis, two males housed
together will develop a dominant-subordinate hierarchy as the result of aggressive interactions. Subordinate males have fewer cells that express arginine vasotocin compared to dominant males (260).

3.3.9 Anterior Hypothalamic Area

The anterior hypothalamic area is a controversial designation. It is recognized by some authors (261) but not by others, who consider this the caudal continuation of the preoptic area (176). Cells are parvo cellular and increase in volume ventrally (200). They express arginine vasotocin, aromatase, androgen & estrogen receptors and tyrosine hydroxylase (38, 130, 174, 193, 200). This area receives projections from the septum and preoptic area proper (52). It projects back to the septum (180). Projections from the preoptic area to the anterior hypothalamic area express brain photoreceptors (225). In addition, fibres projecting to the anterior hypothalamic area express androgen receptors and calcitonin gene-related peptide (108).

In a temperature sex-determined species, females incubated at a ‘masculinizing’ temperature and males incubated at the same temperature have the same metabolic capacity in the anterior hypothalamic area, which is higher than that either sex incubated at a ‘feminizing’ temperature (139, 163, 197, 262). In contrast, at a ‘feminizing’ temperature, males have a higher metabolic capacity than females (163). Castration decreases metabolic capacity in individuals incubated at a ‘masculinizing’ temperature, but not those incubated at a ‘feminizing’ temperature (141, 163). Testosterone implants rescue this decrease in metabolic capacity (163).

Metabolic activity in the anterior hypothalamic area increases with social experience in males but not in females (139, 147, 163). Androgen receptor expression is greater in late vitellogenic females than in previtellogenic females (168). During copulation, there is an increase in activity in cells expressing tyrosine hydroxylase, but only in cells proximal to the third ventricle (164). Aggressive males have less serotonergic activity than nonaggressive males (110). In two closely related species, the species with sexual reproduction has more cells that express tyrosine hydroxylase than the parthenogenic species (193).
3.3.10 Ventromedial Hypothalamic Nucleus

This nucleus is divided into a central core of cell bodies and a surrounding shell of the dendritic projections from the core (128). Cells of this nucleus express androgen, estrogen & progesterone receptors as well as 5α-reductase, aromatase, dopamine receptors and bradykinin potentiating peptide (38, 39, 53, 90, 148, 154, 160, 212, 263). It receives projections from the ventral anterior, ventromedial, dorsal, medial and lateral amygdaloid nuclei, as well as additional projections from the dorsal cortex, lateral & anterior, posterior pallial, and inferior septal nuclei, bed nucleus of the terminal groove, nucleus of the anterior commissure, lateral habenula, dorsomedial thalamic nucleus posteroverentral thalamic nucleus, preoptic area, suprachiasmatic nucleus, posterior hypothalamic nucleus, lateral hypothalamic area, supraoptic nucleus (bilateral), mammillary nuclei (bilateral), contralateral ventromedial hypothalamus, posterodorsal nucleus, isthmal nucleus, ventral tegmental area, torus semicircularis (bilateral), superior raphe (bilateral), and laterodorsal tegmental nucleus (51, 53, 98, 128). Some of these projections express nitric oxide, cholecystokinin and corticotropin releasing hormone (47, 93).

The ventromedial hypothalamic nucleus is heavily implicated in female reproductive behaviour. Lesions to this nucleus abolish female reproductive behaviour, and estrogen implants to this region elicit such behaviour (246, 264). It is one of the main regions of interest in the study of the links between behaviour, the endocrine system and the brain.

The overall volume is larger in males in Anolis carolinensis but larger in females in Cnemidophorus inornatus and sexually monomorphic in Eublepharis macularius and Thamnophis sirtalis (160, 252, 263). However, another study found no difference in volume between male and female Anolis (153). In artificially sex-reversed male Cnemidophorus, that are genetically female but have been made male with an aromatase inhibitor, the ventromedial hypothalamus is female-sized (253).

In general, the ventromedial hypothalamus is larger and expresses more estrogen receptors in the breeding season compared to the non-breeding season (153), (157, 160). Cell density, cell volume, metabolic activity, noradrenergic activity, aromatase expression, androgen receptor expression and estrogen receptor expression are all greater in females, however progesterone receptor and 5α-reductase expression are
greater in males (56, 57, 149, 154, 160, 162, 233, 240, 252). Metabolic activity in females goes down after courtship and copulation, while androgen receptor expression goes up (168, 254). Estrogen downregulates estrogen receptor expression in males but upregulates it and progesterone receptor expression in females (160, 233, 246). Progesterone decreases estrogen and progesterone receptor expression in females; it also inhibits female reproductive behaviour (246, 265). In both females and males testosterone increases estrogen and progesterone receptor abundance as well as nitric oxide expression (165, 233). In males, testosterone also blocks the sensitivity of progesterone receptor expression to estrogen. In castrated males, like in females, estrogen upregulates progesterone receptor expression (266). Testosterone blocks this effect in males but not females (266). Males created from genetic females through hormone manipulation during embryogenesis respond like females (267). Testosterone increases 5α reductase expression in females and decreases it in males during the breeding season (154, 157). Finally, males housed with females have higher metabolic capacities than males housed in isolation (147).

Expression of 5α reductase appears to be lateralized in the ventromedial hypothalamus. It is generally restricted to the lateral pole of the nucleus, and is greater is the right hemisphere than the left during the non-breeding season (95). 5α reductase expression in the right hemisphere decreases in the breeding season to that of the left hemisphere (95). The ventromedial hypothalamus increases in metabolic activity after aggressive encounters (268). Aggression is also lateralized to the right hemisphere, and testosterone does mediate aggressive behaviour, so a link between these two effects should be investigated.

Castration in males increases the volume and estrogen receptor density of the ventromedial hypothalamic nucleus, and testosterone reverses the volumetric effect but not the receptor density (141, 233). In females, ovariectomization also increases estrogen receptor density, and estrogen treatment exacerbates this effect (233). Metabolic activity decreases in castrated females (141). Gonadectomization decreases progesterone receptor expression in both sexes, and testosterone reverses this effect (233).

In a sexually dimorphic species, males have more cells that express androgen receptors than females. However, in a closely related monomorphic species, there is no difference
in androgen receptor expression between males and females (203). Androgen receptor expression is greater in a species without elaborate courtship and territorial behaviours in comparison to one with these behaviours (257).

In a species with temperature-dependent sex determination, males incubated at a ‘masculinizing’ temperature have lower metabolic capacities, smaller overall volumes and fewer cells expressing tyrosine hydroxylase than males incubated at a ‘feminizing temperature’ (140, 163, 197). Females from a ‘masculinizing’ incubation temperature have lower metabolic capacity than females from a ‘feminizing’ incubation temperature (139, 140). However, only females from a ‘masculinizing’ temperature show increased metabolic capacity with sexual experience (197). The effects of gonadectomy and testosterone on metabolic capacity are heavily dependent on incubation temperature (163). The overall volume is larger in younger animals and in males housed with females compared to males housed in isolation (139).

Parthenogenic females express fewer progesterone receptors but more estrogen receptors than females of their ancestral sexual species (91, 233). Estrogen is more effective at inducing reproductive behaviour, estrogen receptor expression and progesterone receptor expression in the ventromedial hypothalamic nucleus in parthenogenic females as compared to females of the sexual species (169, 269). Metabolic activity and estrogen receptor expression decrease in postvitellogenic females, which display male pseudocopulatory behaviour, compared to previtellogenic females, which display female pseudocopulatory behaviour (183, 246). The opposite pattern is seen with progesterone receptors, which are expressed more during postvitellogenesis (169). Observing either male or female courtship behaviours induces expression of estrogen and progesterone receptors (270). Investigation in the serotonergic control over female reproductive behaviour seems to only have been conducted in parthenogenic females. In these females, injection of serotonin into the ventromedial hypothalamus, but not other brain regions, suppresses female reproductive behaviour (258). Testosterone suppresses serotonergic activity, and previtellogenic lizards express fewer serotonin receptors than late vitellogenic lizards (258).
3.3.11 Lateral Hypothalamic Area

Cells of this sparsely populated region express progesterone receptors, calcitonin gene-related peptide, nitric oxide, dopamine receptors, tyrosine hydroxylase and gonadotropin releasing hormone (47, 53, 91, 108, 164, 192). This area receives projections from the dorsal cortex, anterior, dorsal & lateral septal nuclei, straitoamygdalar transition area, anterior dorsal ventricular ridge, the medial, ventral anterior and ventromedial amygdaloid nuclei, accumbens nucleus, nucleus of the diagonal band, nucleus of the accessory olfactory tract, preoptic area, suprachiasmatic nucleus, posterior hypothalamic nucleus, nucleus of the paraventricular organ, dorsomedial hypothalamic nucleus, mammillary nuclei, posteroventral thalamic nucleus, ventral tegmental area, torus semicircularis, ventral isthmal nucleus, superior raphe (bilateral), and solitary nucleus (152, 167, 180, 192). Some innervating fibres express orexin, thyrotropin releasing hormone, tyrosine hydroxylase, dopamine, serotonin, nitric oxide, neuropeptide-FF and nitric oxide (47, 53, 54, 96, 125, 192). Serotonergic projections come from the nucleus of the paraventricular organ, and the lateral hypothalamic area sends dopaminergic projections to the forebrain, including the amygdala (53, 192). Additional projections innervate the red nucleus, dorsomedial thalamic nucleus, dorsolateral thalamic nucleus and ventromedial thalamic nucleus (128). Metabolic capacity is greater in males housed with females as compared to males housed individually (139, 147).

3.3.12 Posterior Hypothalamic Nucleus

The distinction between the posterior hypothalamic nucleus and the periventricular hypothalamic nucleus is unclear. Both Cruce (261) and Greenberg (176) recognize two distinct nuclei, however they reverse them. What Greenberg calls the periventricular hypothalamic nucleus, Cruce labels the posterior hypothalamic nucleus, and vice versa. In the past twenty years, no paper has mentioned both nuclei except one that uses both terms to refer to the same nucleus (233). Therefore, here we have treated both nuclei together. Furthermore, the dorsomedial hypothalamic nucleus is sometimes considered to be within this nucleus, however that nucleus is distinct enough to be treated separately (128).
The small, round or fusiform cells of this nucleus express neurophotoreceptors, substance-P, cholecystokinin, neuropeptide-Y, nitric oxide, aromatase, androgen, estrogen & progesterone receptors, 5α-reductase, galanin, dopamine receptors, somatostatin, orexin, tyrosine hydroxylase, thyrotropin-releasing hormone, calcitonin gene-related peptide, neuropeptide-FF and the R1α regulatory kinase subunit (37, 38, 41, 45, 46, 53, 54, 57, 95, 96, 108, 125, 130, 138, 202, 225, 228). Somatostatin-expressing cells are arranged in one or two layers around the third ventricle, and project their apical dendrites into the cerebrospinal fluid (45). The posterior hypothalamic nucleus has the most intense estrogen receptor expression of any brain region (130). Fibres innervating this nucleus express dopamine, serotonin, neuropeptide-Y, orexin, androgen & progesterone receptors, galanin, somatostatin, neuropeptide-FF, gonadotropin releasing hormone and nitric oxide (22, 37, 45, 46, 54, 90, 91, 96, 228, 270). This nucleus projects to the septum, the accumbens nucleus, ventromedial hypothalamus, and hypoglossal nucleus (52, 128, 166). It also receives septal projections (180).

Metabolic capacity is greater in females than in males, and in older females compared to younger females (139, 140). Ovariectomazation reduces metabolic capacity, and testosterone increases it (141). Androgen receptor expression is upregulated in late vitellogenic females compared to previtellogenic females (168). Observation of male or female sexual behaviour upregulates both estrogen and progesterone receptor expression in females and progesterone receptor expression is upregulated by estrogen (233, 270). In males, cohabitation with females decreases metabolic capacity (139).

3.3.13 Nucleus of the Infundibular Recess

This parvocellular nucleus contains small, round cells and larger, fusiform cells (45, 54, 93). It projects to the dorsomedial thalamic nucleus and the dorsal hypothalamic area (98, 167). These cells express galanin, somatostatin, orexin, mesotocin, corticotropin releasing hormone, androgen receptors, dopamine receptors, dopamine, serotonin, vitamin D3 receptors, and neuropeptide-FF (45, 48, 53, 54, 93, 96, 192, 228). Fibres innervating this area express galanin, somatostatin, orexin, calcitonin gene-related peptide, corticotropin releasing hormone (45, 54, 93, 108, 228). Orexinergic cells project dendrites to the lateral infundibulum and into the fluid of the third ventricle (54).
3.3.14 Mammillary Nuclei

Mammillary neurons express estrogen & progesterone receptors, aromatase, nitric oxide, calretinin, parvalbumin, and calcitonin gene-related peptide (38, 47, 57, 91, 108, 206, 240). The mammillary nucleus has bilateral, reciprocal connections with the septum (180) 1997). It also projects to the medial and dorsal cortices as well as to the dorsomedial and dorsolateral thalamic nuclei, the dorsal hypothalamic area, the ventromedial hypothalamus and the lateral hypothalamic area (52, 98, 128, 167). Anterior to the mammillary nucleus, neurons of the premammillary nucleus express androgen receptors (130).

3.3.15 Supramammillary Nucleus

The supramammillary nucleus expresses progesterone receptors, dopamine, cholecystokinin and calcitonin gene-related peptide (52, 59, 91, 108). Reciprocal connections with the septum are as per the mammillary nucleus, except these connections are ipsilateral (52, 180). This nucleus also projects to the ipsilateral medial cortex, dorsal cortex and red nucleus (59).

3.4 Pretectum

3.4.1 Thalamic Lentiform Nucleus

The thalamic lentiform nucleus can be divided into two parts: the “folded” plicata part, and the “extended” extensa part. The plicata part sparsely expresses progesterone receptors and receives projections from the septum (91, 180). The extensa part projects weakly to the red nucleus (216). This nucleus receives projections from the dorsomedial and dorsolateral thalamic nuclei as well as from the posterior medial nucleus of the thalamus (98, 134). In the plicata part, progesterone receptor expression is greater during vitellogenesis than postvitellogensis (169). This is the opposite pattern from other brain regions that show vitellogenic shifts in progesterone receptor expression, and is more typical of estrogen receptor expression.
3.4.2 Pretectal Geniculate Nucleus

This nucleus is made up of a medial plate of projection neurons and a dense lateral neuropil (206). A few neurons in the cell plate express calcitonin gene-related peptide (108). This region receives retinotopic projections from the retina and projects to the red nucleus and optic tectum (214). Projections from the retina are regenerated after optic nerve lesion (217).

3.4.3 Posterodorsal Nucleus

The posterodorsal nucleus expresses neuropeptide-Y and tyrosine hydroxylase, and the surrounding neuropil expresses calretinin (37, 96, 164, 200, 206). This nucleus receives projections from the retina and projects are sent to the ventromedial hypothalamus (128, 206, 214). Fibres innervating this nucleus express neuropeptide-Y, neuropeptide-FF and calcitonin gene-related peptide (37, 96, 108).

3.4.4 Nucleus of the Basal Optic Tract

This nucleus, which appears to be greatly enlarged in chameleons, receives input from the contralateral retina, but the projections do not form a retinotopic map (214, 271). After damage to the optic nerve, innervation to this nucleus regenerates temporarily but is subsequently lost (217). It receives additional input from the accumbens nucleus (195).

3.4.5 Nucleus of the Posterior Commissure

The nucleus of the posterior commissure is divided into magnocellular and parvocellular subregions. These neurons express parvalbumin and calcitonin gene-related peptide (108, 206). This nucleus projects to the dorsolateral thalamic nucleus and the red nucleus (98). It receives projections from the lateral geniculate nucleus (216).
3.4.6 Pretectal Nuclei

The pretectal nuclei consist of three closely associated cell clusters: the dorsal, medial, and ventral pretectal nuclei. The dorsal and ventral parts are also referred to as the principle pretectal and subpretectal nuclei respectively. Medial nucleus neurons express parvalbumin, 5α reductase, and androgen receptors and the surrounding neuropil expresses calbindin (56, 95, 206). The subpretectal neurons express parvalbumin, estrogen receptors, and nitric oxide (97). The subtectal neuropil expresses parvalbumin and receives dense innervation expressing nitric oxide (206). It receives projections from the optic tectum and projects to the rotund nucleus (218).

3.4.7 Mesencephalic Lentiform Nucleus

This nucleus is often assigned to the mesencephalon, but it is diencephalic and lies within the pretectum (207). Neurons express parvalbumin, substance-P and neuropeptide-FF and receive projections from the optic tectum and the retina (96, 202, 206, 214). It projects to the dorsolateral thalamic nucleus and receives projections from the lateral geniculate nucleus and the optic tectum (98, 215).

4 Midbrain

4.1 Tectum

4.1.1 Optic Tectum

The optic tectum is the primary retinorecipient structure in the squamate brain. It receives visual information, primarily from the contralateral retina, retinotopically: dorsal, ventral, temporal and nasal retinal projections innervate the lateral, medial, rostral and caudal tectum respectively (213, 219, 272). The optic tectum is divided into between six and fourteen distinct layers. Of the six main layers, the two most superficial are the optic layer and the superficial grey & fibrous layer. The optic layer is occupied by optic fibre tracts, which synapse primarily on the cell bodies and dendrites of the superficial grey & fibrous layer (273). Below those layers lies the central grey layer, a layer of cell bodies that receive projections primarily from the superficial grey & fibrous layer. Below that is the central white layer, which is occupied by the axons of
the cells in the central grey layer. Below that is the periventricular grey layer, which receives projections from the central grey layer. Finally, the periventricular fibrous layer contains projection axons before they exit the tectum.

The optic tectum expresses abundant progesterone receptors, serotonin receptors (B/D type) and protein kinase C (91, 94, 274). GABA is expressed by small interneurons interspersed among the large projection neurons in all grey layers, but are more abundant in the superficial grey layer (275). The cells of the superficial grey & fibrous layer also express tyrosine hydroxylase and vitamin D3 receptors (48, 53). The central layers receive input expressing thyrotropin releasing hormone and scarce orexin (54, 108). The neurons of the central grey layer express aromatase, neuropeptide-Y and parvalbumin (37, 38, 218). Neurons of the periventricular grey layer express dopamine receptors serotonin receptors (A1 type), tyrosine hydroxylase, estrogen receptors, nitric oxide, 5α reductase and the R1 α regulatory kinase subunit (41, 53, 57, 94, 95). The periventricular layers receive fibres expressing androgen receptors, somatostatin, gonadotropin releasing hormone and thyrotropin releasing hormone and scarce orexin and calcitonin gene-related peptide (45, 54, 56, 108).

The superficial layers of the optic tectum receive incoming connections. Primarily, these are glutamatergic projections from the retina, but additional projections are received from the septum, entopeduncular nucleus, pretectal geniculate nucleus, substantia nigra, descending trigeminal nucleus and lateral descending trigeminal nucleus (180, 214, 219, 275-277). Projections from the descending trigeminal tract project somatosensory information to the contralateral central grey and white layers (277). The central and periventricular grey layers then project to the contralateral optic tectum, other brain regions and motor neurons of the spinal cord (278). The most massive projections from the optic tectum project to the rotund nucleus. Other brain regions that receive tectal projections include the medial posterior nucleus (bilateral), lentiform mesencephalic nucleus, tectal grey area, lateral geniculate nucleus, perirotundal belt, suprapenduncular nucleus, ventrolateral & ventromedial thalamic nuclei, dorsomedial & dorsolateral thalamic nuclei, dorsolateral hypothalamus, triangular area, and the nucleus of the ventral tecto-thalamic tract (98, 119, 218). Uniquely, the dorsolateral geniculate nucleus receives projections from the superficial grey & fibrous layer (215).
In infrared-detecting snakes, visual and infrared sensory inputs converge on the optic tectum from the retina and thermosensory facial pits (121). Infrared information is received from the contralateral lateral descending trigeminal nucleus in boiids and the contralateral reticularis caloris in vipers (276). Both visual and infrared projections synapse onto the same tectal neurons, integrating both into one combined receptive field (276, 279).

After the optic nerve is severed in an Agamid lizard, regenerated fibres reinnervate the optic tectum and even reform a rough retinotopic map (217, 272, 280, 281). However, the lizard remains functionally blind despite regeneration of the optic nerve, and eventually these newly formed connections degenerate (272, 281). This may be due to the co-expression of NMDA and AMPA receptors during reinnervation. During normal development, the superficial layers express only NMDA receptors, which help establish retinotopic projections from the retina to the optic tectum. In adulthood, these layers only express AMPA receptors. After a lesion to the optic nerve, the superficial layers express both NMDA and AMPA receptors, which may help explain why a retinotopic map begins to form but is then lost (280). However, if there is only an incomplete severance of the optic nerve, the nerve regenerates and retinotectal topography is restored (282). In geckos, retinal projections to the optic tectum fail to regenerate following damage to the retinal nerve (283).

4.1.2 Torus Semicircularis

The torus semicircularis is the primary auditory processing center of the brain. It consists of a central cell nucleus surrounded by a laminarly organized cell layers. The torus expresses androgen, progesterone and estrogen receptors as well as somatostatin and nitric oxide (45, 56, 57, 91). Cells in the central nucleus express dopamine receptors, which are associated with fibres expressing tyrosine hydroxylase as well as calbindin (53, 221). The central nucleus has three subdivisions: the lateral subdivision has strong calretinin-expressing innervation, the ventral subdivision has a parvalbumin-expressing neuropil, and the remaining subdivision has sparse, calretinin-expressing bipolar cells (221). The laminar nucleus expresses neuropeptide-FF (96).

The torus is innervated by fibres expressing cholecystokinin, neuropeptide-Y, arginine vasotocin, orexin, calcitonin gene-related peptide (37, 54, 108, 138). The torus receives
projections from the septum and the lateral geniculate nucleus (180). It projects to the
dorsolateral & dorsomedial thalamic nuclei, medial nucleus, posterior medial nucleus,
medial posterior nucleus, dorsal hypothalamic area, ventromedial hypothalamic nucleus,
lateral hypothalamic area, red nucleus and posterocentral nucleus (98, 128, 134, 167).

Metabolic activity in the torus increases with age in females, and decreases with social
experience in males (139). In a temperature sex-determined species, males from the
‘feminizing’ temperature and females from the ‘masculinizing’ have higher metabolic
capacities than the same sex from the alternative incubation temperature (163, 262). In
such females, gonadectomy decreases the metabolic capacity, and testosterone rescues
this effect (163). Estrogen upregulates estrogen receptor expression but not
progesterone receptor expression (169). Progesterone receptor expression is higher in
postvitellogenic females compared to vitellogenic females (169). Estrogen receptor
expression is upregulated by estrogen (169).

4.1.3 Tectal Grey Area

Neurons and fibres of the tectal grey area express neuropeptide-Y (37). This area
receives visual projections from the retina, lateral geniculate nucleus and the optic
tectum and projects to the red nucleus dorsolateral thalamic nucleus (180, 214). Retinal
projections reinnervated the tectal grey area after optic nerve lesions (217).

4.2 Tegmentum

4.2.1 Oculomotor Nucleus

This nucleus expresses estrogen receptors, 5α reductase, aromatase, and corticotropin
releasing hormone (57, 93, 95). It receives projections from the dorsolateral vestibular
nucleus (284).

4.2.2 Red Nucleus

The red nucleus is the main premotor nucleus involved in limb movement, and is very
poorly developed in snakes (285). The magnocellular neurons of this nucleus express
androgen receptors, estrogen receptors, 5α reductase, and acetylcholine (56, 57, 95,
The strongest projections to the red nucleus come from the ipsilateral pretectal geniculate nucleus and bilateral ventrolateral geniculate nuclei (216). Additional projections come from the ipsilateral ventromedial thalamic nucleus, ventrolateral thalamic nucleus, entopeduncular nucleus, suparpenduncular nucleus, dorsolateral hypothalamic nucleus, lateral hypothalamic area supramammillary nucleus, nucleus of the posterior commissure, lentiform thalamic nucleus, torus semicircularis, profound mesencephalic nucleus, reticular formation, and descending vestibular nucleus (167). In addition, the red nucleus receives contralateral projections from the lateral cerebellar nucleus (285).

4.2.3 Substantia Nigra

The substantia nigra is one of the two main dopamine producing nuclei of the brain, except in snakes where there does not seem to be a division between the dopaminergic nuclei (286). It consists of the two parts: a reticulated, cell-poor part and a compact, cell-dense part (37). In addition to dopamine, neurons express neuropeptide-Y and nitric oxide (37). The substantia nigra projects to the optic tectum, posterior medial nucleus, red nucleus, accumbens nucleus and septum and has reciprocal connections with the dorsal striatum (52, 113, 134). It receives input expressing dopamine receptors, cholecystokinin, neuropeptide-Y, orexin, nitric oxide, neuropeptide-FF and thyrotropin releasing hormone (37, 53, 54, 125, 138).

Activity in this region increases during copulation (164). In subordinate males, but not dominant males, serotonergic activity decreases during an aggressive interaction (114). Viewing dominant colouration or aggressive display behaviours increases dopaminergic, serotonergic and noradrenergic activity (111, 287). After an aggressive interaction, dopaminergic activity increases in the dominant male (72). The density of dopaminergic cells increases with the frequency of male courtship displays (183). The number of dopaminergic cells also increases in late vitellogenic females compared to previtellogenic females (193).

4.2.4 Ventral Tegmental Area

The ventral tegmental area is one of two primary dopaminergic brain nuclei (286). It also expresses progesterone receptors, nitric oxide, and calcitonin gene-related peptide.
The ventral striatum has reciprocal connections with the ventral tegmental area (113). Bilateral input comes from the habenula and it receives additional input from the septum and accumbens nucleus (180, 195, 207). Innervating fibres express dopamine receptors, nitric oxide, cholecystokinin, thyrotropin releasing hormone, neuropeptide-FF and orexin (53, 54, 96, 125, 138). It sends projections to the septum, anterior dorsal ventricular ridge, diagonal band of Broca, olfactory tubercle, accumbens nucleus, olfactostriatum, dorsomedial & dorsolateral thalamic nuclei, dorsal hypothalamic area, ventromedial hypothalamic nucleus, lateral hypothalamic area, (52).

Activity in this region increases during an aggressive interaction and during courtship (164). When faced with an unfamiliar opponent, dominant males show an increase in norepinephrine in the ventral tegmental area (117). In contrast, when faced with a familiar opponent, dopamine, norepinephrine and epinephrine increase in the subordinate male (111, 117). Dopamine, norepinephrine & serotonin activity also increase when viewing a dominant opponent (287). During an aggressive encounter, there is decreased serotonergic activity in the subordinate male (114). After an aggressive interaction, dopamine activity increases in the dominant individual (72). Stress increases serotonergic, dopaminergic, noradrenergic and adrenergic activity (116). Males from a ‘feminizing’ incubation temperature upregulate dopaminergic cell number in response to testosterone, while males from a ‘masculinizing’ temperature do not (198).

4.3 Isthmus

4.3.1 Central Grey

The central grey expresses tyrosine hydroxylase and the R1α regulatory kinase subunit (41, 164). Projections come from the hypoglossal nucleus (166). It sends reciprocal connections to the septum and projects to the ventromedial hypothalamic nucleus (52, 128, 180). It is innervated by fibres expressing galanin, orexin, thyrotropin releasing hormone, and (sparsely) calcitonin gene-related peptide (54, 108, 125, 228).
4.3.2 Raphe Nuclei

The raphe consists of a superior nucleus and an inferior nucleus. It is the primary serotonin-producing region of the brain (216). Both nuclei also express progesterone receptors, dopamine receptors, nitric oxide and 5α reductase (53, 91, 95). Serotonergic neurons receive projections expressing orexin (54). Dopamine receptor expressing cells receive input from fibres expressing tyrosine hydroxylase (53). The superior raphe receives additional thyrotropin-releasing hormone projections (125). The inferior raphe expresses androgen and estrogen receptors (56, 57, 90). Both nuclei receive projections from the septum and project to the olfactostriatum, septum and dorsal cortex (52, 180). While both nuclei project to the accumbens nucleus, only the the superior raphe receives reciprocal projections from the accumbens nucleus (195). The superior raphe also projects to the dorsomedial and dorsolateral thalamic nuclei, dorsal hypothalamic area, ventromedial hypothalamic nucleus, and lateral hypothalamic area (98, 128, 167).

Serotonergic activity inhibits aggression, and activity in the inferior nucleus increases during aggressive interactions (110, 164). During an aggressive encounter between two males without an established dominance hierarchy, serotonergic and dopaminergic activity are both reduced (112). After an aggressive interaction, serotonergic activity in subordinate male decreases and it either decreases slightly or increases in the dominant male (114, 288). When a male views a subordinate opponent, serotonergic activity in the raphe decreases and noradrenergic activity increases (287). When a male views a dominant opponent, serotonergic and dopaminergic activity increase (287). Serotonin synthesis increases with long-term alcohol consumption, more in the right hemisphere than in the left (289). The right hemisphere is associated with aggressive behaviour in lizards, and this is reduced following chronic alcohol consumption (289). Stress increases serotonergic, dopaminergic, noradrenergic and adrenergic activity (71, 116). Exercise also increases serotonergic activity (104).

4.3.3 Locus Coeruleus

Neuronal cell bodies express progesterone receptors, noradrenaline and nitric oxide (91, 125). Orexin-, nitrix oxide-, neuropeptide-FF- and thyrotropin releasing hormone-expressing fibres innervate the locus coeruleus (54, 96, 125). This is the main noradrenergic brain nucleus, sending noradrenergic projections to rest of the brain,
including the dorsolateral thalamic nucleus, septum and accumbens nucleus (52, 98, 194).

When a male encounters a threatening, unfamiliar opponent, serotonergic activity in the locus coeruleus increases (117). During an aggressive interaction, serotonergic activity increases and dopaminergic activity decreases when observing a dominant male, whereas serotonergic activity decreases and noradrenergic activity increases when observing a subordinate male (114, 287). After an aggressive encounter, serotonergic and dopaminergic activity increase in both the subordinate and dominant males (72, 112, 114, 287). Serotonergic activity also increases in animals when they are under physical exertion (104). In response to restraint stress, for example by manually restraining a lizard, serotonergic, dopaminergic, noradrenergic and adrenergic activity all increase (71, 104, 116).

4.3.4 Trochlear Nucleus

This nucleus expresses estrogen receptors and 5α reductase (57, 95, 207).

4.3.5 Isthmal Nuclei

There are two isthmal nuclei: the superior isthmal nucleus is magnocellular and the inferior isthmal nucleus is parvocellular. Both nuclei express vitamin D3 receptors and receive projections expressing calcitonin gene-related peptide and neuropeptide-FF, and the parvocellular nucleus also receives cholecystokinin, tyrosine hydroxylase, and nitric oxide projections (48, 53, 96, 108, 138). The tyrosine hydroxylase expressing fibres surround dopamine receptor expressing cells (53). The superior isthmal nucleus projects to the ventromedial hypothalamic nucleus and the posterior medial nucleus of the thalamus, while the inferior isthmal nucleus projects to the dorsal hypothalamic area (128, 134, 167).

4.3.6 Interpeduncular Nucleus

This nucleus contains serotonin-expressing cell bodies (216). It receives projections from the septum, accumbens nucleus, medial habenula (180, 195). It receives projections expressing cholecystokinin, thyrotropin releasing hormone, calcitonin gene-
related peptide, mesococin, corticotropin releasing hormone and gonadotropin releasing hormone (22, 93, 108, 125, 138). The neuropil expresses nitric oxide (127).

5 Hindbrain

5.1 Cerebellum

The cerebellum is the only region outside the telencephalon known to have constitutive neurogenesis in adults (29, 73). Though there is some indication the neurogenesis occurs in the habenula and third ventricle (204, 205). It consists of a dorsal cellular layer and a ventral plexiform layer and projects to the red nucleus (97, 216). Cells express nitric oxide synthase, vitamin D3 receptors, progesterone receptors and the R1α regulatory kinase subunit, and receive somatostatin, gonadotropin-releasing hormone and calcitonin gene-related peptide input (41, 45, 48, 91, 108, 239). There is some evidence for mosaic evolution of cerebellum volume (290).

5.2 Nucleus of the Solitary Tract

The bipolar neurons of this nucleus express tyrosine hydroxylase, dopamine receptors, cholecystokinin, and nitric oxide (53, 138). Tyrosine hydroxylase cell bodies receive dense innervation from fibres expressing orexin, thyrotropin releasing hormone, dopamine receptors, nitric oxide, and neuropeptide-FF (53, 54, 96, 125).

5.3 Principle Sensory Trigeminal Nucleus

This nucleus consists of small and medium size neurons interspersed among the trigeminal fibres (220). This nucleus receives somatosensory information, with crude somatotopic organization, from the head and neck (220). Trigeminal neurons include touch, temperature, and mechanical nociceptive neurons, which have varying physical and electrical properties (291). For example, touch-sensitive neurons tend to be large with large axons, while temperature and mechanical-sensitive neurons are smaller with smaller axons (291). It projects somatosensory information to the periorotundal belt (bilateral), dorsolateral nucleus (ipsilateral), rotund nucleus (contralateral), posterior medial nucleus of the thalamus and lentiform thalamic nucleus (220). It receives
projections expressing nitric oxide, tyrosine hydroxylase and thyrotropin-releasing hormone (125, 134).

5.4 Descending Trigeminal Nucleus

This nucleus is divided into three subnuclei: oralis, interpolaris and caudalis (220). Its neuropil expresses calcitonin gene-related peptide and nitric oxide (108). This nucleus receives head and neck somatosensory information from the trigeminal ganglion and projects to the contralateral optic tectum, bilateral periorbital belts, posterior medial nucleus and bilateral lentiform thalamic nuclei (134, 220). It also projects to the hypoglossal nucleus, which controls the musculature of the tongue (166).

5.5 Lateral Descending Trigeminal Nucleus

This nucleus exists only in infrared-detecting snakes (276). It receives ipsilateral input from heat-sensing pit organs and projects to the contralateral optic tectum (276, 292). In crotaline snakes, the reticular heat nucleus acts as a relay between the lateral descending trigeminal nucleus and the optic tectum (276).

5.6 Dorsolateral Vestibular Nucleus

This nucleus of small-to-medium sized cells expresses calretinin, calbindin and nitric oxide (284). It receives calbindin-expressing projections from the vestibular nerve and additional gonadotropin-releasing hormone expressing projections (239, 284). It projects to the oculomotor nucleus, the hypoglossal nucleus and the dorsolateral thalamic nucleus (166, 167, 284).

5.7 Ambiguous Nucleus

The ambiguous nucleus consists of the nuclei of cranial nerves IX and X, the glossopharyngeal and vagal nerves respectively (159, 293). These nerves innervate the ceratohyoid muscle, which controls dewlap extension and vocal production (90, 157, 159, 293, 294). It expresses calcitonin gene-related peptide and the vagal portion expresses androgen receptors (56, 90, 108, 293). Though there is no sexual dimorphism
in neuronal number, soma volume is larger in males than females during the breeding season (293, 294).

5.8 Motor Nucleus of the Facial Nerve

This nucleus expresses glutamate, calcitonin gene-related peptide, calbindin and parvalbumin (108, 221). This nucleus can be divided into dorsal and ventral parts. The ventral part projects to the ipsilateral ceratothyroid muscle, which controls dewlap extension (157, 221, 293). During the breeding season, neuron cell bodies are larger in males than in females (157, 221, 293).

5.9 Superior Olivary Nucleus

This nucleus has dorsal and ventral parts, both of which express calbindin and parvalbumin and contain glutamatergic cells (221). The superior olivary nucleus receives bilateral, calretinin-expressing projections from the auditory nuclei, and additional projections expressing cholecystokinin (138, 221). It projects to the posterior medial nucleus of the thalamus and the hypoglossal nucleus (60, 134).

5.10 Nucleus of the Lateral Lemniscus

This nucleus is divided into anterior and posterior parts, where the posterior part contains many more glutamatergic cells compared to the anterior part (220). Both parts contain cells that express neuropeptide-Y, calbindin and parvalbumin (37, 221). Both parts receive dense glutamatergic input as well as projections expressing calbindin, calretinin and parvalbumin(221). The nucleus of the lateral lemniscus receives projections from the cochlear and auditory nuclei, and projects to the amygdaloid complex and the posterior medial nucleus of the thalamus (134).

5.11 Hypoglossal Nucleus

The motor neurons of the hypoglossal nucleus coordinate and control tongue-flicking behaviour (166). The nucleus receives projections from the brainstem and hypothalamus. In the brainstem, the central grey, isthmal reticular nucleus, reticular formation, deep mesecephalic nuclei, reticular nuclei, descending trigeminal nucleus,
dorsolateral vestibular nucleus, superior olive and dorsal motor nucleus of the trigeminal nerve all project to the hypoglossal nucleus (166). Its most significant hypothalamic projections come from the lateral posterior hypothalamic nucleus and it receives additional projections from the posterior hypothalamic and preoptic nuclei (166). The lateral posterior hypothalamic nucleus, in turn, receives projections from the medial amygdala (166).

6 Future Directions

In the past twenty years there has been a great expansion in squamate brain research. This literature can be broadly grouped into two distinct areas. First, many neuroscience researchers continue fundamental research on the structure, connectivity, and neurochemistry of the squamate brain from a predominantly anatomical perspective. These studies use methods such as tract-tracers and immunohistochemistry to examine histology. This work is of immense value and it provides the foundation from which we can examine possible homologies between the nervous system of squamates and other better-known vertebrate groups. Second, a new stream has emerged that focuses on linking brain structure and chemistry with behaviour, sociality, cognition and learning. By identifying particular aspects of behaviour, ecology or evolution in squamates, we can “work backwards” to try to link these to underlying brain function. Much of this work has focused on the neurobiological control of reproductive behaviour in three model systems: Anolis, Eublepharis and Thamnophis. Unique aspects of ecology, for example “she-males” in Thamnophis, of development, such as temperature-dependent sex determination in Eublepharis, and of social structure, such as the dominant-subordinate system in Anolis, create ideal systems for examining the neural basis of sexual behaviour and its evolution. As our appreciation for the behavioural and cognitive complexity of reptiles increases, new opportunities to study the neural underpinnings of the evolution of behaviour and cognition are constantly emerging. While great progress has been made, our understanding of squamate reptile brain anatomy and function lags behind other major vertebrate groups (24). Here, we outline broad areas of future research into both structural and functional aspects of squamate neuroscience that will help redress this gap in knowledge and hopefully accelerate the current pace of research.
6.1 Structural Directions

6.1.1 Atlases

The most widely used atlases for squamates date from 30 years ago, of which the most cited is Greenberg (176). It is an excellent resource, although by modern standards, what are available online are low-quality black-and-white scans of the atlas with poor resolution, poorly labelled structures, and poor longitudinal resolution. It also includes only the forebrain, leaving the midbrain and hindbrain structures completely unknown. A full brain atlas of *Tupinambis* was included in Donkelaar's review of reptile brains (1). This atlas is advantageous because it not only shows cell body clusters using Nissl stained sections, but also shows fibre tracts using Kluver-Barrera and Bodian-stained sections. To the best of our knowledge, it is the only modern brain atlas to show fibre tracts, although some stylized fibre tracts are drawn over the Nissl-stained images of the Greenberg atlas. Despite these advantages, it does not appear that this atlas is widely used based on citations of Greenberg (176).

The difficulty with both of these atlases, and others that are currently available (261, 295-298) is the absence of any rationale for the delineation of structures. The current squamate brain atlases indicate different structures by drawing lines to or placing text on top of cell clusters, without indicating how the delineation between different structures was determined. While modern atlases of other species may not, in themselves, discuss the rational for delineating different structures, they are supported by a vast supporting literature of detailed anatomical studies. Such a foundation is missing from squamate neuroanatomical research. This may be a source of confusion that results in inconsistencies between atlases, such as the distinction between the medial preoptic area and the anterior hypothalamus (see section 3.3.10), and the reversal of the posterior and periventricular hypothalamic nuclei (section 3.3.13). Furthermore, it becomes very difficult for people working on squamate brains to use these atlases to measure structures as it is unclear what the consensus is in delineating these structures, and how much consistency in delineation exists between research groups. While our review will be useful to anyone attempting to identify brain nuclei using protein expression profiles, the ability to identify the borders of brain regions, even fairly evident ones such as the lateral cortex and the amygdala, in the absence of extensive biochemical data, remains extremely difficult.
A modern and detailed atlas accompanied by a rationale for the delineation of squamate neural structures is necessary for neurobiological research in squamates to advance. In comparison to available bird and mammal atlases, the existing squamate atlases lag far behind in terms of size, in-plane resolution and longitudinal resolution. We need a squamate brain atlas that is consistent with the modern standards set by the mouse, rat and human atlases (299-301), which are regularly updated. In our opinion, the most useful candidate species for an updated atlas is *Anolis carolinensis*, the most widely used model squamate (Figure 3). *Anolis* is also ideal because the *Anolis* genome is now available (302) and there is a vast literature pertaining to the behavioural, ecological, physiological, endocrinological, and evolutionary aspects of *Anolis*. The congeneric *Anolis evermanni* is capable of behavioural flexibility (25) and recent comparative neurobiological work also has been conducted using *Anolis*, while also highlighting the current difficulty in studying squamate brains (290). The authors of this study indicated that they were unable to delineate the boundary between the mesencephalon and the rhombencephalon. In other vertebrate groups, where much better atlases are available, such delineation would be possible. The detailed atlas that we need for *Anolis* should greatly expand on the atlases already available. The basic essential is a large, clear, high-resolution series of Nissl-stained coronal sections of the *Anolis* brain with much finer longitudinal resolution. Such a series of images should clearly show the various nuclei of the *Anolis* brain. It would need to be accompanied by drawings of each section that clearly outline the various structures visible in the section, and distances based on a clear stereotaxic coordinate system. Greenberg (176) and Del Corral (296) use a stereotaxic coordinate system anchored by external features of the lizard head. Such a coordinate system needs to be updated to be consistent with that of other brain atlases by using the bregma and lambda points of the skull as the basis for stereotaxic coordinates, similar to the coordinate system recently proposed by Wang et al. (303).
Figure 3. Papers published on the neurobiology of various squamate species since 1995. Though there is broad phylogenetic coverage of squamate reptiles in the neuroscience literature, *Anolis carolinensis* has received the most attention based on number of papers published per species since 1995.
The Nissl-stained coronal atlas is the most basic, most essential guide necessary for navigating the brain, but it is not the only one. Both Greenberg and Donkelaar represented, to some extent, fibre tracts in addition to cell nuclei in their atlases (1, 176). A complete atlas of fibre bundles is as important as a Nissl atlas. A library of in situ hybridization atlases for different genes would be an excellent resource alongside the Anolis genome. Atlases based on the expression of proteins such as aromatase, acetylcholinesterase, or substance P, also would be useful. As we continue to move into new, more advanced technologies to ask novel questions in neuroscience, it will become increasingly useful to have brain atlases available in these same formats. For example, the insect brain atlas released this year includes an extensive MRI-based atlas, and such an atlas will prove useful as squamate neuroscience continues to develop (304, 305).

6.1.2 Nomenclature

6.1.2.1 Current Issues with Nomenclature

Nomenclatural inconsistency is an important issue in neuroscience and one that has hampered cross-fertilization across model systems and slowed progress generally. A recent systematic review of neuroscience nomenclature in insects found it severely deficient and an updated naming system was developed for the entire insect brain (305). Similarly, we have identified three issues in squamate neuroscience nomenclature that we think need to be resolved.

First, authors switch between the English and Latin names for the same brain region, obscuring keyword search results. The same paper may use English words for some brain regions and Latin words for others. For example, Dias et al. (38) use twenty-two English and six Latin names for different lizard brain regions. In contrast, Hoogland & Vermeulen-Vanderzee (50) use six English names and fourteen Latin names, and switch between English and Latin names in the same paper. This results in confusion because there is no consistency between papers as to which terms should be in English and which should be in Latin.

Second, there are some genuine cases where multiple different names are being used for the same brain region (Table 1). This use of synonymous terms is rare, and mostly originates when homologous structures are named differently in different species. In
this review we have identified synonyms based on location in the brain, connectivity, and chemical profile and explicitly stated which synonym should be used in the future based on the criterion of common usage over the past 20 years.

Third, there is inconsistency in how names are abbreviated. For example, we have found 12 ways to abbreviate “medial cortex” in the literature we reviewed: MC, CxM, CM, Mc, MCX, MCTX, M, CXM, Cxm, m, MCx, and cm. This inconsistency in abbreviations increases the likelihood of different papers using the same abbreviations to refer to different structures. For example, AME has referred to both the external and medial amygdaloid nuclei (section 2.3.3.4).

6.1.2.2 Proposal for a single system of nomenclature in squamate reptile neuroscience

We propose that future studies should adopt the nomeclatural system used in the mouse, rat, macaque, human, and chick brain atlases (299-301) and adopt the following conventions:

1. Names are consistently written in English, using anglicised terms (“dorsal lateral geniculate nucleus” instead of “nucleus geniculatus dorsolateralis”).

2. Abbreviations are in the order of words as spoken in English (dorsal lateral geniculate nucleus = DLG).

3. The capital letter representing the first letter of a word in a nucleus is followed, if necessary, by the lower case letter most characteristic of the word (Striatum = St).

4. Compound names of nuclei have a capital letter for each word (Nucleus of the Paraventricular Organ = PvO) excluding extremely common words such as “nucleus” and “thalamus”.

5. Fibre tracts and fissures (not covered in this review) are abbreviated in lower case characters (Medial Forebrain Bundle = mfb).
6. Arabic numerals are used instead of Roman numerals in identifying cranial nerves, cranial nuclei and layers of the spinal cord (not covered in this review). Cranial nerve abbreviations should carry a prefix “n” to distinguish them from the nucleus of the same nerve (Trochlear Nerve = n4; Nucleus of the Trochlear Nerve = 4). Spinal cord layers should carry the prefix “l” (layer 4 = l4).

We have included a list of proposed abbreviations for brain regions covered in this review (Table 1).

**6.1.2.3 Realignment of reptile brain nomenclature with bird brain nomenclature**

In the last ten years there has been a recognition amongst those who study neuroscience in avian systems that the standard nomenclature used for the structures of the avian brain were based on flawed assumptions of homology to mammalian brains. This was recognised to promote a large-scale misunderstanding of the organization of the avian brain and its functional, anatomical, and evolutionary relationships to the mammalian brain. In 2002, the Avian Brain Nomenclature Forum was held to bring together leading avian neuroscientists to devise an entirely new nomenclature to describe the structures of the avian brain (346,347,348). This nomenclature has now been widely adopted by those who study bird brains.

Reptile brain nomenclature, however, has never undergone a radical shift in nomenclature and terminology derived from the mammalian brain remains standard, and is what we have described in section 6.1.2.2. Birds are, strictly speaking, reptiles, and the squamate brain shares many more homologies with the bird brain than it does with the mammalian brain. However, the bird brain is not simply and elaboration of the reptile brain, as if often erroneously stated. Nonetheless, we think it would be appropriate to convert to using the avian brain nomenclature for reptiles, including squamates, as the same reasons as those that inspired in the creation of the revised bird brain nomenclature are still problematic in reptile brain nomenclature. However, converting to the bird brain nomenclature would involve simultaneous detailed anatomical study of the brains of reptiles and birds, and would likely be best served by concurrent comparisons of bird, squamate, crocodilian and turtle brains in order to capture variation in brain structure over the full evolutionary radiation. Such a study is
beyond the scope of this review. Our proposal, therefore, is for consistency of terminology until someone undertakes this much larger proposal.

6.1.3 Variation

6.1.3.1 Brain Size Variation

Relative brain size in birds and mammals has long been used as a proxy for neural complexity and associated behavioural complexity. In birds and mammals, robust comparative studies require brain-size measures from hundreds of species, and several specimens per species. This is possible largely because in birds and mammals the brain is almost completely enclosed in a brain case (306). It is easy to get a measure of brain volume simply by filling the brain cases of museum skulls with lead shot and weighing the quantity that fills the brain case. In this way brain size data has been accumulated rapidly for thousands of birds and mammals.

Unfortunately, obtaining similar volume estimates in squamates is difficult. The skulls of most lizards, and especially snakes, are greatly reduced and may be fragile (307). Squamate brains are not completely protected by bone, and therefore filling brain cases with lead shot is not a viable option for measuring squamate brain size. In birds, how closely the volume of the brain case reflects the volume of the brain is also a point of contention, but it does appear that the volume of the brain case can be used as an accurate proxy for brain volume (306, 308). In reptiles, to our knowledge, the relationship between brain case volume and brain volume has never been investigated.

The only method that has been used to gain data on the volumes of reptilian brains has been to extract the brains and weigh them directly. This, unfortunately, cannot be done using museum specimens and instead requires fresh tissue, making it much more expensive in terms of both time and money. Until recently, the extent of encephalization data available for squamates amounted to data from the 1970s for 50 species (309, 310). Encephalization values for six *Anolis* species were published in 2012, but this new data highlights a universal flaw with encephalization data (290). While Platel did not perfuse his specimens, Powell and Leal (290) did. In addition, fixation times and solutions differ between the two studies. It is unknown how these differences may influence brain weight and subsequently, values of encephalization. This is a problem with obtaining
encephalization data from the literature, and increasingly researchers are taking all encephalization data themselves to ensure consistent methodology for all measurements (308, 311). Researchers measuring squamate brain size should be careful to maintain as consistent as possible methods in order to maximize cross-study compatibility of data.

6.1.3.2 Brain Structure Variation

Squamate reptiles are a highly diverse and species rich group that display tremendous variation in their morphology, life history, behaviour and ecology. Like other major radiations, they probably also display great variation in cognition and brain structure. Unfortunately, most of what we know about the structure, chemistry, and function of the squamate brain comes from single-species studies and it is unknown how these results vary between species and higher-order groups. For most protein expression and connectivity patterns, any variation between species is unknown. In the rare cases where similar investigations have been carried out in multiple species, there are often variations found, which we have detailed in our review. For example, there is sexual dimorphism in androgen receptor expression in *Sceloporus* but not in *Eublepharis* (55, 130, 168). That different species have different protein expression patterns is not surprising, however it does highlight the need to look beyond single species studies and ask questions about how much brains vary between squamate species. For a long time there did not seem to be any available analysis as to why this variation might exist, or how it may reflect interspecific variation in behaviour, cognition, ecology or evolution. This is starting to change (203, 257, 290). These types of cross-species comparative studies of brain structure and brain chemistry have great potential help to draw links between the brain and behaviour and cognition, and how natural and sexual selection can act to influence brain structure over evolutionary time. Finally, variation in environmental variables such as temperature and moisture during embryonic development in the egg can have profound effects on brain structure as we detailed above. In viviparous species, while mothers can still buffer these effects, being ectotherms they are nonetheless constrained by fluctuations in environmental temperature. Taken together, squamates may exhibit far more intraspecific variation in cognition and brain structure in particular compared to endotherms, than we appreciate.
6.2 Functional Directions

6.2.1 Neural basis of reproduction

Neural control of reproductive behaviour has traditionally been an area of focus in squamate neuroscience research. *Anolis, Eublepharis, Cnemidophorus and Thamnophis* are all used as model systems for studying the role of steroid hormones, their metabolizing enzymes and receptors in reproductive behaviour. Behaviours associated with reproduction, such as male-male competition, territoriality, courtship and copulation, are associated with changes in well-studied neural circuits (112, 115, 288). This means that, more than for any other regions, we have data for multiple species on many aspects of neurochemistry, structure and function in the brain regions involved in these circuits, primarily the preoptic area (section 3.3.8) and the ventromedial hypothalamus (section 3.3.10). Having so many comparisons reveals just how much variation there can be between species. For example, gonadotropin-releasing hormone seems to be expressed in the preoptic areas of *Thamnophis, Eumeces* and *Sceloporus*, but not in *Anolis* (22, 239). Similarly, the ventromedial hypothalamus is larger in males in *Anolis*, larger in females in *Cnemidophorus*, and is not sexually dimorphic in *Eublepharis* and *Thamnophis* (160, 252, 263). What are the functional implications of these differences? Through a more comprehensive comparative approach we may be able to link these variations in structure with variations in reproductive behaviour and sexual selection.

6.2.2 Genomics and gene expression

The first full squamate genome is now available for *Anolis carolinensis* (302). This presents a remarkable and completely untapped opportunity as *Anolis* is also a principle squamate model system in behaviour, cognition, and neuroscience (Figure 3). Furthermore, *Anolis* remains the most frequently cited squamate brain atlas, and we have argued for making a modern squamate brain atlas of the *Anolis* brain. Therefore, *Anolis* represents the ideal system in which to study reptilian neurogenomics. Neurogenomics is the study of how the genome as a whole contributes to the evolution, development, structure and function of the nervous system (312). This field is completely new to squamate research, and represents immense potential. Using a comparative approach to study the evolution of gene expression in the brain is an
important avenue of research in neurogenomics. As new model organisms in neuroscience are developed across the vertebrate phylogenetic tree, it will become possible to compare gene expression patterns across groups as diverse as fish (zebrafish), reptiles (Anolis), birds (zebra finch, chicken, etc), and mammals (mouse, chimpanzee, human, etc). Extensive genome sequencing is accelerating the availability of entire genome sequences for the model organisms of neuroscience. Ultimately, this will facilitate a much more solid understanding of how gene expression underlies the evolution, development, and differentiation of the nervous system.

Furthermore, as genome sequencing becomes fast, cheaper, and more routine, genomes will become available for more squamate species. This is an excellent opportunity to link variation in squamate behaviour, cognition, ecology and evolution back not only to the nervous system, but also to genetics. There is a very basic need in neuroscience to understand just how, when and where genes, and the proteins they encode, are expressed in the brain (312). We still have a tenuous grasp on how to link gene expression with neuronal structure, and then with brain function and ultimately behaviour and cognition. Because squamates display such variability in behaviour and cognition within species, and between closely related species, they represent an ideal system for using a comparative approach not only to examine variations in the nervous system, but also variations in gene expression that underlie these cognitive and behavioural differences.

6.2.3 Evolutionary developmental neuroscience

We have avoided the topic of reptilian neural development because this is reviewed in depth by Nomura et al. (313), who make a similar case to ours, that reptiles offer a new and promising system in which to study the brain, particularly brain evolution. However, in contrast to our review, they focus primarily on embryonic and postnatal neural development in reptiles. By examining neurogenesis, cellular differentiation and cellular migration during brain development in reptiles, we can further understand characteristics that are difficult to study in mammals, such as postnatal neurogenesis and the development of the vomeronasal system (314). The mechanisms regulating neural development during embryogenesis are relatively easy to study in squamates since many lay leathery, porous eggs. Factors influencing neural development such as hormones and incubation temperature are more readily manipulated in embryos.
developing in eggs compared to inside their mother (315, 316). Reptiles are the only vertebrates where closely related species may lay eggs or have live birth; in a few cases the same species adopts both strategies. This presents a unique opportunity to study relative maternal influence on embryonic development. The *Anolis* genome provides further opportunities to study the links between genes, hormones, environmental factors such as temperature, and neural development, and it provides additional incentive to study neural development across the vertebrate phylogeny. By making links between neurodevelopmental patterns in reptiles, birds and mammals, we can gain a more robust and fundamental understanding of how the brain develops during embryogenesis and the mechanisms that control neural development (313).

**6.2.4 Sexual differentiation in animals without sex chromosomes**

Among amniotes, temperature-dependent sex determination is a uniquely reptilian sex-determination mechanism whereby the temperature at which eggs incubate determines their sex (317). It has been used extensively to study environmental, endocrine and genetic modulators of sexual differentiation since all embryos have the potential to become male or female (197, 248, 318, 319). We have referred to ‘masculinizing’ and ‘feminizing’ incubation temperatures throughout our review. Temperature-sex determination experiments have shown that while individuals from different incubation temperatures may develop primary and secondary sex characteristics that are clearly male or female, behaviourally and neurally there is less rigid division between the sexes. Males from ‘feminizing’ incubation temperatures – temperatures that produce mostly females and only a few males – show relatively female-like brains and some counterintuitive behaviours, such as increased frequency of male courtship behaviour (248). Females from ‘masculinizing’ temperatures have relatively male-like brains and are more aggressive and less receptive to male courtship (248). Functional connectivity within the limbic system varies depending not only on sex but also on incubation temperature (320). Systems such as this have great potential for teasing out details of how the brain controls various behaviours, and how sensitive these neural systems are to top-down endocrine control.
6.2.5 Neurogenesis

Reptiles are a well-known model system for the study of neurogenesis. They are the only amniotes capable of fully regenerating damaged regions of the brain (73, 105). For this reason they have been an intense area of focus for the study of neural regeneration and repair, for example in the cortex (section 2.1) and in the visual system. The visual system is particularly valuable because certain features, such as connectivity, are regenerated while others, such as retinotopographic organization, are not, and vision is not restored (321). This mystery as to why regeneration occurs without sight restoration provides a valuable opportunity to dissect the regeneration process. Furthermore, lizards have the most extensive constitutive neurogenesis and neural proliferation in adults of any amniote, including the cortex, olfactory bulbs (section 2.2) and cerebellum (section 5.1) (29). In mammals the study of neurogenesis is an area of intense research due to the obvious medical implications for new treatments for neurodegenerative diseases, neural injury, and reparation of developmental defects (18). However, post-natal and adult neurogenesis in mammals is limited (322). Many authors are indicating that breakthroughs in our understanding of neurogenesis are likely to come from beyond mammalian systems (323). An increasingly informative approach to understanding neurogenesis is through comparing information gained in studies across fishes, amphibians, reptiles, birds and mammals, (322, 324-326). Reptiles are particularly ideal for such studies as they combine phylogenetic proximity to mammals with extensive neurogenic capabilities (44, 73). Recent studies have started to shed light on the control of neural proliferation and migration, particularly on the involvement of glial cells (105, 327). As these processes are seasonal in some, but not other species, lizards represent an ideal opportunity to examine external involvement in neurogenesis regulation, such as changes in temperature and hormones. And with the new Anolis genome, lizards represent an ideal opportunity to more fully understand the gene networks involved of neural stem cell proliferation and subsequent specialization. Furthermore, the precise function of the new neurons produced by constitutive neurogenesis remains unknown (328). Studies using mammals and birds have consistently failed to find a link between neurogenesis and proficiency at various behavioural tasks (328). Particularly due to high levels of constitutive neurogenesis in areas of sensory and higher-level processing, squamates are ideal for studying the relationship between behaviour or cognition and neurogenesis (73, 329).
6.2.6 Climate change and neural development

In reptiles, incubation temperature affects a wide range of phenotypic traits including body size, head size and locomotor performance (330, 331). Of direct relevance to neuroscience is that the thermal environment experienced by developing embryos has a profound effect on both learning ability and neurobiology in lizards (332, 333). Hatchling lizards from hotter nests learnt a series of tasks faster than those from cooler nests and these differences were also reflected in forebrain organisation. Specifically, lizards incubated at higher temperatures had greater neural density and more neurons compared to hatchlings incubated at lower temperatures, which had significantly larger neurons and forebrains (absolute and relative to body size). Based on the important finding that environmental temperature influences cognition in lizards, the possibility that climate change could profoundly influence cognition in large numbers of populations and species is real. Lizards are only partially able to compensate for temperature increases due to climate change, and therefore as the climate shifts, cognition and intelligence may shift with it (332, 334). Such shifts are necessarily coupled with underlying changes in brain development. Questions about how ambient temperature affects brain development during embryogenesis and beyond are therefore now far more relevant than ever before. The mechanisms underlying this developmental plasticity may help us understand and predict how rising global temperatures may affect complex brain organisation and function.

6.2.7 Evolution of sociality and cognition

One avenue of neurobiological evolution research where the potential of squamates remains completely untapped is ‘social’ brain research. Social intelligence theory—the idea that social behaviour is a precursor for increasingly complex cognition—has been proposed as a general explanation for the evolution of large brains and cognition (335). The Australian lizard clade *Egernia* may be a particularly appropriate system with which to investigate the role of sociality on social learning. This group appears to be unique among lizards because of the high frequency of species that live in family groups (336). Furthermore, they also exhibit varying degrees of sociality from solitary species to extended family groups and although many species exhibit basic parental care, they are socially less complex than previously studied model species, such as birds
and primates (336). Therefore, they may represent a unique ‘intermediate’ state appropriate for understanding the early evolution of socially-based cognition.

6.2.8 Sexual selection, mating systems, alternate reproductive tactics

Squamates, and lizards in particular, adopt a multitude of reproductive tactics that are either condition-dependent or fixed. In the case of sexually dichromatic species, males typically show high levels of precopulatory sexual selection by defending territories or mates, and females may be choosey, although female choice in lizards is contentious and poorly understood (337, 338). Alternatively, females may be promiscuous and mate multiply, setting the stage for postcopulatory sexual selection through sperm competition or cryptic female choice (338). Sexual dimorphism in both body size and coloration is common among lizards and has evolved many times independently (337, 339). Furthermore, male colour polymorphism is not uncommon and may reflect alternate reproductive tactics. Perhaps the best known example of this is the side-blotched lizard (*Uta stansburiana*) in which throat colour morphs represent alternate reproductive tactics as follows: males with orange throats are aggressive and defend large territories encompassing many females; blue-throated males defend smaller territories containing fewer females and mate-guard; and yellow-throated males do not defend territories (‘sneakers’) and range over a much larger area and may resemble females (340). Interestingly, the demands imposed by varying levels of territory size are reflected in the volume of the dorsal cortex which is greatest in orange morphs, intermediate in blue, and lowest in yellow (62). Lizards offer many such opportunities for testing hypotheses about brain structure and organisation in relation to mating and social tactics. Finally, lizards adopt a multitude of mating systems from long-term monogamy (lifetime in the sleepy lizard, *Tiliqua rugosa*) to polygyny and polyandry (341). We need more studies examining the relationship between sexual selection, mating system, social and alternate reproductive tactics and the brain.

6.2.9 Links to Medicine

The basal ganglia is highly conserved between squamates, birds and mammals in structure and connectivity (94, 188). Compared to mammals and birds, the small cortex in squamates allows for the study of basal ganglia function in the relative absence of interference from the cortex (113). The basal ganglia is heavily involved in cognitive
and behavioural responses to stress, and research in squamates has provided valuable insight into how stress affects the basal ganglia resulting in stress-mediated changes in motivation and behaviour (15, 19, 112, 115, 342). Lizards are also a valuable system for examining the relationship between stress, aggression, sociality and monoamine activity in the basal ganglia (343, 344). The basal ganglia is responsible for the simple, repetitive behaviours in lizards known as fixed action patterns or stereotyped displays (113). This is also the brain region thought to be involved in the repetitive, stereotyped behaviours associated with obsessive-compulsive disorder in humans (188). The basal ganglia in *Anolis* has been used successfully as a model for obsessive-compulsive disorder, particularly by examining how stress and the disruption of serotonergic function in the basal ganglia affects the display of fixed action patterns (113). These are similar to the ways in which stress and serotonin are believed to be involved in the pathology of obsessive-compulsive disorder (113, 188). Similarities between the basal ganglia of squamates and mammals has lead to the suggestion that squamates may be useful in studies related to other disorders of the basal ganglia, such as Parkinson’s disease and Tourette’s syndrome, and even the behavioural effects of alcohol consumption (113, 345).

7 Conclusion

Lizards and snakes represent a valuable opportunity for furthering our understanding of the brain and the selective forces shaping its evolution. The amazing array of behaviours, life history strategies, reproductive tactics, morphology and general ecology of reptiles mean that the cognitive abilities exhibited by squamates (lizards in particular) are likely to be constrained in a myriad of ways that are quite different from more traditional mammal and bird models. Questions about the link between social intelligence and brain size, the neural basis of monogamy and pair-bonding, and even the poorly understood link between brain function and cognitive ability all have the potential to be investigated more readily and more thoroughly in squamates. In particular, the potential for studies in brain function, particularly using comparative approaches, is vast.

In order to realize this potential, we must first bring the quality of the tools needed to study squamate brains in line with those available in birds and mammals. We need a model brain atlas, preferably of an *Anolis* lizard, which represents a strong model
organism in behavioural ecology, genomics, and neuroscience. We need a consistent terminology with which to discuss the reptilian central nervous system, and we need a better understanding as to how that nervous system varies within the squamates. Outside of neuroscience, we need to accelerate the pace of genome sequencing in squamates to enable comparative neurogenomics. Furthermore, we need to target ecological and behavioural studies to systems that can be used to answer fundamental and important questions in neuroscience, such as *Egernia*. Squamates are an underutilized resource in neuroscience and hold great potential for the advancement of such diverse fields as neuroendocrine function, brain evolution, and the neural basis of cognition. We hope that in providing this resource we are able to help enable the realization of this potential.
Chapter 2

A perfusion protocol for lizards, including a method for brain removal
Abstract

The goal of fixation is to rapidly and uniformly preserve tissue in a life-like state. Perfusion distributes fixative quickly throughout the entire organism by pumping fixative directly through the circulatory system, achieving optimal fixation. Standard perfusion techniques were developed primarily for application in mammals, which are traditional neuroscience research models. Increasingly, other vertebrate groups are also being used in neuroscience. Following mammalian perfusion protocols for non-mammalian vertebrates often results in failed perfusions. Here, I present a modified perfusion protocol suitable for lizards. Though geared towards standard brain perfusion, this protocol is easily modified for the perfusion of other tissues and for various specialized histological techniques.

Abbreviations

PBS: Phosphate-buffered saline

PFA: Paraformaldehyde
Method Details

Reliable fixation of nervous tissue is a prerequisite for valid histological investigations. Transcardial perfusion utilizes the circulatory system to distribute fixative throughout the organism efficiently. The animal, traditionally a mammal, is opened up just below the thoracic cavity, which is then entered through the diaphragm (1). The heart is exposed, incisions are made into the right atrium and left ventricle, and a specialized, blunt-tipped needle (perfusion needle) is inserted into the left ventricle or the aorta. Fixative is then perfused throughout the animal. This has the added advantage of removing all blood, which may otherwise obscure histological features.

Recently, there have been significant advances in our understanding of the neurobiology of squamate reptiles (lizards and snakes), with particular emphasis on lizards (2-4). Although perfusion is a commonly used method of fixation in lizard neuroscience, there is no published methodology for perfusing lizards. Lizards differ from mammals in anatomical and physiological ways that affect the perfusion method. Here, I have taken a standard mammalian perfusion protocol and modified it for lizards. All materials necessary for this procedure are listed in Table 1.

1 Prepare Buffered Solutions

1.1 Prepare stock solution, 10x phosphate-buffered saline (PBS):

Ingredients
80.0 g NaCl
2.0 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
Distilled Water to 1000 mL

Directions:
1. Dissolve all salts into distilled water.
2. Store at room temperature.
Table 1: Supplies needed for lizard perfusion, with suppliers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Catalog or Model Number</th>
<th>Steps</th>
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<td>3.2</td>
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<td>Vials for lizard heads</td>
<td></td>
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</table>
The dissection microscope I use was manufactured by Wild Heerbrugg, which is now part of Leica. A currently available microscope equivalent to the one I use is listed.

I made my own perfusion needle from a glass pipette pulled to fit the lizard's aorta and glued over a butterfly needle. Depending on the size of the lizard's aorta, a commercial perfusion needle may suffice, one may need to be made.
1.2 Prepare prefix perfusate, heparinized PBS:
Ingredients:
100 mL 10x PBS
10 units Heparin
Distilled Water to 1000 mL

Directions
1. Add heparin to 10x PBS, then add water to 1000 mL.
2. Check pH. If necessary, add NaOH or HCl until the solution has a pH of 7.4.
3. Store at 4°C.

1.3 Prepare fixative perfusate, 4% paraformaldehyde (PFA):
Ingredients:
40.0 g PFA
100mL 10x PBS
Distilled water to 800 mL
NaOH pellets (or 10 M NaOH)
Additional distilled water to 1000 mL

Directions:
1. Add PFA to 10x PBS.
2. Add distilled water to 800 mL.
3. Stir vigorously and heat to 60-65°C. Do not allow the solution to heat above 65°C, this will denature the PFA and ruin the solution.
4. Add three pellets of NaOH and stir for 30 minutes
5. If the solution has not cleared, add two additional pellets of NaOH and stir for an additional 15 minutes. Repeat until the solution has cleared.
6. Add distilled water to 1000 mL
7. Adjust pH to 7.4 using HCl.
8. Filter.
9. Store at 4°C for up to one week.

1.4 Prepare storage buffer, azious PBS:
Ingredients:
100 mL 10x PBS
0.090 g NaN₃
Distilled Water to 1000 mL

Directions:
1. Add NaN₃ to 10x PBS, then add water to 1000 mL.
2. Stir until dissolved.
2. Adjust pH to 7.4 with NaOH and HCl.
3. Store at 4°C.

1.5 Buffer solutions used during perfusion are usually kept at 4°C prior to and during perfusion. However, for some mammal perfusions solutions are warmed to 37°C (mammalian body temperature). Lizards are ectotherms and as such room temperature is their body temperature. If a protocol calls for perfusion of solutions at body temperature, warm buffers to room temperature prior to perfusion.

2 Prepare Apparatus

Two types of devices are commonly used to perfuse liquids into the circulatory system: those depending on gravity to propel solutions, and those that use a pump system. I use a pump system, which is generally sufficient for protocols such as Nissl staining and immunohistochemistry. However for some purposes, such as electron microscopy, a gravity system may be more appropriate. Furthermore, gravity systems are useful in the field, as they do not require electricity.

2.1 Run distilled water through the perfusion tubing and perfusion needle to rinse.

2.2 Draw prefix perfusate into the perfusion tubing. 2 mL per 50 g of lizard is sufficient to flush the blood from the circulatory system. Take care to avoid any bubbles forming in the tubing.

2.3 For a small lizard, the amount of prefix needed is so small that it is possible to store the entire amount in the perfusion tubing. In this case, measure out the required amount of prefix, draw it into the tubing, and mark off the appropriate place on the tubing with a permanent marker. Then continue to fill the tubing with fixative until there is no air left in the tubing. Always take care not to leave any air bubbles in the tubes. For larger
lizards, as with mammals, the switch from prefix to fixative has to occur during the perfusion.

2.4 Affix the perfusion needle to the outlet end of the tubing.

2.5 Turn on the perfusion pump and adjust the flow rate until there is a weak but even flow of liquid out the end of the perfusion needle (no dripping). Flow rate is heavily dependent on the gauge of the needle and the model of perfusion pump. To find the correct pressure, start with a pressure at which liquid drips from the needle. Increase pressure slowly until the stream flows steadily. I use pressures between 75 and 100 mmHg.

3 Anaesthesia

3.1 Prepare a syringe with sodium pentobarbital, dose: 100 mg/kg (5). I do not recommend inhalation anaesthetics, although I have seen it used. Lizards are able to hold their breath for long periods of time and so determining the dose of anaesthetic an animal has inhaled is difficult (5, 6).

3.2 Draw an equal volume of lignocaine into the syringe.

3.3 Administer to the lizard via intraperitoneal injection. I recommend cooling the lizard to room temperature first by removing all heating elements from the lizard’s enclosure 12 hours prior. This makes injection easier as cooler lizards are calmer and have lower heart rates.

3.4 Complete anaesthesia occurs less than ten minutes following an injection of anaesthetic. Once the lizard appears unconscious, test the level of anaesthesia by pinching the lizard’s toe sharply. If the animal is sufficiently anaesthetized, it will show no response.
4 Surgery & Perfusion

I highly recommend using a dissection microscope for all of sections 4 and 5. Relative to their body size lizard hearts and brains are smaller than those of mammals, and some steps are quite intricate and delicate (7, 8).

4.1 Place the animal abdomen-up on the dissection pad.

4.2 Pin each foot to the dissection pad (Figure 1A).

4.3 Open the abdomino-thoracic cavity, exposing the visceral organs. To do this, cut through the skin, abdominal wall, and peritoneal membrane just below the ribs, at the posterior end of the sternum (Figure 1B).

4.4 There is no barrier between the visceral organs and the lungs (no diaphragm) (9), so immediately start cutting up the two sides of the chest, through skin, abdominal wall and ribs, to the level of the clavicle (Figure 1C). As with all perfusions, while cutting through the ribs and body wall be careful not to sever any large blood vessels or damage organs such as the lungs, liver and intestines. A good way to do this is to orient the scissors up and away from the lizard while cutting. The pericardium may be attached to the body wall. If the heart does not detach easily, it can be gently pushed down using a small spatula.

4.5 Pin back the flap of skin (Figure 1D). This should provide a clear view of the heart and major blood vessels.

4.6 In lizards the pericardium is stronger than in mammals and must be cut away manually. Carefully lift the pericardium away from the heart with forceps to create a tent of empty space and then cut the pericardium open with very small scissors. Use two pairs of forceps to pull the pericardium and enlarge the hole, exposing the heart. Cut the heart free of the pericardium by cutting the gubernaculum cordis, which attaches at the base of the ventricle.

4.7 Make a small incision in the posterior end of the ventricle (Figure 2).
Figure 1. Proper preparation of an anaesthetized lizard for perfusion. A: the lizard is pinned abdomen-up to a dissection pad (step 4.2). B: a horizontal incision is made just below the sternum (step 4.3). C: two parallel incisions are made up from the horizontal incision to the clavicle (step 4.4). D: the abdomen is lifted, inverted, and pinned to the dissection pad, exposing the heart (step 4.5).
Figure 2. A schematic diagram of a lizard heart showing correct placement of the **perfusion needle**. Prior to needle insertion, incisions are made into the ventricle (step 4.7) and right atrium (step 4.8). The perfusion needle is then inserted though the incision in the ventricle into the right aorta (step 4.9). The tip of the perfusion needle is visible in the right aorta below the branch point of the right systemic artery.
4.8 Make a small incision in the right atrium (Figure 2).

4.9 Insert the perfusion needle through the incision in the ventricle into the correct aorta (Figure 2). In mammals, the perfusion needle is inserted into the left ventricle or aorta. This is impossible in lizards, because, unlike mammals, lizards have a single ventricle and two aortas (Figure 2) (10, 11). The two aortas and the pulmonary artery all emerge as a single trunk from a ventricular structure called the bulbar ring (10, 12). For brain perfusion, the perfusion needle must be inserted into the right aorta, from which the carotid arteries emerge (10). The base of the right aorta is obscured, so needle placement can be difficult (Figure 2). When viewed under a dissection microscope, the tip of a correctly placed perfusion needle is visible through the transparent wall of the right aorta (Figure 2). To perfuse tissues other than the brain, it may be appropriate to insert the perfusion needle into the left aorta or pulmonary artery.

It is important that there is no back flow of liquid out of the ventricle, so it is critical to choose a gauge of perfusion needle that fits securely into the aorta. In larger lizard species it may be possible to clamp the needle in the aorta.

4.10 Pin the heart in place. Carefully insert a pin through the ventricle, lateral to the perfusion needle and below the level of the bulbar ring. Push the pin down through the lizard’s dorsum and into the dissection pad. Avoid the spinal cord. Pins may also be used to stabilize the perfusion needle.

4.11 Turn on the perfusion pump, which should have been set to the correct pressure in step 2.5.

4.12 Allow the perfusion to proceed until about 2500 mL fixative per kg lizard has been used.

4.13 Turn off the perfusion pump, remove the perfusion needle, and then remove the lizard from the dissection pad.

4.14 Remove the head posterior to the tympanal membranes (if visible) and the jaw bone.
4.15 Pin the head to the dissection pad (Figure 3A). Two pins are inserted through the external nares and two more pins through the upper temporal fenestras (Figure 4).

4.16 Cut away the skin and muscle to expose the base of the spinal column (Figure 3B).

4.17 Find the small opening at the base of the skull, where it attaches to the spine. In this space, cut away the membranes covering the brain, which are exposed in this gap (Figure 3C).

4.18 Remove the head from the dissection pad and place the head in fixative for 24 hours at 4°C.

**5 Brain Dissection**

5.1 Pin the head to the dissection pad (Figure 4). The lizard skull is both smaller and far more flexible than the mammal skull and the brain is not fully encased in bone (13). This means that pressure applied to the head of a lizard is more likely to result in damage to the brain. Pinning the head removes the risk of applying manual pressure to the head.

5.2 Cut the skin up both sides of the head (Figure 3D). Cut through the postorbital bone (Figure 4) and into the eyes.

5.3 Lift up the flap of skin and cut it away.

5.4 Pull the spine away from the spinal cord by grasping the spine firmly and pulling it straight backwards, away from the head (Figure 3E, 4).

5.5 Pull the muscle away from the sides of the skull. There is no bone protecting the sides of the brain, so work carefully.

5.6 Carefully remove the posterior portion of the skull (Figure 4).
Figure 3. The process of removing a lizard brain. A: the head is pinned to the dissection pad (step 4.15). The anterior pins are inserted through the nostrils, and the posterior pins through the upper temporal fenestrae. B: the posterior dorsal skin is removed, exposing the spinal column and base of the skull (step 4.16). C: the membranes covering the brain are cut away from the gap between the skull and the spinal cord, exposing the brain (step 4.17). D: the skin is removed from the dorsal surface of the head, including the eyes (step 5.2). E: the spinal cord is exposed by removing the spine (step 5.4). F: the skull is removed, exposing the dura-covered brain (steps 5.6-5.8). G: the dura is removed (step 5.9). H: the brain is carefully lifted and the ventral nerves severed (steps 5.12-5.13). The brain is now free of the head and can be removed.
Figure 4. Lateral, dorsal, and caudal views of a standardized lizard skull which highlight the bones that must be removed to extract the brain.

Prior to the removal of the skull, the head should be pinned through the external nares and upper temporal fenestrae (step 5.1). First, the postorbital bone is cut, represented by the red lines (step 5.2). Second, the spinal cord, shown in purple, is removed (step 5.4). Third, the posterior skull, in green, is removed (step 5.6). Fourth, the dorsal skull, in orange, is removed (steps 5.7-5.8). Finally, the ear bones, in yellow, are removed (step 5.11).
5.7 Carefully remove the top portion of the skull (Figure 4). The lizard skull is thicker than the mouse skull, and the brain is smaller relative to body size (8, 13). To break apart and remove the skull I use a corneoscleral punch instead of the more traditional rongeurs (Supplementary Materials 2). I find the corneoscleral punch to be more adept at the delicate task of removing the thick skulls of lizards without damaging small brains. For very large lizard species, rongeurs may be useful.

5.8 Carefully remove the bone covering the olfactory tract and the olfactory bulbs (Figure 4). While the brain sits just posterior to the eyes, the olfactory bulbs rest anterior to the eyes (14). They are connected to the rest of the brain by a pair of fiber bundles known as the olfactory tracts, which are delicate and prone to tearing. Extreme care should be taken when removing the bone covering the olfactory tracts (the frontal bone) as the olfactory tracts may be attached to it. If the olfactory bulbs are not necessary for study, it is much more efficient to sever the olfactory tracts at their base and skip steps 5.8 and 5.10.

5.9 Cut away the dura (Figure 3G) using the same method as for the pericardium in step 4.6. This membrane may be blackish (Figure 3F) and is much stronger than in mammals.

5.10 Cut the olfactory bulbs away from the vomeronasal and olfactory nerves. Be careful to keep the olfactory tracts intact. This step can be skipped along with step 5.8 if the olfactory bulbs are not necessary for study.

5.11 Remove the ear bones by crushing them and then pulling them laterally away from the brain (Figure 5).

5.12 Lift the brain carefully to expose the nerves attached to the brain, including the optic nerves.

5.13 Carefully sever the nerves, keeping the scissors well clear of the brain (Figure 4H).

5.14 Grasp the spinal cord with tweezers and lift the brain from the lizard’s head.
6 Post-fixation and Storage

6.1 Place the brain in fixative for 24 hours at 4°C.

6.2 Wash the brain in phosphate-buffered saline by replacing the solution three times, swirling gently between each exchange.

6.3 Store the brain in storage buffer at 4°C.

7 Additional Information

There are many important differences between mammals and reptiles that affect perfusion protocols, which I have outlined above. One additional difference between mammals and reptiles is that heart beat is maintained for longer after anaesthesia in reptiles compared to mammals, even once the heart is exposed (pers. obvs.). This allows more time to follow this procedure carefully and to make sure that the perfusion needle is inserted properly into the correct aorta.

With this protocol lizard brains can be successfully perfused, with complete blood clearance and full fixative penetrance. However, perfusions are delicate. Accidental cutting of a major blood vessel, slippage of the perfusion needle, air bubbles in the perfusion tubing and tachycardia prior to perfusion all may reduce the penetrance of fixative into the circulatory system and leave blood in the tissue (15). As problems may occur without being noticed during the procedure, it is important to verify that the perfusion was successful. Once the brain has been extracted, it can be examined for any visibly red blood vessels along its surface (Figure 5). Even if no blood vessels are visible, a yellowish or pinkish brain is the result of an unsuccessful perfusion, while a successful perfusion will result in a white brain (Figure 5).

In mammals, two commonly used indicators of successful perfusion are loss of colour in the visceral organs, particularly the liver, due to blood clearing; and muscle-twitching when the fixative (paraformaldehyde) is initially pumped through the animal (1). In lizards, however, the visceral organs do not perfuse and the liver will not decolour. This is because lizards have two aortas which circulate blood to the visceral organs (Figure 2), but only the right aorta also circulates blood to the head, including the brain (10, 11).
A successful perfusion of the brain need only perfuse through the right aorta (Figure 2) and does not fully perfuse the visceral organs. As the head is perfused, however, the mouth lining and tongue will be white after a successful perfusion and pink after an unsuccessful one. For the same reason twitching may occur throughout the body, or may be restricted to the head region.

This is a basic protocol, suited for simple histological procedures such as Nissl staining. This procedure is easily adapted for different types of tissue analysis, such as immunohistochemistry, electron microscopy, and live tissue extraction. Each procedure has its own specific modifications that would be made to the standard mammalian perfusion protocol (1), and those are also easily applied to this lizard perfusion protocol. My goal is to make lizard neuroscience more accessible to researchers, and with this protocol I hope to help interested researchers study neuroscience in lizards.
Figure 5. Dorsal (A, B & C) and ventral (D, E & F) views of successfully and unsuccessfully perfused brains. In unsuccessfully perfused brains (A, B, D & E), congealed blood is clearly visible in blood vessels on the surface of the brains, and the brains have an overall yellowish or pinkish tinge. A well-perfused brain (C & F) shows no visible blood vessels and the tissue is white.
Chapter 3

A 3D MRI-based atlas of a lizard brain
Abstract

Magnetic resonance imaging (MRI) is an established technique for comparative morphological analysis of the brain, particularly in the medical sciences. Despite its prevalent use in related fields, use in evolutionary neuroscience is in its infancy. Few magnetic resonance brain atlases exist outside the standard model organisms in neuroscience, such as mice, rats, monkeys and humans, and no magnetic resonance-based atlas has been produced for a reptile brain. Here, we present a magnetic resonance-based atlas for the brain of an agamid lizard, the tawny dragon (*Ctenophorus decresii*). We used a high-field 11.74T magnet, a paramagnetic contrasting-enhancing agent and minimum-deformation modeling of the brains of thirteen adult male individuals. From this, we created a high-resolution three-dimensional model of a lizard brain and we use it to thoroughly illustrate and describe the neuroanatomy of an exemplar lizard species. Magnetic resonance imaging presents a promising tool for comparative neuroscience, including in the study of evolution neuroscience using reptilian model systems.
1 Introduction

The reptile brain remains the most poorly known of any vertebrate group and this may be due in part to slow progress in the modernization of reference atlases. The most recent reptile brain atlases (1, 2), while technically excellent for their time, are now outdated because they are lacking in resolution and precision compared to modern atlases available for other vertebrate groups (3, 4). Addressing these deficiencies is important as reptiles are an excellent system with which to study evolutionary neuroscience (5-7). For example, reptiles are an ideal group with which to study the evolution of cognition (5, 8, 9) and its relationship with the evolution of sociality (10, 11). Interest in the neurobiology of squamates, including both single-species studies (12, 13) and comparative studies (6, 14, 15), is also increasing. However, the absence of modern anatomical references is hindering progress in reptilian neuroscience.

Brain atlases are traditionally based on histological studies (generally Nissl stained coronal sections) and are still produced for prominent model systems in neuroscience such as mice (3), rats (4) and humans (16). Recent histological brain atlases for reptiles include two for cryptodire turtles (2, 17), however most recently published atlases are for squamate reptiles. These include: iguanids (18, 19), lacertids (20), teiids (2, 21), dactyloids (1) and geckonids (22). While highly informative, histological studies are time consuming, introduce fixation artifacts, and only provide an in vitro view of the brain with respect to the head and braincase and so are not useful for all fields of brain research (23).

Magnetic resonance imaging (MRI) is a noninvasive technique that can visualize brain anatomy in situ in three dimensions without dissection, histological sectioning, or staining. However, it should be noted that imaging brains in situ results in a lower level of resolution compared to extracting the brain and imaging it in vitro. Whole brains imaged in vitro have been imaged to resolutions as low as 10 µm (24) but they are more frequently imaged at resolutions of 30–100 µm, particularly in vivo (23). MRI has become an established technique in neuroanatomical research and MRI-based neuroanatomical studies are available for the brains of many vertebrate groups, including teleost fish (23-25), chondrichtian fish (26), birds (27), and mammals (28-31). To our knowledge, only one study has imaged the brain of a reptile using MRI, the
snake *Thamnophis sirtalis* (32), however low resolution meant that only major neural subdivisions could be distinguished.

In this study, we present a detailed description of the brain of a squamate reptile using high-resolution MRI. We selected for study an Australian agamid lizard (the tawny dragon, *Ctenophorus decresii*) that is a generalist species without any obvious anatomical specializations. The three-dimensional nature of this atlas allows for viewing through any plane, and automated scrolling through the three standard orientations simultaneously. This atlas therefore provides the means to understand the structure and connectivity of the reptile brain at a level of detail far beyond what has been previously available.

2 Methods

2.1 Specimen Acquisition

Thirteen male tawny dragons (*C. decresii*) were collected from the southern Flinders Ranges, South Australia. We euthanized each lizard with an injection of 100 mg/kg sodium pentobarbital and an equal volume of 2 mg/mL lignocaine. Each lizard was then intracardially perfused with 1 µL/mL heparin in pH-neutral phosphate-buffered saline followed by a pH-neutral solution of 0.1% Magnevist (gadopentate dimeglumine, Bayer) and 4% paraformaldehyde in phosphate-buffered saline. Magnevist was used to maximize image contrast in magnetic resonance imaging (23). The lizards were subsequently decapitated and the heads were post-fixed for 24 hours in 4% paraformaldehyde. We then dissected the brains from the heads and post-fixed the brains for 24 hours in 4% paraformaldehyde. Finally, the brains were stored at 4°C in a solution of 0.1% Magnevist and 0.05% sodium azide in phosphate-buffered saline. The Australian National University’s Animal Experimental Ethics Committee approved all research under protocol number A2011-49.

2.2 Magnetic Resonance Imaging

Whole-brain images were acquired using a Bruker Avance 11.74 Tesla wide-bore spectrometer (Ettlingen, Germany) with a micro-2.5 imaging probe capable of generating magnetic gradients of 1.50 T/m. Brains were immersed in Fomblin
(perfluoropolyether, Grade Y06/6, JAVAC, Sydney, Australia) and placed in a 10 mm
diameter Wilmad tube using a custom-built plastic holder. The brains were buoyant in
Fomblin and the plastic holder was designed to hold them submerged. Parameters used
in the scans were optimized for gray-white matter contrast in the presence of Magnevist.
We used a gradient echo (T2*-weighted) 3D fast gradient-echo sequence (FLASH), with
a repetition time = 40 ms, echo time = 8 ms field-of-view = 11 × 11 × 16 mm and
matrix size = 110 × 110 × 160, producing an image with 50 µm³ isotropic voxels.

For comparison, fixed brains were embedded in agarose and sectioned at 70 µm using a
vibratome. We stained all sections for 5 min using SYBR-green (Life Technologies
Australia, Melbourne, Australia), rinsed them for one hour in phosphate-buffered saline,
and mounted them in Fluoro-Gel (ProSciTech, Brisbane, Australia). Fluorescence was
visualized using an Olympus BX63 microscope. Images were captured using an XM10
digital camera and the imaging-stitching function of the Olympus CellSens software
package.

2.3 Model Generation and Analysis

To ensure consistent measures of brain morphometry all images were first manually
masked such that consistent coverage of brain structures and nerve endings was
achieved. In the tawny dragon the olfactory bulbs are separated from the brain on long
stalks and we were unable to stabilize their location in the Wilmad tube. Therefore, the
olfactory bulbs were included in the masked regions. The manually masked areas were
then set to the background value such that they were not included in subsequent
calculations.

Thirteen brain image datasets of 50 µm³ resolution were first re-oriented to standard
rostro-caudal orientation. All images were then B0 intensity inhomogeneity corrected
using the N3 algorithm (33). An image with a good signal to noise ratio and no obvious
artifacts was then manually selected from the group to create an initial model by
blurring. All images were then recursively matched to this evolving model of average
structure to create a minimum deformation average with a resulting resolution of 20
µm³. The details of the model creation process can be found in Janke & Ullmann (34).
The fitting stages in this case started at a resolution of 1.28 mm and finished with a
resolution of 80 µm³. The model finished with a resolution of 23.5 µm³.
The major subregions of the brain were manually segmented from the 3D model. The telencephalon, diencephalon, optic tectum, tegmentum, cerebellum, and rhombencephalon were identified using the criteria of ten Donkelaar (2). Because no atlas exists for the tawny dragon brain or any close relative, a variety of references were used to identify brain areas (1, 2, 18-22, 35-41). Amira software (FEI, Hillsboro, Oregon) was used to manually segment the model, generate the 3D surface renderings and the 2D images.

3 Results

Our tawny dragon brain model represents the average spatial positioning and intensity of each neural structure based on the non-linear averaging of thirteen tawny dragon brains. Using the intrinsic three-axis nature of MRI-atlases (24), we have established a coordinate system with x-coordinates running rostro-caudally, y-coordinates running medio-laterally and z-coordinates running ventro-dorsally (Figure 1). The midline of the brain, which divides the two hemispheres, has been designated as the plane y = 0.

We delineated the six major neural subdivisions (Figure 2), and in addition we were able to identify visible nuclei, fiber tracts and ventricles, totaling over 200 structures (Table 1). However, we were not able to delineate all neuronal regions, and have taken a conservative approach to brain region delineation. Figures 3-19 show our atlas in sequential coronal sections, figures 20-28 show sequential horizontal sections, and figures 29-34 show sequential sagittal sections. Where possible, the terminology of ten Donkelaar (2) is used, however it is translated to English where possible, as per modern convention (3, 4).

Tissue regions with the highest water content are generally the neuropil layers and these therefore appear lightest, while nuclei have lower water content and appear darker. Generally, fiber tracts are the darkest due to extensive hydrophobic myelination, however signal intensity varies extensively due to differences in cell size, extent of myelination, and neurochemistry in each region. Different nuclei and fiber tracts are differentiated based on differences in signal intensity. This model is viewable in coronal, horizontal, and sagittal orientations online at www.tissuestack.com.
Figure 1. Coordinate system for the lizard brain. A three-dimensional view of the lizard brain model with coordinate axes indicated (a), a sagittal section through the lizard brain model at y=0.00 mm (b), and a coronal section through the lizard brain model at x=7.75 mm (c).
Figure 2. Lateral view of an MRI model of the brain of the tawny dragon (*Ctenophorus decresii*). The six major brain regions of the brain have been identified by colour. Light green = telencephalon, dark green = optic tectum, red = diencephalon, purple = cerebellum, blue = mesencephalon, yellow = medulla.
Table 1. Brain regions identified herein. Each region is identified in the figures by its abbreviation as indicated here. Figures in which each brain region has been delineated are indicated.

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<td>Abbreviation</td>
<td>Coronal</td>
<td>Horizontal</td>
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<td>vcf</td>
<td>15, 16</td>
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Figures 3-19. Coronal sections through an MRI model of the brain of the tawny dragon (*Ctenophorus decresii*). Figures are in rostro-caudal order and each section is 25 voxels or 587.5 µm caudal to the previous section. The plane of each section in our coordinate system is indicated in the top left corner. The bar in the top right corner = 250 µm. A list of abbreviations is found in Table 1.
Figure 5.

Figure 6.
Figure 9.

Figure 10.
Figure 11.

Figure 12.
Figure 13.

Figure 14.
Figure 15.

x = 8.93 mm

Figure 16.

x = 9.51 mm
Figure 17.

Figure 18.
Figure 19.
Figures 20-28. Horizontal sections through an MRI model of the brain of the tawny dragon (*Ctenophorus decresii*). Figures are in ventro-dorsal order and each section is 25 voxels or 587.5 µm dorsal to the previous section. The plane of each section in our coordinate system is indicated in the top left corner. The bar in the top right corner = 500 µm. A list of abbreviations is found in Table 1.
Figure 21.
Figure 25.
Figure 26.
Figure 27.

z=5.29 mm
Figures 29-34. Sagittal sections through an MRI model of the brain of the tawny dragon (*Ctenophorus decresii*). Figures are in latero-medial order and each section is 25 voxels or 587.5 µm medial to the previous section. The plane of each section in our coordinate system is indicated in the top left corner. The bar in the top right corner = 500 µm. A list of abbreviations is found in Table 1.
Figure 30.
Figure 31.
Figure 32.

y = 1.76 mm
Figure 33.
3.1 General Morphology

A volumetric rendering of the tawny dragon model brain can be seen in Figure 2. Paired olfactory bulbs are not visible as they were not included in the model, but are located anterior to the rest of the brain, attached by long stalks known as the olfactory peduncle (Figure 34). The paired cerebral hemispheres are the most rostral structures in the model. Caudal to the hemispheres, the diencephalon emerges ventrally followed by the optic tectum dorsally. Caudal to the optic tectum is the cerebellum, which rests over the posterior surface of the optic tectum. This is unique to squamates, in all other vertebrates the cerebellum points caudally, away from the optic tectum (2). Caudal to the diencephalon is the mesencephalon (excluding the optic tectum), followed by the medulla.

3.2 Cerebral Hemispheres

The telencephalon, excluding the olfactory bulbs and olfactory peduncle, consists of the paired lobes known as the cerebral hemispheres. The cerebral hemispheres contain three major components: the pallium, the striatum and the septum. In reptiles, the pallium has two distinct parts: the laminar cerebral cortex, located medial, dorsal, and lateral to the lateral ventricle; and the dorsal ventricular ridge, which emerges ventrally and protrudes up into the lateral ventricle. In our model, the lateral ventricles have collapsed anteriorly, however their location is apparent due to the extremely dark alveus, a fiber layer that runs along the dorsal surface of the lateral ventricle. In posterior sections of the cortex the lateral ventricles have not fully collapsed and the space of the lateral ventricle is visible as a hyperintense region. The choroid plexus, which produces cerebrospinal fluid from blood, is visible as a dark region within the lateral ventricle. The lateral ventricles are absent from the most posterior aspect of the cortex.

3.2.1 Olfactory Peduncle

Through the majority of the olfactory peduncle the olfactory tracts are a single dark region situated dorsocentrally in the peduncle, surrounded by the lighter outer granular layer. At the posterior end of the olfactory peduncle, close to when it joins the cerebral hemispheres, the granular layer expands and forms the anterior olfactory nucleus. The olfactory tracts themselves being to diverge and the medial, lateral and accessory
olfactory tracts are distinguishable as separate light regions. Towards the posterior end of the anterior olfactory nucleus, the olfactory tubercle forms along the ventral surface. Subsequently, the intermediate olfactory tract diverges from the medial olfactory tract. The olfactory ventricle has collapsed throughout most of the visible olfactory peduncle in our model, however close to the cerebral hemispheres it is visible as a hyperintense ventromedial region. This is the point at which the pallium first appears, on the dorsal surface of the olfactory tract. The rostral pole of the pallium has been identified as tissue belonging to either the lateral (22, 42) or medial (43, 44) cortex. In our model no layers are distinguishable in the pallium at this level and therefore we have avoided assigning it to a specific cortical region. The olfactory peduncle itself transitions into the septum and the striatum, while the olfactory canals connects with the lateral ventricles.

3.2.2 Cerebral Cortex

The laminar cerebral cortex contains three cell layers, defined by their variations in cell density. The outer and inner plexiform layers appear relatively light, while the central cell layer appears dark compared to the plexiform layers (Figure 35). The dark alveus runs deep within the inner plexiform layer and is seen to connect with the anterior and posterior pallial commissures. A cell layer, the periventricular layer, exists along the surface of the lateral ventricle, but is not visible as a distinct layer from the alveus in our model.

The laminar cerebral cortex is divisible into four regions, which are distinguishable based on cell density and morphology in the central cell layer: the medial, dorsomedial, dorsal and lateral cortices. In MRI, the cortex is relatively homogenous in contrast. Little, if any differentiation of the four cortical regions can be accomplished from MRIs of single brains. In our model, the different regions are subtly visible, however the limits between the four regions are indistinct. We have estimated these regions based on subtle differences in contrast, gaps where the cell layer has the same intensity as the surrounding plexiform layers, and comparisons with histological studies.
Figure 35. Coronal sections through an MRI model (grey scale) and a fluorescence DNA-stained brain (green) compare the resolution of the layers of the medial and dorsomedial cortices (a) cerebellum (b) and optic tectum (c). Although layers are distinguishable using both imaging methods, resolution is superior in the histological sections. opl = outer plexiform layer, cl = cell layer, ipl = inner plexiform layer, a = alveus, LV = lateral ventricle, EZ = ependymal zone, gl = glomerular layer, Pl = Purkinje layer, ml = molecular layer, pwl = periventricular white layer, pgl = periventricular grey layer, cwl = central white layer, cgl = central grey layer, sgfl = superficial grey and fibrous layer, ol = optic layer
The medial cortex is the clearest region; it lies above the septum and is characterized by a cell layer that is distinctly darker than the surrounding plexiform layers and therefore provides the clearest three-layered structure in the cortex. Where the medial cortex transitions into the dorsomedial cortex the cell layer widens and becomes lighter in intensity. The cell layer of the dorsomedial cortex also appears slightly convex. The dorsal cortex regains the distinct three-layered structure with a thin, prominent cell layer anteriorly that becomes less distinct as the inner plexiform layer decreases in intensity posteriorly. The lateral cortex is the most indistinct of the three cortices. The cell layer of the lateral cortex is diffuse and provides little contrast to the plexiform layers in our model. We have delineated the lateral cortex based on the absence of features that define surrounding structures, such as the three distinct layers of the dorsal cortex.

There is a distinct thickening of the cerebral cortex at the level of the dorsomedial cortex. This corresponds to a layer of large cells visible in histological sections and we have tentatively labeled this the dorsomedial interposition.

### 3.2.3 Dorsal Ventricular Ridge

The dorsal ventricular ridge is a pallial region ventral to the lateral ventricle. The majority of telencephalic tissue in the lizard is dorsal ventricular tissue, and it is divided into two divisions: the anterior dorsal ventricular ridge and the posterior dorsal ventricular ridge or amygdaloid complex. The anterior dorsal ventricular ridge is relatively homogenous in contrast and it is dark compared to the striatum and septum due to the significant projections it receives from the dark fibers of the lateral forebrain bundle, located ventrolaterally to the anterior dorsal ventricular ridge. The projections from the lateral forebrain bundle into the anterior dorsal ventricular ridge are some of the clearest projections visualized in our model.

The amygdaloid complex, or posterior dorsal ventricular ridge, occupies the posterior pole of the dorsal ventricular ridge, and extends ventro-caudally to the striatum. The central amygdalar nucleus appears ventral to the posterior pole of the anterior dorsal ventricular ridge and is delineated by a subtle increase in intensity. Below the central amygdalar nucleus lies the spherical nucleus, which is clearly visible as the lightest amygdaloid region. The remaining nuclei are delineated only as estimations in comparison to histological sections and published atlases. They are the external
amygdalar nucleus, the lateral amygdalar nucleus, and the medial amygdalar nucleus. The cell plate of the medial amygdalar nucleus is clearly visible as a relatively dark region ventral to the spherical nucleus, but the delineation between the medial and external amygdalar nuclei is not clear. Further caudally the spherical nucleus descends and is isolated from the remaining amygdalar nuclei. At this point it is not possible to distinguish the remaining amygdalar nuclei.

3.2.4 Striatum

The striatum appears ventral to the anterior dorsal ventricular ridge and anterior to the amygdaloid complex. It is easily distinguished from the anterior dorsal ventricular ridge by the cell-free dorsal-medullary lamina, which appears as a light layer, however the lamina is partially obscured by the dark projections from the lateral forebrain bundle to the anterior dorsal ventricular ridge. Due to the many dark fiber bundles coursing through the striatum, including the lateral and medial forebrain bundles, medial, intermediate, lateral and accessory olfactory tracts and stria medullaris, the individual nuclei of the striatum are mostly obscured.

One region clearly distinguishable amidst the dark fiber tracts is the light accumbens nucleus, which appears surrounding the ventral extension of the lateral ventricle. Further caudally the accumbens nucleus is replaced by the similarly light striatoamygdaloid transition area.

Most of the fiber bundles in the basal forebrain are not readily distinguishable from each other but instead appear as one continuous dark zone. However, the lateral forebrain bundle is clearly distinguishable, and we have used it as an approximate boundary between the dorsal striatum and ventral striatum as per ten Donkelaar (2) and Smeets (22).

3.2.5 Septum

The septum is located along the medial wall of the telencephalon from a point just posterior to the expansion of the lateral ventricle rostrally to a point just posterior to the anterior commissure caudally. It is bounded laterally by the lateral ventricle and dorsally by the medial cortex. Ventrally the septum is continuous with the structures of
the olfactory tubercle and ventral striatum. Towards the posterior end of the telencephalon the septum is ventrally bound by the anterior commissure. The septum is a relatively light region but it is innervated by dark fibers from the medial olfactory tract and medial forebrain bundle coursing upwards from the subpallium. They produce a clear dorsoventral intensity gradient in the septum, with the dorsal aspect of the septum being the lightest and the ventral aspect being the darkest. The septal nuclei are not distinguishable in our model, except for the inferior septal nucleus as it is bordered dorsally, laterally, and ventrally by the anterior pallial commissure.

3.3 Diencephalon

The diencephalon is located between the telencephalon and the mesencephalon and is generally divided into five regions: epithalamus, preoptic area, dorsal thalamus, ventral thalamus, and hypothalamus. The rostral pole of the diencephalon can be identified by the appearance of the preoptic recess of the third ventricle, which coincides with the start of the optic chiasm. The posterior pole of the diencephalon can be identified as the point at which the mammillary bodies terminate. The preoptic recess of the third ventricle connects caudally to the third ventricle proper. The third ventricle runs along the midline of the mesencephalon and is continuous with the subarachnoid space dorsally. In our model it has mostly collapsed, however it is visible as a dark vertical line along the midline.

3.3.1 Epithalamus

The epithalamus consists of the habenula, stria medullaris, and epiphysis. The habenula is divided into medial and lateral parts. They are visible at the dorsal aspect of the diencephalon, ventral to the posterior pallial commissure. The lateral habenula appears as a round, dark region jutting up from the diencephalon into the posterior pallial commissure. The medial habenula appears as a light region in contiguous with the midline and dorsal to the third ventricle.

The stria medullaris is an epithalamic fiber bundle connecting the two cerebral hemispheres. Its fibers cross the midline through the habenular commissure, which appears at the caudal end of the habenula.
Though the epiphysis is neural tissue, it is not considered part of the brain and is generally not included in neural atlases. In our model, it is visible as a dark region between the caudal poles of the telencephalon and the rostral poles of the optic tecta.

### 3.3.2 Dorsal Thalamus

The dorsal thalamus lies ventral and caudal to the epithalamus, rostral to the pretectum, and dorsal to the ventral thalamus and hypothalamus. Its major nuclei are the dorsomedial thalamic nucleus, dorsolateral thalamic nucleus, dorsal part of the lateral geniculate nucleus, rotund nucleus, and medial thalamic nucleus.

The first appearance of the dorsal thalamus is just caudal to the appearance of the habenula, where the dorsomedial nucleus appears against the wall of the third ventricle and then expands ventrally. At the caudal end of the dorsomedial thalamic nucleus it is bound dorsally by the posterior commissure, and the commissural fibers coursing through the dorsomedial thalamic nucleus result in an intensity gradient. At this level, the dorsomedial thalamic nucleus is lightest ventrally and darkest dorsally. Caudally, the dorsomedial nucleus is replaced by the plicata part of the thalamic lentiform nucleus.

The dorsolateral thalamic nucleus appears lateral to the dorsomedial thalamic nucleus, ventral to the habenular nuclei and medial to dorsal part of the lateral geniculate nucleus. It is darker than the dorsomedial thalamic nucleus but lighter than the lateral habenula. This nucleus is divisible into two components, a dorsal, lighter parvocellular part and a ventral, darker magnocellular part.

The dorsal part of the lateral geniculate nucleus lies lateral to dorsolateral thalamic nucleus and medial to the optic tract dorsally and the ventral part of the lateral geniculate nucleus ventrally. This nucleus consists of a clearly visible as a dark region, the cell plate, surrounded by a lighter neuropil. The neuropil is also lighter than the surrounding nuclei.

The rotund nucleus begins caudal to the other dorsal thalamic nuclei, posterior to the anterior fascicle of the lateral forebrain bundle. It is bounded dorsomedially by the dorsomedial thalamic nucleus, dorsolaterally by the lentiform thalamic nucleus,
ventromedially by the medial thalamic nucleus, and ventrally by the ventral thalamus. The rotund nucleus is somewhat darker than the other dorsal thalamic nuclei. It expands caudally to occupy a large central area of the dorsal thalamus and is clearly delimited by a dark neuropil-free area.

The medial thalamic nucleus appears with the rotund nucleus posterior to the anterior fascicle of the lateral forebrain bundle. It is a small, light nucleus lying ventromedial to the rotund nucleus and dorsal to the hypothalamus along the third ventricle.

### 3.3.3 Ventral Thalamus

The ventral thalamus begins further rostrally than the dorsal thalamus, at the level of the start of the habenula. It is composed of the interstitial nucleus, triangular area, oval nucleus, ventrolateral thalamic nucleus, ventromedial thalamic, entopeduncular nucleus, the ventral part of the lateral geniculate nucleus, the medial posterior nucleus and the posterocentral nucleus.

As the rostral limit of the third ventricle expands ventrally, the interstitial nucleus forms an arc over the forebrain bundles and merges ventrally with the anterior hypothalamus. The interstitial nucleus is separated from the anterior hypothalamus by the dark fibers of the medial forebrain bundle. The interstitial nucleus is darker than the anterior hypothalamus but lighter than the forebrain bundles.

The oval nucleus, a light, round region, is present at the same level as interstitial nucleus and lies dorsal to it. The nucleus is located rostrally and slightly medially to the dorsal part of the lateral geniculate nucleus.

Dorsolaterally and caudally, the interstitial nucleus is replaced by the darker triangular area, which rests over the forebrain bundles but is more lateral and ventral than the interstitial nucleus. The triangular area is bordered dorsally by the dorsal thalamus and laterally by the cell plate of the ventral portion of the lateral geniculate nucleus.

Ventral to the triangular area is the entopeduncular nucleus, which is a light area within the most dorsal part of the lateral forebrain bundle.
Lateral to the triangular area is a small, dark, cell free area, which is followed by the rostral pole of ventral part of the lateral geniculate nucleus. It has a distinct layered appearance, with a darker cellular layer medial to a lighter neuropil layer. The ventral part of the lateral geniculate nucleus is bounded laterally, dorsally and ventrally by the optic tracts and dorsomedially by the dorsal part of the lateral geniculate nucleus. The ventral part of the lateral geniculate nucleus extends further caudally than the dorsal part. At its caudal pole, this nucleus is bounded dorsally by the pretectal geniculate nucleus and medially by the ventrolateral thalamic nucleus.

The ventrolateral thalamic nucleus lies ventromedial to the ventral part of the lateral geniculate nucleus, lateral to the ventromedial thalamic nucleus and dorsal to the dorsal peduncle of the lateral forebrain bundle. The dorsal and ventral parts of the ventrolateral thalamic nucleus are separated by a dark, cell-free layer. The ventromedial thalamic nucleus lies ventral to the rotund nucleus and lateral and dorsal to the hypothalamus. The final two nuclei of the ventral thalamus, and the thalamus as a whole, which we have been able to delineate are the medial posterior and posterocentral nuclei. These nuclei are located caudally to all other thalamic nuclei, at the level of the posterior and tectal commissurae. They are relatively dark nuclei located ventral to the pretectum and dorsal to the hypothalamus. The medial posterior nucleus is a round, medial light area while the posterocentral nucleus is more laminar, pointing ventrolaterally from the medial posterior nucleus to the nucleus of the basal optic root.

3.3.4 Preoptic Area

The preoptic area is a rostral extension of the hypothalamus and the most rostral part of the diencephalon. It sits along the ventral midline above the optic chiasm. The delineation between the preoptic area and hypothalamus has been noticeably inconsistent between authors. Here, we follow Cruce (21) as he provides an extensive justification for his delineations and a comparison to other methods. Cruce defines the preoptic area as the area ventral to the anterior commissure. The preoptic area is replaced by the hypothalamus when the anterior commissure terminates.

In our model, three regions are distinguishable within the preoptic area by subtle variation in intensity. All regions appear dark due to the abundance of anterior
commissural fibers projecting through the preoptic area. Surrounding the preoptic recess of the third ventricle is the periventricular preoptic nucleus. This is the darkest and smallest of the preoptic regions. Surrounding the periventricular preoptic nucleus is the medial preoptic nucleus, and surrounding that is the lateral preoptic area, which is bound laterally by the medial forebrain bundle’s projections to the septum.

The nucleus of the anterior commissure is located in the preoptic region but is associated with the anterior commissure, a subpallial structure, and therefore it is unclear whether the nucleus of the anterior commissure is a telencephalic or diencephalic structure. Cruce (21) conservatively does not place it in either region. Smeets (22) and Greenberg (1) place it in the telencephalon while Northcutt (19) places it in the diencephalon. This nucleus is visible in our model as a light region dorsal and to the dorsal pole of the third ventricle and ventral to the anterior commissure. The border between the commissure and the nucleus is not well defined as the cells of the nucleus are scattered throughout the fibers of the anterior commissure, as well as surrounding it, creating a dark-to-light dorso-ventral gradient within the nucleus of the anterior commissure. If the nucleus of the anterior commissure is part of the preoptic area, it is its caudalmost component.

3.3.5 Hypothalamus

The rostral limit of the hypothalamus is at the level of the termination of the anterior commissure, where it consists of the anterior hypothalamus, a light area surrounding the rostral limit of the third ventricle. Caudal to the optic chiasm, this nucleus is continuous with the periventricular hypothalamic nucleus. Other major nuclei of the hypothalamus are the supraoptic nucleus, ventral hypothalamic nucleus, lateral hypothalamic nucleus, dorsomedial hypothalamic nucleus, dorsolateral hypothalamic nucleus, and the mammillary nuclei.

Ventral to the anterior hypothalamus is the supraoptic nucleus. Caudally, the supraoptic nucleus shifts dorsolaterally along the border of the optic tracts before being replaced by the ventral thalamus.

The lateral hypothalamic area is a relatively cell-poor area rich in fibers of the supraoptic decussation and the forebrain bundles. Therefore, it appears dark compared
to the remaining hypothalamic nuclei, but light compared to the fiber tracts. It appears lateral to the periventricular hypothalamic nucleus posterior to the supraoptic decussation and continues to the caudal pole of the hypothalamus, where it is replaced by the pretectum.

The ventromedial hypothalamic nucleus lies lateral to ventral half of the periventricular hypothalamic nucleus. It develops along a ventral extension of the hypothalamus at the level where the anterior hypothalamus is replaced by the periventricular hypothalamic nucleus. It is the lightest of the principle hypothalamic nuclei. Along its ventral surface is the ventral tuberal nucleus.

The most dorsal of the hypothalamic nuclei are the dorsomedial and dorsolateral nuclei. These two light nuclei are bound laterally and dorsally by the ventral thalamus and medially and ventrally by the periventricular hypothalamus. At the caudal end of the ventromedial hypothalamic nucleus a small round nucleus appears dorsolateral to it; this is the premammillary nucleus. Posterior to this are the mammillary nuclei, located at the caudal end of the hypothalamus, consisting of a medial and a lateral nucleus. The medial and lateral mammillary nuclei are located dorsolateral to the posterior pole of the ventromedial hypothalamic nucleus and caudal to the premammillary nucleus. At this same level, along the midline, is the supramammillary nucleus. These are the most caudal structures of the hypothalamus.

### 3.3.6 Pretectum

The pretectum is an area of transition between the diencephalon and the mesencephalon. As such, it is not always clear whether pretectal tissue is diencephalic or mesencephalic, although recent indications are that it is entirely diencephalic (2). The pretectal nuclei include the pretectal geniculate nucleus, mesencephalic lentiform nucleus, posterodorsal nucleus, thalamic lentiform nucleus, and profound mesencephalic nucleus. These nuclei lie across the base of the optic tectum, part of the mesencephalon, as a caudal continuation of the dorsal thalamus. They replace the dorsal thalamus in a lateral-to-medial fashion so that at the most rostral level of the pretectum, in coronal sections the pretectum is visible laterally while the dorsal thalamus is visible medially. The ventral thalamus and hypothalamus continue caudally through the level of the pretectal region.
The rostral portion of the pretectal geniculate nucleus appears at the level of the rostral tectum, ventrolateral to the lentiform thalamic nucleus, extending along the dorsolateral border of the diencephalon at an oblique angle. It is lighter than the lentiform thalamic nucleus. The pretectal geniculate nucleus is a dorsocaudal continuation of the ventral part of the lateral geniculate nucleus, interrupted in continuity only by a thin, dark cell free zone occupied by medially coursing fibers of the optic tract. The nucleus is also separated from lentiform mesencephalic nucleus, which lies dorsal to it, by a thin dark zone.

The mesencephalic lentiform nucleus lies at the rostral base of the optic tectum and is continuous with the curvature of the tectum. It is lighter than the tectum and the pretectal nuclei ventral to it. Caudally it is replaced by the posterodorsal nucleus.

The posterodorsal nucleus is continuous with the central grey layer of the optic tectum, though it is darker. It is located between the tectal commissure dorsally and the posterior commissure ventrally, and is replaced by the torus laminaris caudally. Below the posterior commissure, the light subcommissural organ is present surrounding the dorsal end of the third ventricle.

The lentiform thalamic nucleus is the largest region of the pretectum. Rostrally, it appears as an elongated region of variable intensity along the dorsolateral limit of the diencephalon, ventral to the rostral optic tectum. Following this nucleus caudally it separates into a folded, light plicata part that curves around a dark band of periventricular white matter and a round, dark extensa part ventral to the plicata part.

Caudally, there are three additional pretectal nuclei: the medial, dorsal, and ventral pretectal nuclei. The dorsal pretectal nucleus is a relatively dark nucleus lying at the level of the fibers of the posterior commissure, which course through it. The medial pretectal nucleus is even darker than the dorsal pretectal nucleus, to which it lies ventromedially. The medial pretectal nucleus also has posterior commissural fibers coursing through it. The ventral pretectal nucleus is located ventrolaterally to the dorsal pretectal nucleus and is continuous with the superficial grey and fibrous layer of the optic tectum. It is the lightest of the pretectal nuclei. The dorsal and ventral pretectal
nuclei are separated by fibers from the central white layer of the optic tectum descending into the hypothalamus.

The most ventral part of the pretectum is the profound mesencephalic nucleus. It is a large nucleus, lighter than the pretectal geniculate nucleus dorsal to it, but darker than the extensa part of the thalamic lentiform nucleus and the medial pretectal nucleus dorsal to it.

3.4 Brain Stem

We follow ten Donkelaar (2) in defining the brainstem as the mesencephalon and rhombencephalon. The two most prominent structures of the brain stem are the optic tectum, which is the largest structure in the *Ctenaphorus* brain after the cerebral hemispheres, and sits caudal to them, and the cerebellum, which rests over the caudal surface of the optic tectum and attaches at the rostralmost point of the medulla.

Along the ventral surface of the brain stem it is difficult to clearly delineate the mesencephalon from the medulla due to the abundance of longitudinal fiber tracts and cell nuclei that pass through both structures, such as the reticular formation and the raphe nuclei. We have estimated an approximate delineation between the two structures at the level where the cerebellum joins the rest of the rhombencephalon and the isthmic nuclei are visible.

3.4.1 Optic Tectum

In reptiles, like in most vertebrates, the neurons and fiber bundles of the optic tectum are arranged in thirteen concentric layers. The layers are then grouped into six layers: the optical layer, superficial grey and fibrous layer, central grey layer, central white layer, periventricular grey layer and periventricular fibrous layer. The optical layer is only slightly darker than the adjacent superficial grey and fibrous layer, but the two can be distinguished by the dark border between them. The central white layer is the darkest layer, while the central grey layer is of intermediate intensity between the central white and superficial layers. The periventricular grey layer is darker than the superficial layers but lighter than the central white layer, and finally the periventricular white layer is as dark as the central white layer, but much thinner.
Anteriorly, only four layers are present: the optic layer, the superficial grey and fibrous layer, the central grey layer, and the central white layer. The periventricular grey and periventricular white layers appear most caudally, at the level of the tectal ventricle (Figure 35). At its most caudal levels, the optic tectum again consists only of the optic layer, superficial grey and fibrous layer, the central grey layer, and the central white layer.

The optic tectum is the primary recipient of optic fibers from the optic tracts. Beginning at the level of the preoptic area, fibers from retina can be seen ventral to the brain, crossing at the optic chiasm and then projecting dorsally around the diencephalon. Some of these fibers can be observed projecting into the diencephalon via the supraoptic decussation, while others are diverted to the accessory optic system, which consists of the basal optic root and its associated nucleus. However, the vast majority of optic tract fibers project into the optic tectum, and this is observed throughout the rostral-caudal extent of the tectum as the optic tracts shift towards the optic tectum and shrink, until they are no longer present at the most caudal extent of the tectum.

3.4.2 Cerebellum

The cerebellum consists of three parts, the cerebellar plate, the auricle or flocculus, and the cerebellar nuclei. It is well developed in Ctenophorus, similar to Iguana and Varanus (45).

The most anterior part of the cerebellum is the anterior pole of the cerebellar plate, which is everted and rests over the caudal surface of the optic tectum. The cerebellar plate continues caudally further than the optic tectum, at which point it is connected to the basal rhombencephalon by the auricles. The dorsal median sulcus is visible on the dorsal surface of the cerebellar plate throughout its rostral-caudal extent. The three cell layers are clearly visible: the dorsal the molecular layer, the central Purkinje layer, and the ventral granular layer (Figure 35). The Purkinje layer is darkest, likely owing to the fact that these are the output cells of the cerebellum and therefore this layer contains many fibers. The granular layer is the lightest.
There are two cerebellar nuclei, a lateral and a medial, however the boundary between them is indistinct, even with histological staining, and therefore they are often referred to together as the “cerebellar nucleus”. In our model, the medial cerebellar nucleus is darker than the lateral cerebellar nucleus and we have used this to estimate a boundary between the two nuclei. They are located lateral to the central grey and medial to nucleus of the lateral lemniscus.

**3.4.3 Torus Semicircularis**

The torus semicircularis is continuous with the periventricular layers of the optic tectum, lying ventral to the tectal ventricle. It appears posterior to the pretectum and terminates just caudal to the terminus of the optic tectum. The trajectory of the torus through the rostral-caudal extent of the brain is interesting because though it is clearly distinguishable at its rostral and caudal poles, in between it is obscured by the central grey, which has a similar intensity. At this intermediate level we have labeled the entire structure the central grey. Anterior to this level the laminar nucleus of the torus is distinguishable, and caudal to it the central nucleus is visible.

The laminar nucleus of the torus semicircularis, visible at its rostral extent, exists at the boundary between the basal mesencephalon and the optic tectum. It is continuous medially with the central grey and laterally with the periventricular nuclei of the optic tectum. The dorsal tectobulbar tract separates the laminar nucleus of the torus from the tectal grey.

The laminar nucleus merges with the central grey caudally as the dorsal tectobulbar tract descends and joins the predorsal bundle, which extends through the rhombencephalon. Though this region, the torus semicircularis is not distinguishable within the central grey. Caudally, the torus reemerges as the central nucleus of the torus, which bulges outwards dorsally.

The central nucleus consists of a large, light, round central layer, and a darker, laminar periventricular layer dorsally. The two layers are separated by a thin, dark fiber tract called the mesencephalic trigeminal tract. The periventricular layers and trigeminal tracts of the two hemispheres are connected at the commissure of the torus.
semicircularis, which is also called the intertoral commissure. The central nucleus of the torus receives projections from the lateral lemniscus.

The nucleus of the lateral lemniscus consists of cell bodies medial to the lateral lemniscus. This nucleus sends secondary auditory projections, via the lateral lemniscus, to the torus semicircularis. The lateral lemniscus itself runs medial to and parallel with the spinal lemniscus and cannot be differentiated from it in our model.

### 3.4.4 Catecholaminergic Nuclei

Catecholamines are neurotransmitters that are important neuromodulators of brain function. The nuclei that produce them are located in the hindbrain, at the caudal level of the optic tectum. The ventral tegmental area occupies the space in between the oculomotor nerves, along the midline dorsal to the interpeduncular nucleus. It is darker than the interpeduncular nucleus but much lighter than the nerves. The substantia nigra is located more laterally, on the ventral border of the brain lateral to the reticular formation. Its darker, caudodorsal extension is called the retrorubral area. Dorsal to the substantia nigra is the darker locus coeruleus and its darker still ventral extension, the subcoerulear area.

### 3.4.5 Interpeduncular Nucleus

The interpeduncular nucleus is the most ventral nucleus in the hindbrain. This nucleus receives habenular projections via the retroflex tract, a very prominent bright tract that runs dorso-ventrally close to the midline. The interpeduncular nuclei themselves are brighter rostrally. There is only the dorsal part of the interpeduncular nucleus at the level of the oculomotor nerve, where it lies ventral to the ventral tegmental area. Subsequently it shifts dorsally and becomes smaller, while the ventral part of the interpeduncular nucleus appears ventral to it. A small cell-free dark zone along the midline separates them. Caudally, the dorsal part ends first, and then the ventral part is replaced by the superior raphe.
3.4.6 Reticular Formation

The reticular formation extends continuously from the level of the pretectum to the spinal cord. It starts at a dorsolateral position ventral to the tectal grey and is known as the pretectal reticular field. The pretectal reticular field is sparsely populated with cells and appears dark in our model. Once the reticular field enters the hindbrain it starts to contain various nuclei and so has a more heterogeneous appearance. The hindbrain portion of the reticular formation is divided into three medio-lateral sections: the median zone, the medial zone, and the lateral zone. The medial zone contains magnocellular nuclei while the lateral zone contains parvocellular nuclei.

The median zone of the reticular formation consists of the superior raphe rostrally and the inferior raphe caudally. The superior raphe is located along the midline dorsal to the interpeduncular nuclei and ventral to the medial longitudinal tract. It extends from the level of the mesorhombencephalic junction to the level of the motor nucleus of the trigeminal nerve. The inferior raphe extends from the terminus of the superior raphe to the start of the spinal cord. However, it is not always clearly differentiated from the medial reticular field. It becomes larger and lighter caudally, and therefore easier to delineate.

The medial and lateral reticular zones are subdivided into three parts: the superior reticular formation, the medial reticular formation and the inferior reticular formation. The most rostral nucleus of the superior reticular formation is the interstitial nucleus of the medial longitudinal tract, which appears at the level of the caudal optic tectum.

The medial reticular formation extends from the level of the trigeminal nerve to the level of the statoacoustic nerve. Caudal to the statoacoustic nerve, the medial reticular field transitions into the inferior reticular field. The inferior reticular formation is divided into two nuclei: a dorsal nucleus and a ventral nucleus. The ventral nucleus is lighter than the dorsal nucleus, and they extend from the level of the trigeminal nerve to the spinal cord.
3.4.7 Cranial Nerves and Nuclei

There are ten cranial nerves that enter the brainstem, nerves II-X and XII. Nerve II is the optic nerve, which runs lateroventrally from the level of the preoptic nucleus to the optic tectum. The cranial nerves are very dark structures, with almost no intensity. Each nerve is associated with a nucleus of cells that appears lighter than the fiber tract and is located proximal to it.

The first cranial nerve to appear after the optic nerve is the oculomotor nerve (III). This is a huge fiber tract that enters the brain ventromedially, with only the ventral tegmental area and the interpeduncular nucleus medial to it. It enters the rostral end of the medial longitudinal tract. The nucleus of the oculomotor nerve appears dorsal to the nerve itself along the midline. The nucleus of Edinger-Westphal, or the accessory nucleus of the oculomotor nerve, lies dorsal the oculomotor nerve along the ventral surface of the central grey.

The trochlear nerve (nerve IV) enters the brain dorsolaterally, along the outside of the lateral surface of the brain rostral to the isthmus, then curves medially below the cerebellum and torus semicircularis and above the brainstem. At the midline it curves ventrally and enters the medial longitudinal tract. The nucleus of the trochlear nerve is located on the dorsal surface of the medial longitudinal tract.

The trigeminal nerve (nerve V) is massive. It courses along the dorsolateral surface of the brain from the spinal cord to the level of the termination of the cerebellum. At this level it enters the brain laterally and divides into two roots: a dorsal sensory root and a ventral motor root. Both project into the medial longitudinal tract. There are several nuclei associated with the trigeminal nerve. The principle nucleus of the trigeminal nerve and the dorsal motor nucleus of the trigeminal nerve sits between the sensory and motor roots. Ventral to the motor root, the ventral motor nucleus of the trigeminal nerve lies laterally. Caudally, the descending motor nucleus sits ventral to the statoacoustic nerve. Two other caudal nuclei associated with the trigeminal nerve are the spinal nucleus of the trigeminal nerve, an elongated nucleus lateral to the dorsal column nucleus, and the medial parvocellular nucleus, which is ventral to the anterior half of the motor nucleus of the vagus nerve. It is darker than the nucleus of the hypoglossal nerve that lies just ventral to it.
The abducens nerve (nerve VI) enters the brain in the same position as the oculomotor nerve, but more caudally, at the level of the infima commissure. This is a much smaller nerve than the oculomotor nerve, and it runs dorso-rostrally until it enters the medial longitudinal tract. The nucleus of the abducens nerve is located ventrolateral to the medial longitudinal tract at the level where the statoacoustic nerve enters the brain.

The facial nerve (nerve VII) enters the brainstem at the anterior most level at which the statoacoustic nerve enters. It is smaller than the statoacoustic nerve and enters ventral to it, projecting medially to the solitary tract. The nucleus of the facial and glossopharyngeal nerves is located caudal to the facial nerve, lateral to the nucleus of the abducens nerve.

The statoacoustic or vestibulocochlear nerve (nerve VIII) enters the brain at about the same position as the trigeminal nerve, but caudal to it. This nerve also has two roots, an anterior root, which enters the brain and the caudalmost level of the cerebellum, and a posterior root, which enters the brain at a slightly more dorsal position than the anterior root. Fibers from both roots enter the medial longitudinal tract. There are two groups of nuclei associated with this nerve: the vestibular nuclei and the cochlear nuclei, all located in the alar plate of the rhombencephalon. The cochlear nuclei are lighter and more dorsal than the vestibular nuclei. The vestibular nuclei start more rostrally, with the large dorsolateral vestibular nucleus appearing first, at the level of the trigeminal nerve. Caudally, this nucleus is replaced by the ventrolateral and tangential vestibular nuclei and then finally the ventromedial and descending vestibular nuclei. The cochlear nucleus appears at the level of the ventrolateral vestibular nucleus. At its most rostral levels, the angular cochlear nucleus is more ventral, with the dorsalmost pole of the rhombencephalon occupied by the dorsal column, however caudally it moves dorsally. The angular cochlear nucleus is intertwined in the fibers of the dorsal column. Ventrolateral to the angular cochlear nucleus is the magnocellular cochlear nucleus rostrally and the posterior cochlear nucleus caudally, which is the lightest of all the cochlear nuclei.

The glossopharyngeal nerve (nerve IX) is a very small nerve located caudal and dorsal to the posterior root of the statoacoustic nerve, where it enters from a point just dorsal to
the entry point of the statoacoustic nerve. The fibers project rostro-medially to the periventricular fiber system, which then courses ventrally to join the solitary tract.

The vagus nerve (nerve X) is another small nerve. It enters posterior to the infima commissure at a dorsolateral position. This nerve does not visibly cross to the medial longitudinal tract, instead, it dissipates into many small rootlets which are not visible in our model. The nucleus of the vagus nerve is located dorsolateral to the medial longitudinal tract from the level of the infima commissure to the start of the spinal cord. It is lighter that the medial parvocellular nucleus located ventral to it, but darker than the nucleus of the solitary tract dorsal to it.

The hypoglossal nerve (nerve XII) enters ventrally, at the same position as the oculomotor and abducens nerves, but at the level of the infima commissure. At this same level the nucleus of the hypoglossal nerve can be seen ventral to the medial parvocellular nucleus, which is equivalent to the mammalian perihypoglossal nuclear complex. The nucleus of the hypoglossal nerve proceeds in this position to the level of the spinal cord.

3.4.8 Additional Nuclei of the Alar Plate

The alar plate occupies the dorsal half of the hindbrain, roughly from the level of the auricles rostrally to the spinal cord caudally. The division between the alar plate and the basal plate is roughly at the level of where the trigeminal nerve bisects the brainstem to enter the medial longitudinal tract. In our model, the nuclei visible in this region, in addition to the nuclei associated with the cranial nerves, include the dorsal column nucleus and the nucleus of the solitary tract.

The dorsal column nucleus is an elongated, slender nucleus associated with the dorsal column, a fiber bundle running rostro-caudally along the dorsalmost pole of the hindbrain. The fiber bundle is sometimes obscured in our model by the nuclei present in this region, such as the cochlear nuclei. The dorsal column nucleus extends from the end of the cochlear nuclei to the start of the spinal cord.

The nucleus of the solitary tract is where the fibers of the solitary tract terminate. The solitary tract carries fibers from the facial, glossopharyngeal, and vagus nerves. This is
one of the lightest nuclei in the hindbrain. It lies lateral to the fourth ventricle for the caudal third of the rhombencephalon. At the level of the infima commissure, the dorsal portion of this nucleus separates to form the nucleus of the infima commissure.

3.4.9 Additional Nuclei of the Basal Plate

The basal plate consists of the tissue ventral to the level of the trigeminal nerve, posterior from the level of the auricle to the start of the spinal cord. It primarily contains motor nuclei, including the superior olive, nucleus of the trapezoid body, and the motor nuclei of the cranial nerves. It does not include the reticular formation.

The superior olive is found in the middle of the basal plate, along the boundary of the lateral vestibulospinal tract. It starts at the caudal end of the isthmus and terminates at the level of the abducens nerve. It consists of a rostral, more ventral portion and a caudal, more dorsal portion. Caudally, the ventral portion of the superior olive differentiates into the nucleus of the trapezoid body, which is slightly darker in intensity.

4 Discussion

This is the first time, to our knowledge, that an MRI atlas of a lizard brain has been produced. MRI is an innovative technique used frequently in the medical sciences and in model organisms such as mice and rats. Here, we have added the first reptile to the growing list of MRI atlases that can be used in comparative studies using nontraditional study organisms.

4.1 Comparison with other lizards

At a broad level, the majority of the *Ctenophorus* brain conforms well to what is known about lizard brain anatomy but it is important to point out that our comparison can only be with histological studies on the anatomy of other squamate brains. Detailed comparative analysis between the *Ctenophorus* brain and other lizard species would require high-quality MRI-based atlases to be available for a large number of lizard species representing broad taxonomic coverage. However, certain qualitative
differences between the *Ctenophorus* brain and the brains of other lizard species are evident from our model, and are outlined below.

The distinct cell layer in the cortex in the inner plexiform layer of the medial, dorsomedial and dorsal cortices was surprising. The cell layer was associated with a distinct thickening of the cortex which resulted in an invagination into the lateral ventricle. Though this thickening is visible in other reptiles, it is not usually associated with its own distinct cell layer. Greenberg (1) identifies a nucleus, the dorsomedial interposition, at a location that would correspond to the medial aspect of the cell layer visible in our model. Northcutt (19) identifies a cell layer that closely corresponds to the cell layer in our model, called the supraventricular layer, but states that this layer has never before been observed and may be unique to *Iguana iguana*. We have tentatively labeled the cell layer in our model as the dorsomedial interposition, but further work is necessary to clarify its identity.

The absence of the pallial thickening in *Ctenophorus* may be related to the presence of the cell layer we have labeled the dorsomedial interposition. The pallial thickening is a cluster of large cells usually present in reptiles at the lateral aspect of the dorsal cortex, and is sometimes considered a subregion of the lateral cortex. Though it is present in most lizards and turtles, it appears to be absent in *C. decresii* and is similarly absent in *Anolis* (1). It seems possible, though unlikely, that what we have labeled the dorsomedial interposition is actually the pallial thickening, which is shifted medially in *Ctenophorus*.

There is significant variation between species in the extent of the spherical nucleus. Generally, anatomical structure in the telencephalon is relatively conserved among lizards at the level at which we are examining, however the spherical nucleus is an exception. This nucleus may occupy almost the entirety of the caudal pole of the subpallium, as in *Tupinambis* (2), or be practically nonexistent, as in *Anolis* (1). In *Ctenophorus* the spherical nucleus sits at the caudal ventrolateral aspect of the subpallium, occupying no more than a quarter of the area of the subpallium in each coronal section it appears. This is similar to the relative volume occupied by the spherical nucleus in *Iguana* (19).
In the thalamus, the magnocellular part of the dorsolateral thalamic nucleus is small, similar to *Iguana* (19), while *Tupinambis* (2) and *Gekko* (22) have larger magnocellular dorsolateral thalamic nuclei. It should be noted that, among this small group of lizards, *Ctenophorus* is most ecologically similar to *Iguana*.

*Ctenophorus* has cochlear nuclei consistent with those of other agamids and chameleons in that there is relatively little differentiation of the cochlear nuclei, and only three nuclei could be identified in our model (2, 38). In some species as many as seven cochlear nuclei are visible, each morphologically distinct within the alar plate. It is unknown why agamids and chameleons have such a reduced number of cochlear nuclei (46).

### 4.2 MRI as a method for studying neuroanatomy

Delineating neuronal structures is difficult and requires a trained eye, whether it is histological- or MRI-based atlases. Neuroanatomical identification is not inherently more difficult with MRI. Furthermore, MRI atlases provide greater flexibility for those creating atlases and provide more information with less effort, and are therefore more efficient than traditional atlases. However the novelty of the method with respect to atlasing may make the methods of region identification seem more daunting and they are not as useful for all avenues of research. Therefore, care should be taken when determining what type of atlas would be most useful.

Despite the tremendous methodological differences between traditional histology and MRI, our lizard brain model produces coronal images that strongly resemble published coronal Nissl stained sections. Nissl staining is the basic procedure used to map brain regions as it specifically stains cell bodies, resulting in clearly visible regions of high and low cell density. However, a range of chemical stains are available in histology in order to selectively stain specific tissue characters, such as dendrites, fiber tracts or neurotransmitters. Though this is not possible with MRI imaging, different image weighting and contrast mechanisms can be used to selectively emphasize certain tissue properties. Different relaxation properties of brain tissue can be emphasized or deemphasized in MRI by altering the relative weighting of different relaxation properties (T1, T2 and T2*) during imaging (47-49). Emphasizing the contrast between white-matter and grey-matter, which is desirable for MRI-based atlasing, requires a T2*
weighted imaging sequence (30), which we have employed here. A different kind of relaxation, diffusion weighted imaging, is excellent for imaging white matter and the directionality of white matter fibers (axons).

MRI provides an advantage over histology in that, as imaging is a 3D process that does not require brain sectioning, the resulting image can be viewed in any plane, coronal, horizontal and sagittal, and all planes can be viewed at once. This allows for brain regions and fiber tracts to be viewed throughout their rostral-caudal extent, facilitating visualization of the true structure of the brain. Traditional histological atlases of reptiles, which consist of coronal sections, have much poorer longitudinal resolution than they do in-plane resolution. Each image in an atlas is typically 100 µm or more caudal to the previous one. Our 3D atlas has the same resolution – 23 µm³ – in all three dimensions. Moving through the brain at such a fine resolution provides a much better understanding of how brain regions change shape, size, and particularly how they are connected and related to each other. This can be clearly demonstrated by scrolling in a rostro-caudal direction through our online atlas and comparing the result with flipping through the figures in this paper.

MRI is also advantageous over histology in that it allows brains to be imaged in situ and even in vivo. Therefore, euthanizing the animal is not necessary, which is not only an ethical advantage, but allows for more complex experimental designs coupling brain measurements with behavioural, physiological and other studies. Longitudinal studies on brain growth and maturation could easily be conducted with MRI. Furthermore, examining brains in situ avoids potential shrinking and distortion of the brain as can often occur with histological processing. In this study, in order to maximize our resolution, we opted to extract the brain from the skull prior to imaging. However, we maintained the reduction of distortion of the brain tissue that MRI provides over histology.

Though our MRI atlas represents a great improvement in longitudinal resolution compared to histological atlases, the in-plane resolution of MRI is far inferior to traditional histology (Figure 35, 35). Histological staining allows for the characterization not only of brain nuclei, but also of individual cells. There is great variation in cell size, shape, and arborization, as well as in fiber tract structure and neuropil layers between brain regions. Using MRI we cannot characterize the neurons of various cell regions, as is possible in histological atlases, nor can we comment
precisely on the extent of myelination of various fiber tracts. Nonetheless, the non-invasive natural of MRI means that it can be used in complement to histological investigations. Magnetic resonance microscopy can be used to inform potential histological investigations prior to the irreversible process of sectioning and staining tissue samples. With histology, time and resource investment increases significantly with the amount of brain tissue being imaged, while the amount of additional investment necessary to image a larger brain region with MRI is low.

Unlike histology, resolution in MRI is dependent on brain size. Signal intensity is a significant factor in determining resolution in MRI, and higher signal intensity comes from larger voxels (50). Larger brains can be imaged with larger voxel sizes, and therefore with improved signal intensity resulting in improved signal-to-noise ratios. For example, an MRI atlas of a monkey brain is able to delineate 720 structures in an image with a 0.5 x 0.5 x 0.5 mm$^3$ voxel size (51), whereas an MRI atlas of a cichlid brain is able to delineate only 54 structures in an image with a 50 µm$^3$ voxel size (52). Though the absolute voxel size in the cichlid atlas is much smaller than the voxel size in the monkey atlas, voxel size relative to brain size is much smaller in the monkey relative to the cichlid. This provides a two-fold benefit to the monkey atlas: the larger absolute voxel size provides greater signal intensity, while smaller relative voxel size provides greater spatial resolution. Together, these factors allow for much more precise structural delineation in larger brains. As the tawny dragon has a very small brain, we used multiple techniques to improve our resolution: use of a paramagnetic contrast enhancer, use of perfusion to distribute the contrast agent evenly throughout the brain, and creating a minimum deformation model of thirteen brains.

Paramagnetic contrast enhancers are frequently used in MRI to enhance relaxation properties and therefore improve resolution (53). By temporarily interacting with water, paramagnetic contrast agents decrease relaxation times, thereby allowing for greater signal-to-noise per unit time, and increased contrast between tissue types (53). In this study, we have perfused Magnevist, a commercial paramagnetic contrast agent, though the Ctenophorus brains and stored the brains in a buffer solution containing Magnevist to improve the quality of our model. Traditionally, brains are placed in a Magnevist solution post-fixation, allowing for passive diffusion of Magnevist into the brain. However, this can result in Magnevist gradients across the brain, introducing artifacts.
into the images (54). Perfusion is preferable as it distributes Magnevist evenly throughout the brain using the circulatory system.

To further enhance the resolution of our atlas, we have used a non-linear image averaging strategy to create an “idealized” *C. decresii* brain (34). This same technique is employed in the creation of the monkey atlas (51), rat atlas (55) and mouse atlas (30), among others. By creating this model from thirteen brains instead of using the image of a single brain, we have greatly improved the signal-to-noise ratio of our atlas (Figure 36). This improved clarity directly enhances our ability to distinguish proximate structures of similar intensity, such as the many nuclei of the diencephalon and the different layers of the telencephalon. The voxel size is reduced by more than half, from 50 µm³ in the original images to 23 µm³ in the model. This process also greatly increases the signal-to-noise ratio and thereby increases image contrast. Furthermore, reproducibility of the images can be verified though comparison between multiple brains. Any structure that is not visible in the majority of the brains is deemed an artifact and dropped from the data. All of these factors contribute to our ability to identify and delineate brain structures, and therefore we are able to delineate far more structures in our model than we would have if we had used an image of a single brain.

With our model of the *Ctenophorus* brain we were able to achieve images with contrast levels and resolution comparable to MRI atlases available for mice (30) and rats (55), however, they are of reduced contrast compared to nuclear-stained histological images. It is important to recognize that histology and MRI image different characteristics of brain structure. Histology stains cells and therefore the resulting images reflect cell density. The greater the intensity of the pixel, the greater the cell density in that brain region. This can clearly be seen in the laminar regions of the brain, such as the cortex, where there is a strong signal from the cell layer and the ependymal layer of the cortex, while the plexiform layers have a much weaker signal (Figure 36). The opposite is true in our MRI model. The outer and inner plexiform layers, which have greater water content, have the strongest signal and appear lightest (Figure 36). The cell layer is darker as it has lower water content, and the alveus, which is strongly myelinated and therefore hydrophobic, has the weakest signal.
Figure 36. Comparison between a coronal section through an MRI image of a single brain (a), through an MRI model of thirteen brains (b), and a fluorescent nuclear-stained histological section (c). The model has far superior resolution compared to the image of a single brain, but in-plane resolution is still lower than the histological section.
4.3 *Ctenophorus decresii* as a model organism

We have selected *C. decresii* as our model organism as it represents a “typical” lizard without any obvious sensory or motor specializations such as limblessness, bipedal locomotion or gliding. The variation between species in lizards in sensory and motor specializations is vast. This provides an excellent opportunity for the study of the evolution of sensory and motor adaptations within the nervous system. Frequently, specific adaptations have evolved independently several times, such as limblessness, directional olfaction using forked tongues, and reduced vision. These provide opportunities to study the neural underpinnings of such specializations using a robust comparative approach. However, the initial challenge is to understand the neural architecture of a “standard” lizard brain, without any extreme motor or sensory shifts. *C. decresii* is an ideal candidate for this purpose.

*Ctenophorus decresii* is also an established model species in behaviour and evolution, providing a foundation for neurological research. Two populations of *C. decresii* are colour-polymorphic and, similar to other polymorphic lizard species, each morph exhibits different social behaviours and reproductive strategies (56, 57). There has been extensive interest in colour polymorphic lizards in behaviour, ecology, evolution, morphology, physiology and endocrinology, and one of the principle findings of this system is the variation in reproduction strategy between colour morphs (58, 59). However, little attention has been paid to neural differences between morphs, despite their obvious implications as the underlying cause of behavioural variation, including reproduction strategy. Preliminary studies have found that there do appear to be neural differences between morphs, in dorsal cortex volume and in medial cortex neurogenesis (13, 60). Further work using colour polymorphic lizard species holds great potential in elucidating the neural underpinnings of different reproduction strategies.

As well as intraspecific comparisons, there is ample opportunity for interspecific comparative neuroscience. Another member of the same genus, *Ctenophorus pictus*, is also polymorphic (61), and the remaining species in the genus are monomorphic. Several species are popular model organisms for behavioural studies in Australia and exhibit unique and complex social behaviours and adaptations to different environments. A well-resolved phylogeny is available for this genus, to control for relatedness in comparative studies (62).
5 Conclusion

This is the first MRI-based brain atlas to be published for any reptile. Additionally, this atlas provides an update and synthesis of our current knowledge surrounding the anatomy of the reptile brain, including advances from the twenty years since the publication of the last reptile brain atlas. The resolution obtained in this atlas is significantly higher than that of other atlases for animals with similarly-sized brains, and our use of a non-linear average of thirteen brains instead of a single brain provides greater confidence that our atlas resembles the “idealized” *Ctenophorus decresii* brain. This atlas is available in three dimensions online at www.tissuestack.com.
Section 2

Evolutionary patterns in dragon brain structure
Chapter 4

Evidence for concerted and mosaic evolution of the brain in dragon lizards
Abstract

The brain, though a single tissue, has a wide variety of functions including behaviour, perception, motor control, and homeostatic maintenance. Each function can experience different selective pressures over the course of evolution, and as selection acts on the outputs of brain function, it necessarily alters the structure of the brain. Two models have been proposed to explain the patterns observed in evolutionary changes in brain morphology. The concerted brain evolution model posits that the brain evolves as a single unit and the evolution of different brain regions are coordinated. The mosaic brain evolution model posits that brain regions are evolving independently of each other in response to different evolutionary selective pressures. It is now well understood that both models are relevant in explaining brain evolution but the relative influence of each mode on brain structure varies between vertebrate groups. Mammals and cartilaginous fishes show primarily a concerted model of brain evolution while birds and bony fishes show primarily a mosaic model of brain evolution. It remains unclear what factors favour concerted or mosaic brain evolution. Reptiles are an ideal group with which to study the patterns and processes of brain evolution due to their phylogenetic position between birds and mammals. We examined the volumes of the six major neural subdivisions across fourteen species of the agamid lizard genus *Ctenophorus* (referred to as ‘dragons’). We found evidence for both mosaic and concerted brain evolution in dragons. Furthermore, we found a pattern of concerted brain evolution with respect to morphological characters and mosaic brain evolution with respect to ecological and life history characters. Reptiles present an ideal system in which to examine the patterns and processes of brain evolution and particularly the evolutionary, ecological, and physiological conditions that favour either mosaic or concerted modes of brain evolution.
**Introduction**

A relatively large and complex brain is one of the major characteristics of vertebrate animals and it is linked with the complex sensory, behavioural, social, and cognitive functions vertebrates display (1). The volume of the vertebrate brain is most strongly linked to body size (2), however many other factors also correlate with brain size including phylogeny (2), social intelligence (3), problem solving ability (4) and metabolism (5). Many factors may be under evolutionary selective pressures that vary between vertebrate groups, for example the ability to learn vocalizations (6) and the ability to maintain a constant internal body temperature (7), and these factors and many others are often linked to evolutionary changes in brain volume (eg. (8-10). Brain volume can be linked to so many disparate evolutionary selective pressures because the brain is composed of functionally distinct but interconnected structures that perform a wide array of disparate tasks (11).

When, over the course of evolution, the brain increases or decreases in volume as a result of a multitude of different selection pressures all acting upon it, what happens to the component parts that make up the brain? Can the discrete and functionally distinct cell groups that make up the brain change volume independently, or only in a coherent manner? How evolutionarily plastic is the brain in response to different selective pressures? There are two principle hypotheses proposed to explain the patterns of brain evolution in response to selection.

The mosaic brain evolution hypothesis posits that, because different species exist in different ecological and social niches, and are therefore under different selection pressures, changes in brain volume as a result of specific evolutionary pressures should be limited to the brain regions directly functionally related to those pressures (12). This hypothesis suggests that volumetric changes in a particular brain region are the result of direct evolutionary selective pressure on the function of that brain region.

Conversely, the concerted brain evolution hypothesis posits that, though the brain is made up of functionally distinct cell groups, these groups are not independent. Brain nuclei are highly interconnected, both physiologically and developmentally, and therefore brain regions should evolve in concert with each other. That means that, as one brain region gets larger or smaller over evolutionary time, the other brain regions
get larger or smaller in proportion (12). The developmental-constraints hypothesis is a mechanistic explanation for concerted brain evolution. This hypothesis suggests that the brain is constrained to evolve more or less as a single unit due to the rules of neural development (13).

It is well understood that brain volume is constrained by body size (12). As animals increase in size, so too do their brains. In this way, all brains evolve under a concerted model of brain evolution (12). However, concerted and mosaic brain evolution are not mutually exclusive processes, and it is now thought that the vertebrate brain is evolving both in concert and as a mosaic (12). Within the constraint of body size, the question remains, how free are different brain regions to respond to specific selection pressures, and how constrained are they to evolve as a single unit? The answer may depend in part on phylogenetic history. In mammals, the volumes of major brain regions appear to be evolving primarily in concert (12, 14, 15), while in birds the major brain regions seem to be evolving as a mosaic (16, 17). However all groups show evidence of both processes, with evidence for mosaic brain evolution in mammals (18-21) and concerted brain evolution in birds (22, 23). Therefore, brain evolution may be thought of as a balance between the two evolutionary patterns. This same contrast exists between the major clades of fishes, with cartilaginous fishes showing primarily a concerted pattern of brain evolution (24) whereas boney fishes seem to be under a primarily mosaic pattern (25, 26).

As the most closely related species-rich vertebrate group between birds and mammals, squamate reptiles (snakes and lizards) are an ideal to examine the evolutionary, developmental and physiological conditions which might favour concerted or mosaic modes of brain evolution. However, brain evolution in reptiles is poorly understood relative to other vertebrate groups. The only study to date to look at the scaling of the major neural subdivisions across squamates found evidence for both concerted and mosaic brain evolution, but did not correlate variations in brain volume with ecological, evolutionary, or life history traits (27). More recently, there have been suggested cases of mosaic evolution in reptiles in the spherical nucleus (28), red nucleus (29) and anterior dorsal ventricular ridge (30), but these have not been examined rigorously. Only two studies have systematically examined the evolution of the major brain regions across multiple reptile species (31, 32). However, they were unable to find evidence for
mosaic or concerted evolution in the volumes of major brain regions across six species of *Anolis* with respect to ecomorph or habitat complexity (31, 32). The question still remains, therefore, as to what patterns and processes underlie brain evolution in reptiles.

The Australian semi-arid agamid genus *Ctenophorus* (referred to as ‘dragons’) displays three distinct ecomorphs: burrow-associated species, which dig burrows, rock-associated species, which inhabit rock crevices, and vegetation-associated species, which do neither (33). Like *Anolis*, each ecomorph is generally associated with a characteristic morphology, or “morphotype” (33). Burrow-associated species are morphologically “diggers”, with short limbs and tails. Rock-associated species are “squeezers”, with dorso-ventrally flattened bodies and heads to squeeze into tight spaces. Vegetation-associated species are “runners”, with long legs and tails (33, 34).

However, the morphotype of a particular *Ctenophorus* species does not always match its observed behaviour. For example, the ring-tailed dragon, *Ctenophorus caudicinctus*, behaves like a “squeezer” but has the morphology of a runner (30). As brain evolution may be influenced differently by the evolution of morphology vs. behaviour, we have included both morphotype and behaviours characteristic of each morphotype in our analysis (Table 1). Furthermore, we have included additional behaviours that do not seem to be characteristic of any morphotype but instead vary across species in ways that seem un-associated with morphotype variation: level of territoriality, reproductive strategy, basking strategy, and population density (Table 1). In addition, we examine brain evolution with respect to two indices of sexual selection.

Our goal here is not to make predictions as to which brain regions may evolve with which character traits. The brain regions examined are too large and perform too many tasks to make such specific predictions. Instead, we are looking for broadscale patterns in how morphological differences, behavioural differences, and differences in sexual selection influence brain evolution. Are the patterns in the way brains evolve, either following a concerted or mosaic model, different when looking at brain evolution in the context of morphology, behaviour or sexual selection?
Table 1. Character states for each species of *Ctenophorus* dragon used in this study.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>N (M/F)</th>
<th>Habitat</th>
<th>Morphotype</th>
<th>Burrower</th>
<th>Territorial</th>
<th>RS</th>
<th>Bask</th>
<th>Density</th>
<th>SD</th>
<th>SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Ring-tailed Dragon</td>
<td><em>C. caudicinctus slateri</em></td>
<td>8/11</td>
<td>Rock</td>
<td>Digger</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>Low</td>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>Bicycle Dragon</td>
<td><em>C. cristatus</em></td>
<td>12/8</td>
<td>Open</td>
<td>Runner</td>
<td>Yes</td>
<td>Yes</td>
<td>Semel</td>
<td>Elevated</td>
<td>High</td>
<td>11</td>
<td>0.03</td>
</tr>
<tr>
<td>Tawny Dragon</td>
<td><em>C. decresii</em></td>
<td>13/7</td>
<td>Rock</td>
<td>Squeezer</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>High</td>
<td>13</td>
<td>0.16</td>
</tr>
<tr>
<td>Peninsula Dragon</td>
<td><em>C. fioni</em></td>
<td>10/11</td>
<td>Rock</td>
<td>Squeezer</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>High</td>
<td>14</td>
<td>0.21</td>
</tr>
<tr>
<td>Mallee Military Dragon</td>
<td><em>C. fordi</em></td>
<td>10/10</td>
<td>Open</td>
<td>Runner</td>
<td>No</td>
<td>No</td>
<td>Semel</td>
<td>Ground</td>
<td>High</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>Gibber Dragon</td>
<td><em>C. gibba</em></td>
<td>11/8</td>
<td>Open</td>
<td>Digger</td>
<td>Yes</td>
<td>No</td>
<td>Itero</td>
<td>Ground</td>
<td>Low</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>Central Military Dragon</td>
<td><em>C. isolepis gularis</em></td>
<td>10/10</td>
<td>Open</td>
<td>Runner</td>
<td>No</td>
<td>No</td>
<td>Semel</td>
<td>Ground</td>
<td>High</td>
<td>12</td>
<td>0.02</td>
</tr>
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<td>Central Netted Dragon</td>
<td><em>C. nuchalis</em></td>
<td>14/3</td>
<td>Open</td>
<td>Digger</td>
<td>Yes</td>
<td>Yes</td>
<td>Semel</td>
<td>Elevated</td>
<td>High</td>
<td>2</td>
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<tr>
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<td><em>C. ornatus</em></td>
<td>9/8</td>
<td>Rock</td>
<td>Squeezer</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Ground</td>
<td>High</td>
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</tr>
<tr>
<td>Painted Dragon</td>
<td><em>C. pictus</em></td>
<td>15/14</td>
<td>Open</td>
<td>Digger</td>
<td>Yes</td>
<td>Yes</td>
<td>Semel</td>
<td>Elevated</td>
<td>High</td>
<td>14</td>
<td>0.09</td>
</tr>
<tr>
<td>Rusty Dragon</td>
<td><em>C. rufescens</em></td>
<td>10/10</td>
<td>Rock</td>
<td>Squeezer</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>Low</td>
<td>5</td>
<td>0.09</td>
</tr>
<tr>
<td>Claypan Dragon</td>
<td><em>C. salinarum</em></td>
<td>10/10</td>
<td>Open</td>
<td>Digger</td>
<td>Yes</td>
<td>Yes</td>
<td>Itero</td>
<td>Ground</td>
<td>Low</td>
<td>4</td>
<td>0.10</td>
</tr>
<tr>
<td>Ochre Dragon</td>
<td><em>C. tjantjalka</em></td>
<td>12/8</td>
<td>Rock</td>
<td>Digger</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>High</td>
<td>14</td>
<td>0.19</td>
</tr>
<tr>
<td>Red-backed Dragon</td>
<td><em>C. vadnappa</em></td>
<td>10/8</td>
<td>Rock</td>
<td>Squeezer</td>
<td>No</td>
<td>Yes</td>
<td>Itero</td>
<td>Elevated</td>
<td>High</td>
<td>14</td>
<td>0.23</td>
</tr>
</tbody>
</table>

RS = Reproductive Strategy, Semel = Semelparous, Itero = Iteroparous, Bask = Basking Strategy SD = Sexual Dichromatism, SSD = Sexual Size Dimorphism
Materials & Methods

Specimen Acquisition, Scoring, and Preparation

We collected 287 lizards from fourteen Ctenophorus species in central and southern Australia. Following capture, they were transported to the Australian National University in Canberra, Australia, were they were maintained in outdoor enclosures with ad-libitum access to food (wild insects) and water, and their diet was supplement twice weekly with domestic crickets. The Australian National University’s Animal Experimental Ethics Committee approved all research under protocol number A2011-49.

We assigned each species to an ecomorph based on Greer (33) and confirmed these assignments by personal observation. However, not all dragon species fit clearly into one ecomorph, but rather are intermediate (34). Therefore, in our analysis, we have split the ecomorph phenotype into three ‘components’: whether the species digs burrows, whether the species lives in rocky or open habitat (habitat preference), and morphotype (“digger”, “squeezer”, or “runner”). Data for each species on the morphotype, habitat preference, and burrow construction, as well as three ecological and life history characters not associated with ecomorph, reproductive strategy (itero/semelparity), basking strategy, and territoriality, were gathered from the literature (33-43).

Data for population density were gathered by personal observation during the collection of specimens. Based on these data we applied a simple and conservative metric to categorize species. Species that were observed in ideal habitat less than once per hour were rated as “low” density, while those observed more than once per hour were rated as “high” density.

We also classified each species based on two indices of sexual selection: sexual dichromatism and sexual size dimorphism. Each species of Ctenophorus was classified on a sexual dichromatism scale from zero (completely monochromatic) to 18 (maximally dichromatic) following Chen et al. (44). We measured the snout-vent length of each lizard, which we then used to calculate sexual size dimorphism ratings for each species using the Lovich-Gibbons sexual dimorphism index: (Male/Female) – 1 (44).
We euthanized each lizard with an injection of 100 mg/kg sodium pentobarbital and an equal volume of 2 mg/mL lignocaine. Each lizard was then intracardially perfused with 1 µL/mL heparin in pH-neutral phosphate-buffered saline followed by a pH-neutral solution of 0.1% Magnevist (gadopentate dimeglumine, Bayer) and 4% paraformaldehyde in phosphate-buffered saline. Magnevist was used to maximize image contrast in magnetic resonance imaging (45). The lizards were subsequently decapitated and the heads were post-fixed for 24 hours in 4% paraformaldehyde. We then dissected the brains from the heads as described in Chapter 2 and post-fixed the brains for 24 hours in 4% paraformaldehyde. We did not include the olfactory bulbs. Finally, the brains were stored at 4°C in a solution of 0.1% Magnevist and 0.05% sodium azide in phosphate-buffered saline.

Calculating Brain Volumes using Magnetic Resonance Imaging

Whole-brain images were acquired using a Bruker Avance 11.74 Tesla wide-bore spectrometer (Ettlingen, Germany) with a micro-2.5 imaging probe capable of generating magnetic gradients of 1.50 T/m. Brains were immersed in Fomblin (perfluoropolyether, Grade Y06/6, JAVAC, Sydney, Australia) and placed in a 10 mm diameter Wilmad tube using a custom-built plastic holder. The brains were buoyant in Fomblin and the plastic holder was designed to hold them submerged. Parameters used in the scans were optimized for gray-white matter contrast in the presence of Magnevist. We used a gradient echo (T₂*-weighted) 3D fast gradient-echo sequence (FLASH), with a repetition time = 40 ms, echo time = 8 ms field-of-view = 11 × 11 × 16 mm and matrix size = 110 × 110 × 160, producing an image with 100 µm³ isotropic voxels. As the geometry was similar for each scan, tuning, matching and shimming (on most occasions) was needed only for the first brain in a sequence. Using this method, four brains could be individually scanned per hour.

One model brain was built for each species using, on average, 20 individuals per species (range 17-29). Equal numbers of males and females were used to create each model. As measurements were taken from a model per species, there are no error bars as these are not “means” in the traditional sense, but rather an idealized version of a brain of the species.
To create the species models, the images were first cropped, re-orientated to standard rostro-caudal orientation and then masked such that consistent coverage of brain structures and nerve endings was achieved. The manually masked areas were set to the background value such that they were not included in subsequent calculations. All images were then B0 intensity inhomogeneity corrected using the N3 algorithm (46). An image with good signal to noise and no obvious artifacts was then manually selected from each group and used as an initial model for that group after blurring. All images within the respective groups were then recursively matched to their own evolving model of average structure to create a minimum deformation average with a resulting resolution of 40 µm. The details of the model creation process can be found in Janke et al. (47). The fitting stages in this case started at a resolution of 1.28mm and finished with a resolution of 80 µm.

To look for both concerted and mosaic evolution in the lizard brain, we examined the volumes of the whole brain and the six major subdivisions of the brain. It is at this level that evidence for both mosaic and concerted brain evolution have been found in reptiles: in avian reptiles (16) and in squamate reptiles (27). These six subdivisions are the telencephalon, diencephalon, optic tectum, mesencephalon (excluding the optic tectum), cerebellum and medulla.

In order to measure the volumes of six regions in each of the models in an automated fashion, we used model based segmentation. First a global model was created and the six regions were manually traced, each of the individual models were then non-linearly aligned to the global model and the manually traced labels were then back-transformed to each of the models. From here all that is required is to count the number of voxels in each of the back-transformed label volumes.

**Statistical Analysis**

Phylogenetic information, including relationships among species and branch lengths, was taken from a time-calibrated molecular phylogeny (44). We fitted phylogenetic linear regression models using a model of trait evolution by Brownian motion (48) in the R package *phylolm* (49). We generated separate models for each response variable: brain volume, telencephalon volume, diencephalon volume, optic tectum volume, mesencephalon volume, cerebellum volume and medulla volume. As independent
factors we entered our seven ecological characters, two sexual selection indices, and morphotype. We also entered snout-vent length into each model as a size correction.

We used the natural model averaging (50, 51) method of the R package *MuMIn* \{MuMInMultimodeli:vn\} to generate models of all possible combinations of our independent factors and to calculate average models where the corrected Akaike information criterion (AICc) (52) was within ten of the lowest AICc ($\Delta$AICc $\leq$ 10). Relative importance takes into account both the number of models in which a factor appears and the quality of those models (based on their AICc) to estimate the importance of a factor in predicting variation in the responding variable (51). In addition, factors that had a significant effect on a response variable have estimates with confidence intervals that do not include zero (50).

To test for phylogenetic signal in the data, we estimated Blomberg’s K (53) using the *picante* package (54) in R (55). We simulated the 95% confidence intervals for K under a Brownian motion model using the R package *phytools* (56).

To visualize our model averaging results, we have taken the residuals of regressions of brain region volume against body size. This removes the effect of body size on the volumes of each brain region, resulting in the variation in volume associated with different ecological and life history variables becoming more evident (Figure 1).
Figure 1. Differences in the volumes of the telencephalon, diencephalon, optic tectum, mesencephalon (excluding the optic tectum), cerebellum and medulla between different character states in *Ctenophorus* dragons. Bars represent means for each character state, based on residuals from regressions of brain region volume against snout-vent length and are presented for illustrative purposes only. Species means of the raw data, not residuals, were used for analysis in the phylogenetic linear regression models reported in Table 3. Differences between character states that are correlated with brain region volumes according to the phylogenetic linear regression models are outlined in black. Semel = Semelparous, Itero = Iteroparous.
Results

The volumes of the brain and all subdivisions (Table 2) were positively correlated with body size, as estimated by snout-vent length. Furthermore, there were significant differences in whole brain volume and the volumes of different subregions with respect to difference ecological variables. These results are presented in Table 3 and summarized below. Our two indices of sexual selection, sexual dichromatism and sexual size dimorphism, did not correlate with the volumes of any brain region. Habitat, territoriality, and density also did not correlate with any volume.

Whole Brain. Morphotype was an important predictor of brain volume (confidence interval, CI $\not\in 0$; relative importance, RI = 54%), the volumes of both the right (CI $\not\in 0$, RI = 75%) and left (CI $\not\in 0$, RI = 54%) hemispheres, and the volumes of all brain regions (CI $\not\in 0$, RI > 35% in all cases) except the cerebellum and the medulla (CI $\in 0$ RI < 35% in both cases). The volumes of the brain and all correlated brain regions were larger in diggers and smaller in squeezers. Runners were not significantly different from either group. No other ecological variable was correlated with whole brain volume or the volumes of either hemisphere (CI $\not\in 0$, RI < 35% in all cases).

Telencephalon. Both the left telencephalon (CI $\not\in 0$, RI = 52%) and right telencephalon (CI $\not\in 0$, RI = 39%) were larger in iteroparous species compared to semelparous species.

Diencephalon. Basking strategy was a significant predictor of both right (CI $\not\in 0$, RI = 92%) and left (CI $\not\in 0$, RI = 58%) diencephalon volume. Species that bask on elevated surfaces had larger diencephalons compared to ground-basking species. Whether or not a species constructed its own burrows was a significant predictor of left diencephalon volume (CI $\not\in 0$, RI = 42%) but not right diencephalon volume (CI $\in 0$, RI = 25%). Species which construct their own burrows had larger left diencephalons compared to species that do not.

Optic Tectum. Whether or not a species constructed its own burrows was a significant predictor of left optic tectum volume (CI $\not\in 0$, RI = 38%) but not right optic tectum volume (CI $\in 0$, RI = 26%). Species which construct their own burrows had larger left optic tecta compared to species that do not.
Table 2. Volumes of each brain region for the left and right hemispheres.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Left Hemisphere</th>
<th></th>
<th></th>
<th></th>
<th>Right Hemisphere</th>
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<td>Di</td>
<td>OT</td>
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<td>Di</td>
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<td>Mes</td>
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Tel = Telencephalon, Di = Diencephalon, OT = Optic Tectum, Mes = Mesencephalon, Cer = Cerebellum, Med = Medulla
Table 3. Effects of morphology, ecology and life history characters, and sexual dimorphism indices on brain region volume in dragon lizards.

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**Right Optic Tectum**

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These results are averages of phylogenetic linear regression models that include all models that were within 10 of the best corrected Akaike Information Criterion (ΔAICc ≤ 10). Confidence intervals (CI) above zero or below (in **bold**) indicate that different levels of the independent factor correlate with differences in the brain region volume. Confidence intervals that include zero indicate that there is no correlation between variation in sexual dimorphism and the response variable. Relative importance (RI) is a percentage that compares independent factors in their ability to account for variation in response variables. Bask = Basking Location, Burr = Burrower/Non-burrower, Morph = Morphotype, RS = Reproductive Strategy, SD = Sexual Dichromatism, SE = Standard Error, SSD = Sexual Size Dimorphism, SVL = Snout-vent Length, Ter = Territoriality
Mesencephalon, excluding Optic Tectum. Iteroparous species had larger left mesencephalons (CI $\not\ni 0$, RI = 58%) and right mesencephalons (CI $\not\ni 0$, RI = 52%) than semelparous species.

Cerebellum. Basking strategy was an important predictor of right cerebellum volume; species that bask on elevated surfaces had larger right cerebellar lobes compared to those that bask on the ground (confidence interval, CI $\not\ni 0$; relative importance, RI = 63%). There were no ecological variables that correlated with left cerebellum volume (RI < 35%, CI $\ni 0$ in all cases).

Medulla. No ecological variables correlated with medulla volume.

Phylogenetic Signal. Most variables displayed values for Blomberg’s K consistent with a Brownian motion model of evolution (Table 4). Habitat and refuge type showed values for Blomberg’s K values greater than expected under a Brownian motion model, however no variables displayed values under those expected under Brownian motion. All values were significant ($p < 0.05$) except for morphotype, perch, and density.

Discussion

We found support for both concerted and mosaic brain evolution among dragon lizards. As expected, the most important factor for predicting brain volume and the volume of all the major neural subdivisions was body size. We found additional support for concerted evolution with respect to morphotype. In this case, the pros- and mesencephalic structures seemed to be evolving in concert with respect to morphotype, while the rhombencephalic structures were not. Therefore, we found evidence for concerted brain evolution only in response to characters that described aspects of the lizard’s morphology. In contrast, we found that all the neural subdivisions, with the exception of the medulla, showed evidence for mosaic brain evolution with respect to differences in behaviour between species. This supports our hypothesis that as brains evolve, they change in ways that can be described as concerted and mosaic simultaneously. Our data suggest that while brains evolve in a concerted fashion with respect to morphology, they evolve in a more mosaic way with respect to behavioural traits.
Table 4. Summary of tests for phylogenetic signal

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The 95% confidence interval for K under a Brownian motion model on the phylogeny used in our analysis is 0.663-1.520. Most variables fall within this interval, some have a stronger phylogenetic signal than expected under Brownian motion, but none have a weaker phylogenetic signal.
We found a concerted pattern of brain evolution in dragons with respect to body size. In this way, the dragon brain follows the same consistent pattern found in all vertebrates of concerted brain evolution with respect to body size (12). Furthermore, the volume of the medulla correlated only with brain volume. The medulla is the most conserved neural subdivision across vertebrates (25) and has been used as a “baseline” brain structure with which to measure mosaic brain evolution in other subdivisions (12). Therefore, it is not surprising that this would be the least evolutionarily labile structure in the dragon brain and show no correlation with any ecological, life history, or evolutionary variable.

Despite being the least labile brain region, evidence of brain evolution in the medulla independent of body size has been found, with the most consistent pattern being correlated evolution between the medulla and the other component of the rhombencephalon, the cerebellum. This pattern has been found in in mammals (19) and in birds (16). We have found this pattern as well, with these structures being the only two showing no difference in volume between squeezer and digger morphotypes. The pros- and mesencephalic regions, the telencephalon, diencephalon, optic tectum, and remaining mesencephalon, are all larger in squeezers compared to diggers. Correlated evolution between tissues derived from the same developmental region of the neural plate is consistent with the hypothesis that brain region evolution is constrained by shared developmental origins (14). Therefore, the pattern of variation we have found between morphotypes supports a developmental-constraints hypothesis of brain evolution.

Platel (27) described the cerebellum and optic tectum as among the most evolutionarily labile brain regions between squamate species. Here, we detect almost no variation in cerebellum or optic tectum volume with any factor except body size, again supporting a more concerted model of brain evolution. This difference may be due to Platel’s broader scope within the squamates. Though he does no formal analysis, Platel associates the variation he found in cerebellum volume with locomotion. Species capable of bipedal locomotion or are arboreal had the largest cerebellum, while species without legs have the smallest cerebellum. All *Ctenophorus* dragons are capable of bipedal locomotion (57, 58), and therefore according to Platel’s hypothesis we would not expect to observe variation in cerebellar volume. Similarly, all dragons are diurnal and heavily vision-dependent, whereas more broadly amongst squamates there is great variation in visual dependence between species (27).
Both the mesencephalon and telencephalon varied in volume with reproductive strategy, which is characteristic of a mosaic model of brain evolution. Both structures are larger in iteroparous species compared to semelparous species. Since iteroparous species are longer lived and take longer to develop and mature, iteroparous species may simply have more time to invest resources in brain structure and, as longer-lived species, have more need for complex information processing in these two regions which are associated with higher-level cognitive processing and neuromodulation, respectively (11).

The diencephalon alone varied with basking height. We included basking height as a factor in our model because *Ctenophorus* dragons are strongly dichotomous between species which bask on elevated surfaces and species which bask on the ground (33). The differences in sensory perception, particularly vision, that would come from being elevated while basking are a potential source of differential selection on specific brain regions involved in sensory processing. Cell groups within the diencephalon are heavily involved in sensory processing, including processing visual input, however it is unclear why other regions heavily involved in visual processing, including the telencephalon and optic tectum, would not show similar patterns of variation if the explanation were that simple.

We also have detected surprising bilateral asymmetries in response to mosaic evolution in the lizard brain. Laterality in the neural control of behaviour is well known in lizards (59-61), including in one of the species included in this study (62), and the habenula is larger in the left compared to the right hemisphere in many lizards (63, 64). Though the habenula is not asymmetrical in *Ctenophorus* (pers. obvs.), we have detected bilateral asymmetries in volumetric changes between species in the diencephalon, optic tectum, and cerebellum. This result is confounding, however, as though bilateral asymmetry is ubiquitous in small brain regions, neurochemistry and behaviour (63), we cannot find a single report of bilateral asymmetry in major brain regions, as we have found here.

It is important to remember that, with the exception of the optic tectum, the brain regions we have measured are large structures made up of functionally distinct, interconnected cell groups. Making assumptions about why a complex, multifunctional neural subdivision such as the diencephalon would vary in volume with a specific
behavioural trait such as basking strategy would be spurious, however we have provided plausible hypotheses for the mosaic brain evolution we have observed. Nonetheless, these are hypotheses, not explanations. To determine the precise nature of the structural variation within each brain region, and ultimately to understand how specific selection pressures result in changes in brain morphology, more detailed studies of behaviour and brain structure are needed.

Here we have shown that, contrary to previous findings (31, 32), the reptile brain is evolving both in concert and as a mosaic both in response to components of ecomorph phenotype as well as other characters. We have found concerted evolution in response to morphological characters: brain size and morphotype. In contrast, we have found evidence for mosaic brain evolution in response to ecological and life history characters: basking strategy, burrowing, and reproductive strategy. Future studies are needed to confirm these patterns of concerted and mosaic brain evolution across a wider range of squamate species and with respect to a wider variety of relevant characters.
Chapter 5

Sexual selection predicts brain structure in dragon lizards
Abstract

It is well understood that phenotypic traits such as ornaments and armaments are shaped by sexual selection, typically selecting for larger and more elaborate males compared to females. But can sexual selection also influence the brain? The role of sexual selection in altering brain organisation and structure over evolutionary time is poorly understood. Previous studies in vertebrates have found contradictory results with no consistent pattern between whole brain volume and the strength of sexual selection. We hypothesize that sexual selection will act in a consistent way on two brain regions that directly control sexual behavior, the medial preoptic nucleus and the ventromedial hypothalamic nucleus. The medial preoptic nucleus controls male reproductive behavior while the ventromedial hypothalamic nucleus controls female reproductive behavior and is also involved in male aggression. To test our hypothesis, we used high-resolution magnetic resonance imaging combined with traditional histology of brains in 14 lizard species belonging to the Australian genus *Ctenophorus* (known as dragons), which vary in the strength of sexual selection they experience. Males belonging to species that experience greater sexual selection had a larger medial preoptic nucleus and a smaller ventromedial hypothalamic nucleus. Conversely, females did not show any obvious variation in these brain regions. We show that the primary brain nuclei underlying reproductive behavior in vertebrates can evolve in a mosaic fashion, differently between males and females, likely in response to the strength of sexual selection.
Introduction

Sexual selection is responsible for the evolution of traits that promote success in competition for mates or gametes (1, 2). Classical examples of premating sexual selection mechanisms include male contest competition and mate choice. These mechanisms favor the evolution of bright colors, structures such as horns, and larger body size among many other traits (3, 4). Underlying all behavior, including male contest competition and mate choice, is brain function. Therefore, brains should experience selection pressures imposed by mate choice and sexual selection more generally (5, 6). Empirical evidence for sexual selection acting on brains is rare, but where it exists, three competing patterns emerge. Different studies across a range of vertebrate species have found evidence that species under strong sexual selection either have smaller (7-10), or larger (11, 12) brains, or brain size does not vary with the strength of sexual selection (13-17). Studies that find positive correlations between sexual selection and brain size argue that sexual selection increases cognitive demands resulting in larger brains (8, 18). Studies that find negative correlations between sexual selection and brain size argue that sexual selection increases energetic demands, resulting in a trade-off between allocating energy to brain tissue and reproduction (7, 8). These hypotheses are not mutually exclusive, nor do they offer criteria that preclude competing explanations, and therefore no unifying theory exists.

Recent studies have suggested that instead of evolving as a unit, the major subdivisions of the brain may be evolving as a mosaic in response to sexual selection (19). However, no consistent pattern has emerged. The cerebellum may be one of the only subdivisions under sexual selection (17) or conversely it may be among the only subdivisions not influenced by sexual selection (18). And neocortical volume may increase with sexual selection (20), decrease with sexual selection (21), or not vary with the strength of sexual selection (18). Ultimately, there may not be any consistent relationship between sexual selection and the volume of the brain or its major subdivisions (8, 22). The complex and diverse functions of the brain mean that variation in major brain subdivisions likely cannot be explained by a single selective pressure (14, 23, 24). We think that a better understanding of the relationship between the brain and sexual selection will come by targeting specific brain nuclei with well-characterized functions involved in reproduction (25) and by selecting a model system of closely related species that differ in key traits and have a well-resolved phylogeny to control for relatedness.
(22). These approaches have already provided important and novel insights into the evolution and the neural underpinnings of vocal learning, including in the context of sexual selection (26-28).

Across all vertebrates, the same brain regions control reproductive behavior. Detailed analysis on structure and function show that the medial preoptic nucleus (MPON) is the key brain region controlling male reproductive behavior while the ventromedial hypothalamic nucleus (VMN) is the key brain region controlling female reproductive behavior (29). Variation in the volume of these regions is associated with variation in sexual activity levels experimentally and in nature. For example, using stress to experimentally manipulate MPON volume in rats showed that MPON volume predicts sexual activity (30). Furthermore, seasonal variation in MPON and VMN volume corresponds to season variation in sexual activity (31).

The sex-specific functions of these nuclei suggest that they are likely to be sexually dimorphic. In some taxa this is true. For example, the MPON is larger in males (male-biased sexual dimorphism) in Japanese quail, Anolis lizards, rats and humans (32-35), even though there is no sex-difference in whole brain volume. However, the MPON is not always sexually dimorphic. It is monomorphic in species such as leopard geckos, mice and macaque monkeys (36-38). Volumetric data on the VMN are rare, but the VMN is larger in females (female-biased sexual dimorphism) in the lizard Cnemidophorus inornatus, monomorphic in several other reptile species, and larger in males in rats and the lizard Anolis carolinensis (39-42). These contrasting examples show that sexual dimorphism in MPON and VMN volume is not consistent across species, and we hypothesize that sexual dimorphism in these brain regions is related to the strength of sexual selection. In a pair-wise comparison between two sister species of voles (Microtus) in which one species is monogamous and the other is promiscuous, the MPON was sexually dimorphic only in the species under strong sexual selection (43), and in a similar comparison between two fence lizards (Sceloporus) only the species under strong sexual selection had sexually dimorphic aromatase-expressing cell counts in the MPON and VMN (44). Based on these studies, we propose that strong sexual selection increases sexual dimorphism in brain regions that control sexual activity, as these regions have a direct impact on reproductive success, the ultimate target of sexual selection.
To test our hypothesis, we have selected closely related lizard species in the Australian genus *Ctenophorus* (known as dragons), which vary in the strength of sexual selection they experience, and for which there is a robust phylogeny (45). We asked whether brain, MPON, and VMN sexual dimorphism is predicted by three indices of precopulatory sexual selection: sexual dichromatism, body size sexual dimorphism, and head size sexual dimorphism (Figure 1). These indices are widely used as indicators of the strength of sexual selection in comparative studies (2, 45-49) and have been empirically shown to be associated with sexual selection in lizards (48, 50-52), including *Ctenophorus* (49, 53-55). We predicted that species under strong sexual selection would have male-biased MPON sexual dimorphism and female-biased VMN sexual dimorphism, but no difference in brain dimorphism. We also measured the volume of the habenula (HB) as a control region because it is not known to be involved in reproductive behavior in lizards (36, 44, 56-58).

**Materials & Methods**

*Specimen Acquisition, Scoring, and Preparation*

We collected 287 lizards from fourteen *Ctenophorus* species. All lizards were collected during the breeding season (September and October) in 2011, 2012 or 2013. Following capture, they were transported to the Australian National University in Canberra, Australia, where they were maintained in outdoor enclosures with *ad-libitum* access to food (wild insects) and water, and their diet was supplemented twice weekly with domestic crickets. The Australian National University’s Animal Experimental Ethics Committee approved all research under protocol number A2011-49.

Each species of *Ctenophorus* was classified on a sexual dichromatism scale from zero (completely monochromatic) to 18 (maximally dichromatic) following Chen *et al.* (45). For all species included in this study, females have cryptic coloration while males may be either cryptic or conspicuous (45, 49). Therefore, the more conspicuous the male, the more sexually dichromatic the species (Table 1).
Figure 1. Ancestral state estimation contrasting the evolution of sexual dichromatism and body size sexual dimorphism across all *Ctenophorus* species. Species analyzed for brain morphology in this study are indicated in bold. Values for species not used in this study are from Chen *et al.* (45). Values for species used in this study, including values for head size sexual dimorphism (not depicted here) can be found in Supplementary Materials 2. This figure was generated using the R package phytools (66).
Table 1. Each species of *Ctenophorus* classified on three indices of sexual selection.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
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<th>HSSD</th>
<th>BSSD</th>
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*SD = sexual dichromatism, HSSD = head size sexual dimorphism, BSSD = body size sexual dimorphism*
We measured the snout-vent length and head width of each lizard, which we then used to calculate body size sexual dimorphism and head size sexual dimorphism ratings for each species using the Lovich-Gibbons sexual dimorphism index (LGSDI); (Male/Female) – 1 (45, 59). A rating of zero indicates no sexual dimorphism and a rating of one indicates that one sex is double the size of the other. Positive values indicate male-biased dimorphism while negative values indicate female-biased dimorphism (Table 1).

*Calculating Whole-brain Volume using Magnetic Resonance Imaging*

Whole-brain images were acquired using a Bruker Avance 11.74 Tesla wide-bore spectrometer (Ettlingen, Germany) with a micro-2.5 imaging probe capable of generating magnetic gradients of 1.50 T/m. Brains were immersed in Fomblin (perfluoropolyether, Grade Y06/6, JAVAC, Sydney, Australia) and placed in a 10 mm diameter Wilmad tube using a custom-built plastic holder. The brains were buoyant in Fomblin and the plastic holder was designed to hold them submerged. Parameters used in the scans were optimized for gray-white matter contrast in the presence of Magnevist. We used a gradient echo (T$_2$*-weighted) 3D fast gradient-echo sequence (FLASH), with a repetition time = 40 ms, echo time = 8 ms field-of-view = 11 × 11 × 16 mm and matrix size = 110 × 110 ×160, producing an image with 100 µm$^3$ isotropic voxels. As the geometry was similar for each scan, tuning, matching and shimming (on most occasions) was needed only for the first brain in a sequence. Using this method, four brains could be individually scanned per hour.

To ensure consistent measures of brain morphometry all images were first manually masked such that consistent coverage of brain structures and nerve endings was achieved. The manually masked areas were then set to the background value such that they were not included in subsequent calculations. Brain volumes were then calculated in the MINC toolkit via a histogram cut-off value for image and background using a minimum error thresholding algorithm (61).

*Calculating Brain Region Volumes using Histology*

Fixed brains were embedded in agarose and sectioned at 70 or 60 µm using a vibratome. We stained all sections for 5 min using SYBR-green (Life Technologies Australia,
Melbourne, Australia), rinsed them for one hour in phosphate-buffered saline, and mounted them in Fluoro-Gel (ProSciTech, Brisbane, Australia). Fluorescence was visualized using an Olympus BX63 microscope. Images were captured using an XM10 digital camera and the imaging-stitching function of the Olympus CellSens software package (Figure 2). The areas of the MPON, HB and VHM were measured, blind to group, by a single person (DH) using the Count & Measure CellSens module. Volumes were calculated by multiplying the areas by the slice thickness (58). We identified and delineated each region (Figure 2) following Cruce (62).

Statistical Analysis

We analyzed 287 brains, however tissue damage to one or more brain regions in some specimens meant that not all brains could be used in all analyses (Table 2). We calculated the mean volume and standard error for each brain region per sex/species and sexual dimorphism was calculated using the LGSDI using these mean values. All means and sexual dimorphism values were log-transformed prior to analysis. Phylogenetic information, including relationships among species and branch lengths, was taken from a time-calibrated molecular phylogeny (45). To test for phylogenetic signal in the data, we estimated Blomberg’s K (63) using the picante package (64) in R (65). We simulated the 95% confidence intervals for K under a Brownian motion model on three different pruned trees using the R package phytools (Table 3; (66)).

We fitted phylogenetic linear regression models using a model of trait evolution by Brownian motion (67) in the R package phylolm (68). We generated separate models for sexual dimorphism, male volumes, and female volumes for each response variable: brain volume, MPON volume, VMN volume and HB volume. As independent factors we entered into the model sexual dichromatism, head size sexual dimorphism, and body size sexual dimorphism. We also entered a factor into each model as a size correction: snout-vent length for brain volume and brain volume for MPON, VMN and HB volumes.
Figure 2. Fluorescent-stained coronal sections (a) and magnetic resonance images rendered transparent (b) depict the locations of the medial preoptic nucleus (MPON), ventromedial hypothalamic nucleus (VMN), and habenula (HB) in the brain of a *Ctenophorus* lizard. Coronal sections are anterior-posterior left-to-right. Magnetic resonance images depict lateral (left) and frontal (right) views.
Table 2. Means and standard errors for each brain region per sex/species. Due to tissue damage that sometimes affected a brain region of interest, not every brain region could be measured in every sample. MPON, VMN and HB means and standard errors (SE) are in um³. Brain volume means and SE are in 10⁶ um³.

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<th>VMN Volume</th>
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N = sample size, MPON = medial preoptic nucleus, VMN = ventromedial hypothalamic nucleus, HB = habenula
We used the natural model averaging (69, 70) method of the R package MuMIn (71) to generate models of all possible combinations of our independent factors and to calculate average models where the corrected Akaike information criterion (AICc) (72) was within four of the lowest AICc ($\Delta$AICc $\leq$ 4). Relative importance takes into account both the number of models in which a factor appears and the quality of those models (based on their AICc) to estimate the importance of a factor in predicting variation in the responding variable (70). In addition, factors that had a significant effect on a response variable have estimates with confidence intervals that do not include zero (69).

**Results**

We tested for phylogenetic signal using Blomberg’s K and found that all variables display values for K that we would expect under a Brownian motion model of evolution. All values were significant ($p < 0.05$) except for MPON and VMN volumes (Table 3).

Sexual dichromatism was an important predictor of both MPON dimorphism (relative importance, RI = 100%) and VMN dimorphism (RI = 81%) and was positively correlated with MPON dimorphism (confidence interval, CI $> 0$) and negatively correlated with VMN dimorphism (CI $< 0$). Since male-biased dimorphism is positive and female-biased dimorphism is negative this indicates that the MPON is larger in males and the VMN larger in females as sexual dichromatism becomes more pronounced. None of the sexual selection indices were important predictors of brain sexual size dimorphism or HB dimorphism (RI $< 50\%$, CI $\ni 0$ in all cases). See Table 4 for details.

To determine the extent to which these differences in dimorphism involved variation in males and females we examined brain, MPON, VMN, and HB volumes separately for each sex. No index of sexual selection was important for predicting brain volume in males or any volume in females (RI $< 30\%$, CI $\ni 0$ in all cases). However, sexual dichromatism had strong predictive value for both the MPON and the VMN in males. As species became more sexually dichromatic, male MPON volume increased (RI = 81%, CI $> 0$), while VMN volume (CI $< 0$) and HB volume (RI = 66%, CI $< 0$) decreased (Figure 3; Table 4).
Table 3. Summary of phylogenetic signal tests. P values < 0.05 are indicated in bold.

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<td>Snout-vent Length</td>
<td>1.071</td>
<td>0.042</td>
</tr>
<tr>
<td>Brain Volume</td>
<td>0.994</td>
<td>0.041</td>
</tr>
<tr>
<td>MPON Volume</td>
<td>0.849</td>
<td>0.404</td>
</tr>
<tr>
<td>VMN Volume</td>
<td>0.967</td>
<td>0.164</td>
</tr>
<tr>
<td>HB Volume</td>
<td>1.080</td>
<td>0.038</td>
</tr>
</tbody>
</table>

SD = Sexual Dichromatism, HSSD = Head Size Sexual Dimorphism, BSSD = Sexual Body Size Dimorphism, MPON = medial preoptic nucleus, VMN = ventromedial hypothalamic nucleus, HB = habenula
Table 4. Correlations between three indices of sexual selection and sexual dimorphism in the brains of *Ctenophorus* lizards.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sexual Dimorphism</th>
<th>Male Volumes</th>
<th>Female Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>RI</td>
</tr>
<tr>
<td>Whole Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.033</td>
<td>0.055</td>
<td>-</td>
</tr>
<tr>
<td>SVL</td>
<td>0.046</td>
<td>0.030</td>
<td>0.32</td>
</tr>
<tr>
<td>SD</td>
<td>0.001</td>
<td>0.001</td>
<td>0.21</td>
</tr>
<tr>
<td>HSSD</td>
<td>0.061</td>
<td>0.041</td>
<td>0.28</td>
</tr>
<tr>
<td>BSSD</td>
<td>0.064</td>
<td>0.039</td>
<td>0.35</td>
</tr>
<tr>
<td>Medial Preoptic Nucleus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.021</td>
<td>0.009</td>
<td>-</td>
</tr>
<tr>
<td>BV</td>
<td>-0.031</td>
<td>0.040</td>
<td>0.13</td>
</tr>
<tr>
<td>SD</td>
<td>0.005</td>
<td>0.001</td>
<td>1.00</td>
</tr>
<tr>
<td>HSSD</td>
<td>0.040</td>
<td>0.075</td>
<td>0.11</td>
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<tr>
<td>BSSD</td>
<td>-0.029</td>
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<tr>
<td>Ventromedial Hypothalamic Nucleus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.007</td>
<td>0.006</td>
<td>-</td>
</tr>
<tr>
<td>BV</td>
<td>0.042</td>
<td>0.023</td>
<td>0.33</td>
</tr>
<tr>
<td>SD</td>
<td>-0.001</td>
<td>0.001</td>
<td>0.81</td>
</tr>
<tr>
<td>HSSD</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSSD</td>
<td>0.075</td>
<td>0.040</td>
<td>0.48</td>
</tr>
<tr>
<td>Habenula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.003</td>
<td>0.004</td>
<td>-</td>
</tr>
<tr>
<td>BV</td>
<td>0.041</td>
<td>0.019</td>
<td>0.64</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>0.001</td>
<td>0.10</td>
</tr>
<tr>
<td>HSSD</td>
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<td>0.036</td>
<td>0.26</td>
</tr>
<tr>
<td>BSSD</td>
<td>-0.027</td>
<td>0.038</td>
<td>0.13</td>
</tr>
</tbody>
</table>

These results are averages of phylogenetic linear regression models that include all models that were within 4 of the best corrected Akaike Information Criterion (ΔAICc ≤ 4). Confidence intervals (CI) above zero (in **bold**) indicate that increases in the independent factor correlate with increases in the response variable. Confidence intervals below zero (in *italics*) indicate that increases in the independent factor correlate with decreases in the response variable. Confidence intervals that include zero indicate that there is no correlation between variation in sexual dimorphism and the response variable. Relative importance (RI) is a percentage that compares independent factors in their ability to account for variation in response variables (70). Snout-vent length (SVL) and brain volume (BV) are included as independent factors for size-corrective purposes. SD = Sexual Dichromatism, HSSD = Head Size Sexual Dimorphism, BSSD = Body Size Sexual Dimorphism.
Figure 3. The volumes of the brain, medial preoptic nucleus (MPON), ventromedial hypothalamic nucleus (VMN), and habenula (HB) in males and females have a variety of different relationships with an index of sexual selection, sexual dichromatism. Male MPON volume is positively correlated with sexual dichromatism, while male VMN and HB volumes are inversely related. Female MPON, VMN, and HB volumes are not correlated with sexual dichromatism. Brain volume is not correlated with sexual dichromatism in either sex. The regression lines do not represent the phylogenetic linear regression models used for the analyses (results shown in Table 1). Points show size-independent log-transformed species means ± standard error. These graphs were generated using the R package ggplot2 (102).
Discussion

As predicted, we did not find any differences in whole brain sexual dimorphism with any index of sexual selection. However, we did find variation in specific brain nuclei correlated with sexual selection. Our results support our hypothesis that sexual dimorphism in the brain regions responsible for reproductive behaviors increase with the strength of sexual selection. Males from more sexually dichromatic species had larger MPONs. While we predicted that females would have larger VMNs, we only found this relationship because there appeared to be selection for smaller VMNs in males as sexual dichromatism increased. Surprisingly, we found that males from more sexually dichromatic species also had smaller HBs.

The MPON was larger in males from species that we estimated are under relatively stronger sexual selection. The MPON is the key brain region necessary for consummatory reproductive behavior (e.g. courtship, mounting, erection, ejaculation) in male vertebrates (29). The MPON also facilitates appetitive male reproductive behavior (sexual motivation or mate seeking) (29). In male vertebrates, larger MPONs are associated with greater strength and frequency of reproductive behaviors (30, 73, 74). This is consistent with our hypothesis that MPONs are larger in species under stronger sexual selection where males also experience more intense competition for mates and greater sexual conflict.

Although we found support for our hypothesis of increasing female-biased VMN sexual dimorphism under stronger sexual selection, the underlying pattern was unexpected: the dimorphism is due to a reduction in male VMN volume, not an increase in female VMN volume. Recent studies show that the VMN controls male-male aggression in mice (75) and likely in lizards (57). In male lizards, as with many other vertebrates, intra-sexual aggression is associated with territory maintenance and establishing dominance, behaviors that strongly influence reproductive success in many systems (76). Therefore, aggression under certain circumstances may be positively sexually selected (2), and this may be why we see male-biased sexual dimorphism in VMN volume in relatively monochromatic species. In contrast, in highly dichromatic species, we see female-biased sexual dimorphism in VMN volume. This mirrors previous findings in lizards, where VMN volume may be larger in males, monomorphic, or larger in females (39-41, 77). This pattern suggests that VMN volume decreases in males under relatively
stronger sexual selection and may be related to the evolutionary costs associated with increased aggression. For example, in the leopard gecko, males incubated at abnormally high temperatures have larger VMNs (36), are more likely to respond to females with aggressive behaviors instead of reproductive behaviors (78), and consequently, experience a fitness cost. It is possible that a reduction in VMN volume may ameliorate the costs of aggressive behavior in circumstances where it is unwarranted.

The VMN facilitates female consummatory reproductive behaviors (29, 79, 80) and if our indices of sexual selection reflected the strength of sexual selection in both sexes then we would expect female VMN volume would be positively correlated with one or more index. However, female VMN volume was not correlated with any index of sexual selection. This suggests that perhaps our indices of precopulatory sexual selection do not reflect the strength of sexual selection in females. This is consistent with mate-choice experiments in lizards generally (81), and in three species of *Ctenophorus* in particular (82-84) which found no evidence for female mate choice. One argument is that female choice in *Ctenophorus* may be postcopulatory (85, 86), and therefore physiological rather than behavioral, thereby precluding sexual selection on the brain (5).

Surprisingly, we found that the HB is smaller in males from more sexually dichromatic species. Previous studies have used the HB as a control region when examining the neural basis for reproductive behavior in lizards, primarily due to the absence of sex hormone receptors (36, 44, 56-58). While the HB does not serve any known function in reproductive behavior in lizards, this is not the case across all vertebrates. In mammals, the HB is involved in both reproductive and maternal behavior (87-90) and there is some evidence that the HB may be responsive to sex hormones in mammals (90, 91) and birds (92). In at least one species of lizard the HB varies in size seasonally (58) and it appears that the HB receives projections expressing androgen receptors in some lizard species but not others (93-95). Considering all this evidence from across vertebrates, our result must serve as a caution against using the HB as a control region in studies of the neural underpinnings of reproductive behavior in lizards. It is also a reminder that brain function, particularly in lizards, is still extremely poorly understood, and research is still needed on the most fundamental aspects of brain function.
These different patterns in the MPON, VMN, and HB suggest that the *Ctenophorus* brain is evolving as a mosaic in response to sexual selection, without corresponding variation in the volume of the brain. The MPON, VMN and HB are small nuclei compared to the volume of the entire brain (Figure 2) and it is easy to see how they may vary in size without significantly influencing brain volume. This is in contrast with the only other study to examine brain evolution in a genus of lizards, which found little evidence for mosaic brain evolution in *Anolis* lizards and instead found a pattern of allometric scaling across the major neural subdivisions and regions of the telencephalon (96, 97). However, our results are consistent with the prevailing view in mammals and birds, that brains in both groups are evolving as a mosaic (18, 22, 98, 99). Therefore, we suggest that the previous absence of evidence for mosaic brain evolution in *Anolis* may be because the ecological differences measured did not place different selection pressures on the brain regions examined (22). For example, the cerebellum is involved in movement coordination (100), which is important for arboreal lizards such as *Anolis*. However, all *Anolis* ecomorphs examined are arboreal, and the differences between ecomorphs in the complexity of their arboreal environments may not have been sufficient to differently impact their need to be well coordinated, and so have a well developed cerebellum. Alternatively, the cerebellum is a major neural subdivision and differences between ecomorphs may be more readily detected at the level of individual cerebellar nuclei, where subtle variation in movement coordination demands between ecomorphs may be more easily reflected. This is the level at which we have found evidence for mosaic brain evolution.

Evolutionary comparative neuroscience continues to be a powerful yet underutilized avenue of neuroscience research (101). Brain research on sexual selection has traditionally taken two paths (14): using a phylogenetic comparative approach to examine variation in brain volume across a large set of species (7-12, 14, 16, 17), or looking at variation in brain structure (43, 44), and more recently, gene expression (5), at a finer scale between two species that are different in some key aspect of their biology. Here, we have combined relatively fine-scale brain analyses with a comparative approach. We carefully selected brain nuclei with specific, well-characterized functions and a hypothesis with a strong foundation in the literature. Using this approach, we show that the primary brain nuclei underlying reproductive behavior in vertebrates can evolve in a mosaic fashion, likely in response to the strength of sexual selection. Given the importance of female choice, sexual deception, and the
general conflict that accompanies so much of sexual selection—and all of which impose a cognitive load—we suggest that the brain is an obvious target of selection (19, 25). Sexual selection has likely played an under-appreciated role in organizing the brain and future studies addressing this deficit could be especially rewarding and enrich a rapidly growing field.
Contributions

General
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Chapter 2
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Chapters 3, 4 & 5
These three chapters use MRI data collected thanks to the facilities and the scientific and technical assistance of the National Imaging Facility, University of Western Sydney and University of Queensland Nodes. B. Moroney (Nanoscale Group, University of Western Sydney) designed the lizard brain holder that made the MRI scanning possible. The MRI scanning was supported by a grant from the National Imaging Facility of Australia.

Chapter 4
Thanks to E. Walsh for her help with data visualization in this chapter.

Chapter 5
This chapter would not have been possible without the support and dedication of D. Birch and N. Vella at the Macquarie University Microscopy Unit. Thanks to A. Mailey and J. Godara for their support with the histology. L. Holman provided advice on the statistical analyses used in this chapter and the Evolutionary Ecology Reading Group provided comments on this manuscript.
Section 3

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