LEARNING UNDER STRESS: SEPARATING THE EFFECTS OF ALLOPREGNANOLONE AND FLUOXETINE IN *CARASSIUS AURATUS*

BY
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A Thesis

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Dedication to

Dinah, Devin, Donald:

My caryatids.

Foremost, to my goldfish.

I'd a monument construe to the folks I knew

but words are hardly true, frenetically flew

from my mind the moment it was you anew.

Lit like passions in somber passing stations,

love like roses in spring-cessation:

you may be mine to root attestations.

Contents

Abstract

This thesis compares the cognitive and affective effects of two anxiolytic compounds in the goldfish, Carassius auratus: fluoxetine (Prozac©), an antidepressant, and allopregnanolone, a neurosteroid native to the brain of vertebrates. There is a literature review on the role of the interactions of stress, the endocrine system, and the brain in both goldfish and humans to stage the hypotheses in question, ultimately predicting a decline in cognition in fish exposed to the anxiolytics unless they are also presented with exogenous stress. The results of the scototaxic trials indicate a significant increase in exploratory behavior in only the allopregnanolone group over the control. Cortisol-treated groups exhibited greater initial avoidance behavior, but this effect diminished in latter trials, resulting in significant impairments by the final block. Those fish treated with anxiolytics and cortisol presented a similar pattern, but with only allopregnanolone+cortisol demonstrating hippocampal-dependent avoidance behavior on par with the control group by the end of the experiment. Throughout the trials, all fish treated with fluoxetine demonstrated substantial impairments, especially in tests that required the retention of a trace stimulus. In conclusion: it is apparent that fluoxetine impedes temporal cognition in goldfish, even when compensating for hypercortisolemia, and that allopregnanolone is capable of ameliorating the cognitive deficits imposed by chronic stress on the hippocampal homologue in goldfish. Additionally, the acute anxiolytic effects of allopregnanolone are likely conserved in the goldfish.

Introduction

Modern psychopharmaceutical therapies are notorious for their inefficacy relative to other medical treatments and often toxic side-effects (Leucht et al, 2012). Given that psychiatric disorders are generally only loosely correlated with biological findings and that the understanding of causality is still a distant goal, it is unsurprising that clinical psychiatry has not yet been realized as clinical neuroscience (Reynolds et al, 2009; Insel and Quirion, 2012; Kraemer, Schultz, and Arndt, 2002). Instead, positive results in clinical trials lead to the adoption of treatment paradigms for complex disorders without a full understanding of the mechanisms involved. An example of this reasoning is the establishment the Serotonin Hypothesis in developing the selective serotonin reuptake inhibitor (SSRI) Prozac© as a treatment for major depression. This approach, as will be addressed later, is highly problematic. Unfortunately, coping with the complexities of diseases that manifest subtle behavioral symptoms isprofoundly difficult and definitive etiology is hard to come by. The careful control of confounding variables may be better managed in animal models, though this solution requires the effective demonstration of analogous behaviors between species composed of homologous organs in order to translate effects to other species (Green, Gabrielsson, and Fone 2011).

This experiment involves the use of an animal model to manipulate possible confounding variables in an attempt to disentangle their possible effects. In any comparison of behavioral effects, there is an array of known and unknown factors that may interact to affect experimental results, especially complicating the investigation of the profile of various treatments. The use of a fish model in the laboratory permits an exacting level of control of the subjects' environment and the administration of experimental treatments that would not be feasible with human subjects. However, extrapolation to the human condition must, as with any model, be exercised

with care. To provide a basis for possible analogies to function and homologies to structure, a review of previous literature on the physiological systems of vertebrates that regulate the stress response and its effects on learning is carried out.

Ultimately, this thesis aims to explore the relative effects of the popular pharmaceutical fluoxetine (Prozac©) and the neurosteroid allopregnanolone, on cognition in goldfish. Since the former substance has been correlated to lowered performance in shuttle-box avoidance learning (Beulig et Fowler, 2008) and the latter has been shown to attenuate performance on similar tasks in rats (Johanssonet al, 2002) and humans (Kask et al, 2007), it is predicted that both anxiolytics may have negative effects for the acquisition of operant conditioning. To dissect these possible cognitive deficits from their value as antidepressants, the performance of the fish administered these treatments will be compared to a parallel cohort with the same dosing schedule plus cortisol, the principle regulator of chronic stress in fish and humans. It is hypothesized that the negative effects on learning in the non-stressed paradigm will be overpowered by the benefits provided by these antidepressants in this cortisol-implanted model of chronic stress, resulting in greater avoidance behavior over the cortisol-alone group.

By initially investigating the compounds' effects on exploratory behaviors, this design aims to explore the acute affective changes produced by these treatments, which may be predictive of the long-term affective profiles of these compounds. It is predicted that both fluoxetine and allopregnanolone without cortisol will promote significantly greater exploratory behavior given the anxiolytic profiles of these drugs. Cortisol should be roughly the same as the control group, as the hyperarousal of this corticosteroid competes with its regulation of anxiety response, likely summing to produce no overall effect on exploratory behavior.

The differenetial performance of these animals in a task with and without a temporal delay between the presentation of the conditional and unconditional stimuli (CS and UCS) will be utilized to determine the effects of the treatments on hippocampal-dependent behavior. Improvements in avoidance performance in fish that must retain memory of the preceding stimulus over a delay during which the CS is absent (the "Trace" condition) require use of the lateral zone of the dorasalis telencephali, whereas those animals with this region ablated perform identically to controls in non-delayed learning paradigms (Portavella et al, 2004). This region is purportedly homologous to the hippocampal formation in mammals a region essential to memory function and implicated in the development of pathologies such as depression (Northcutt, 2008; Sheline et al, 1996). Cortisol, a glucocorticoid of the endocrine stress axis in humans and fish but not rats, has been shown to lead to atrophy of the hippocampus when present in excess (Zhang et al, 2006). By demonstrating the selective effects of substances with similar antidepressant properties in hypercortisolemic animals versus controls, it may be possible to understand the actions of the chemicals in a context resembling major depression in humans. In major depression, cognition is significantly impaired largely through inhibition and atrophy of the hippocampus (Lupien et al, 1998; Sapolsky, 2000). The results of this experiment suggest the efficacy of allopregnanolone compared to the well-researched Prozac© as a treatment for depression that may have greater efficacy and fewer side-effects.

Stress, the Endocrine System, and the Brain

Defining Stress

> ***Everybody knows what stress is and nobody knows what it is. The word stress, like success, failure, or happiness, means different things to different people and, except for a few specialized scientists, no one has really tried to define it although it has become part of our daily vocabulary. Is it effort, fatigue, pain, fear, the need for concentration, the humiliation of cen-***

> ***sure, loss of blood, or even an unexpected success that requires complete reformulation of one's life? The answer is yes and no. That is what makes the definition of stress so difficult. Every one of these conditions can produce stress, and yet none of them can be singled out as "it" since the word applies equally to all other as well. (Selye, 1976)***

Stress has been a topic surrounded by controversy even before the first rodents were injected with topsoil by the famous Hans Selye. Walter Cannon was the first to employ the term "stress" as biologists use it today, as a threat to homeostasis, inclusive of the previous definitions that focused on emotional upset as well as a physiological phenomenon (Cannon, 1935). Cannon urged his peers in medicine to address psychological stress as an underlying issue for patients of other maladies: "The doctor is properly concerned with the workings of the body and their disturbances, and should have, therefore, a natural interest in the effects of emotional stress and in the modes of relieving it." (Mason, 1975). While Cannon was the first to expand the definition of stress and advise a practical role for stress research in medicine, he was relatively unheeded due to the absence of published findings and a lack of pre-established models of stress research.

Hans Selye, an endocrinologist and most memorable researcher in this field who elaborated the theory of General Adaptation Syndrome, observed a series of common pathologies that resulted from severe stress to an organism's metabolism or tissue across species (Selye, 1946). This led Selye to investigate the roles of the various hormones and organs involved in stress, essentially the modern HPA axis, and he began to recognize a pattern of arousal of non-specific stress that we now term HPA-axis activation. Measuring the short-term consequences of extreme stress, such as the atrophy of the liver and thymus after the injection of sublethal doses Selye contrasts this generalized demand with the specific effects of particular "stressors";

Relevant HPA/HPI axis hormones and drugs to humans and teleosts								
Dexamethsone (DEX)	**Epinepherine/ Norepinepherine**	**Croticotropin Releasing Hormone (CRH)**	**AdrenoCorticotropic Releasing Hormone (ACTH)**	**Corticosterone**	**Cortisol**	**Urocortin I (mammals) / Urotensin I (teleosts)**	**Size / Classification**	
392.461 g/mol Exogenous glucocorticoid	183.20 g/mol Catecholamine/ 169.18 g/mol Catecholamine	4.75 kDa (human) 41 AAs	4.54 kDa (human) 39 AAs	346.46 g/mol Steroid, glucocorticoid	362.46 g/mol Steroid, glucocorticoid	4.707 kDa (human) 40 AAs		
artificial	Chromaffin cells, adrenal[13]	Paraventricular nucleus of the hypothalamus[20]	Anterior Pituatary[16]	*Zona fasciculate*, adrenals	*Zona fasciculate*, adrenals	Pituitary gland[1]	**Origin (Humans)**	
artificial	Chromaffin cells, interrenal[12]	Olfactory bulbs, Dd, Dc, SCN, PVN, …[21]	Epsilon cells of the *pars distalis*, Pituatary[11,17]	Interrenal cells[14]	Interrenal cells[12]	Urophysis[3] Forebrain[4] Pituatary[11]	**Origin (Teleosts)**	
Binds GR with greater affinity than endogenous GCs[32]	Increases LTP[31] Decreases recall[31] Improves attention, wakefulness[31]	Stimulates ACTH, endorphins; POMC[19]	Stimulates steroidogenesis[18] Increases cortisol production[18]	Similar to cortisol in rodents, less prevalent in humans and teleosts[13]	Amygdalar hypertrophy[14] Hippocampal atrophy[14] PFC atrophy[14]	Downregulates CRH expression[7] Corticotropin-releasing[11]	**CNS effects**	
Suppresses HPA/HPI response to acute stress[32]	Vasoconstrictive[29] Motor activity[26] Sympathetic PNS activation[27,31] Energy mobilization[28]	Regulates the interactions of CRH-BP and CRHRs[20]	Product of POMC[16] Diel regulation[16] Induces catecholamines[18]	Similar to cortisol with less affinity in most teleosts, humans[15]	Inhibits ACTH, CRH[14] Mobilizes FFA, glucose, proteinolysis[12,13]	c-Fos induction via CRH_2R[2]	**Other effects**	
GRs Lesser:MRs	Adrenergic R's: α_1, α_2, β_1, β_2, β_3	CRH_1R[6] CRH_2R[6]	MC_2R[18]	MRs[12,13] GRs[12,13]	MRs[12,13] GRs[12,13]	CRH_1R[6] CRH_2R[6]	**Ligand affinities**	
DEX suppression test[32] Anti-inflammatory[32] Symptom management[32]	Glycogenolysis[12] Exercise Adaption[26,29] Enhances learning[25,13] Activates adaptive immunity[30]	Anxiogenic, depressive[22] Acute stress response[23,24]	Mobilizes steroidogenesis on a quotidian and acute basis[16] Stimulates glucocorticoids[18]	Diel regulation[15] Feeding[15] Functions as glucocorticoid[13]	Glycogenolysis[12, 15] Immunosuppressant[12, 13] Stress response[12,13] Memory consolidation[12,13]	Regulator of appetite (anorexigenic)[7] Coronary vasodilator[8] Gastric antiinflammatory[9]	**Proposed functions**	

TABLE 1: HPA/HPI HORMONES

1, Iino et al, 1987; **2**, Vaughan et al, 1995; **3**, Bern, 1985; **4**, Conlon, 2007; **5**, Lederis, 1985; **6**, Bernier, 1999; **7**, Bernier et al, 2001; **8**, Lenz et al, 1985; **9**, Chatzaki et al, 2003; **10**, Lederis et al, 1982; **11**, Fryer et al, 1983; **12**, Mommsen et al, 1999; **13**, Herbert et al, 2006; **14**, Sangalang et al, 1972; **15**, Kuhn et al, 2004; **16**, Aguilera, 1994; **17**, Ball and Olivereau, 1965; **18**, Rae et al, 1979; **19**, Jessop, 1999; **20**, Huising et al, 2004; **21**, Alderman et al, 2007; **22**, Holsboer, 1999; **23**, To et al, 1999; **24**, Merali et al, 2004; **25**, Mazeaud et al, 1977; **26**, Stone et al, 2003; **27**, Wortsman, 2002; **28**, Sorrels and Sapolsky, 2007; **29**, Floras et al, 1988; **30**, Kohm and Sanders, 2000; **31**,Aston-Jones et al, 2009; **32**, Arana et al, 1985

in his earliest studies these specific effects were the actions of various drugs and, later, the behaviors aimed at avoiding the noxious stimuli in the future. (Mason, 1975)

Only after the discovery of the role of glucocorticoids as a useful hormonal indicator by the Nobel Laureates Reichstein, Kendall, and Hench in 1944 did the science of stress truly escalate. It was quickly realized that elevations of glucocorticoids followed a diverse array of stimuli which range from mild (e.g., exposure to novel objects) to moderate (e.g., restraint) to severe (e.g., prolonged social isolation). Selye went further to characterize stressors into two general categories of "eustress" (literally, "good stress") and "distress" based on whether the organism was capable of surmounting the difficulties presented or not, respectively. This *prima facie* picture of stress and glucocorticoids became muddled as glucocorticoids were found to have a multiphasic daily regulation that corresponded not only with acute stress but also the circadian cy-

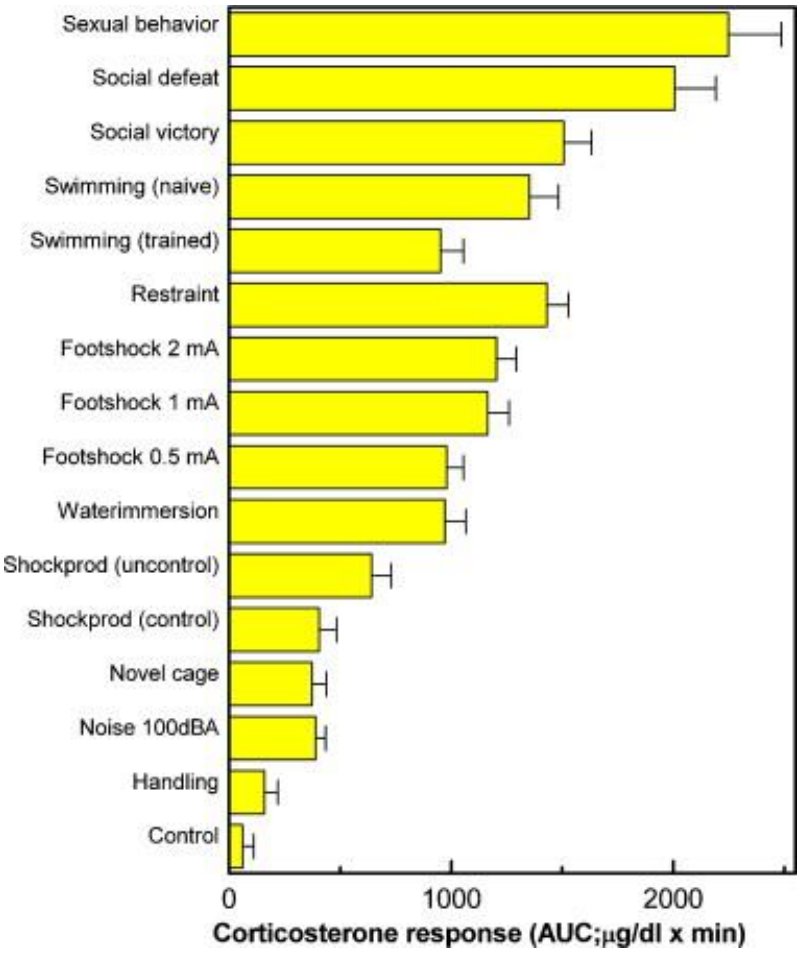


FIGURE 1: RELATIVE ELEVATIONS OF CORTICOSTERONE (SUMMED AREA UNDER CURVE) IN THE SAME RATS EXPOSED VARIOUS STIMULI;

TESTS WERE ADMINISTERED IN SERIES, WITH BASELINE SAMPLES FOLLOWED BY 15 MINUTES OF EXPOSURE AND THEN ONE HOUR OF RECOVERY;

CONTROL ESTABLISHED FROM HOME CAGE SAMPLE DURING REST, HALF-WAY BETWEEN TOTAL TESTING.

(ADAPTED FROM KOOLHAASA ET AL, 2011)

cle (Buckley and Schatzberg, 2005).

Furthermore, glucocorticoids are also mobilized in response to seemingly appetitive scenarios, such as during social victory and sexual activity. Koolhaasa and colleagues, perturbed by these inconsistencies, call the traditional model of stress into question: they insist that stress is best defined as an unpredictable and uncontrollable stimulus and response (2011). They cite that the sympathetic adrenomedullary (SAM) system and hypothalamic-pituatary-adrenal (HPA) axis, while indisputably activated during stress reactions, also serve to facilitate general behaviors that cannot be defined as stress, such as the mobilization of glucose and localization of oxygen during bouts of increased metabolism. When one defines stress as Cannon and Selye do, a "threat to homeostasis", we are faced with the reality that most actions result in alterations of homeostasis, from affective shifts to essential and stereotyped behaviors.

Elaborating on this model, *Koolhaasa et al.* review a number of recent studies on rodents. They assert that most models of stress research suffer from diminishing magnitude of effect as the animals attenuate their responses to the increasingly predictable stress; it has not been established whether or not this represents an inherent physiological habituation process or a cognitive recognition of patterns that leads to the termination of an anxiety response (Grissom and Bhatnagar, 2009). Investigators originally employed chronic, mild stress (CMS) to induce chronic hypercoticosteronemia, though it was found that with modifications to vary the daily stressors (e.g., shaking the home cages one day and exposure to loud noise the next) or randomizing intervals of exposure, that it was possible to induce a state considered akin to depression (Willner, 2005). Recently, it has even been found that the use of predictable CMS (PCMS) induces the opposite changes in rodents, improving affect, memory, and hippocampal volume (Parihar et al, 2011).

Controllability refers to the capability of the organism to mitigate the stressor. In the case of learned swimming, rats demonstrate consistently lessened circulating adrenaline, glucose, and corticosterone, simultaneous with increased free fatty acids and noradrenaline after learning the activity (Scheurink et al, 1999). The lessening of the glucocorticoid may be seen as adaptive as the role of elevated corticosteroids is considered hazardous to long-term health (Sapolsky et al, 2002; Bodo, 2010); it is likewise useful to the organism to limit elevations in blood sugar, instead relying on free fatty acids, given that elevations in glucose contribute to tissue deterioration, deplete glycogen stores, and ultimately limit the organism's endurance (Halliwell et Gutteridge, 1986; Holloszy et Coyle, 1984). Controllability in a psychological context is demonstrable in the paradigm of learned helplessness, in which an experimental subject is conditioned to elicit "helpless" behavior via an inescapable stimulus (Abramson et al, 1978; Maier et al, 1976). The effects of this paradigm in rats typically results outcomes traditionally associated with prolonged distress: less exploratory behavior, decreased body weight, loss of sucrose preference, impairment in spatial learning, declining hippocampal volume/function, chronic elevation of corticosterone, and decreased expression of brain-derived neurotrophic factor (BDNF) and cAMP-response element binding protein (CREB) (Raizer et al, 2003; Songa et al, 2006; Malberg et al, 2003).

Acute Allostatic Induction

Koolhasa and colleagues suggest that the combination of unpredictability and uncontrollability, allows for a more specific model of stress that is defined by the induction of alternate mode of homeostasis, termed "allostasis" by Dr. Bruce McEwen. McEwen describes allostasis as "maintaining stability, or homeostasis, through active change" which is accomplished through alterations to the organism's hormonal profile, generally to the benefit of the organism (2004). The induction of the SAM system is one of the first responders to stress, followed by the more

gradual release of corticosteroids and cytokines, eventually returning to a less allostatic state over time assuming that the stressor attenuates. In the CNS, the actions of catecholamines potentiate the release of cAMP and promote LTP in the limbic structures whereas the effect of glucocorticoids depends on the amplitude and duration of their exposure, sometimes encouraging LTP and under other conditions inhibiting it severely (McEwen and Sapolsky, 2002; McEwen, 2007; Ryan et al, 2010).

During states of acute allostasis associated with HPA/HPI and SAM activation, there is a common constellation of physiological changes that vary little across vertebrates (Denver, 2009). When an organism is exposed to a predator, for example, the uncontrollable and unpredictable situation is transduced via the senses and transmitted via various thalamic and a few extrathalamic tracts to the brain stem nuclei (specifically the locus coeruleus and raphe nuclei), amygdala, and the bed nucleus of the stria terminalis (BST) (Herman and Cullinan, 1997). The locus coeruleus, the source of norepinephrine in the CNS which projects broadly, connects directly to the paraventricular nucleus (PVN) of the hypothalamus, the convergent point of HPA/HPI neurosecretion for all acute stressors. Upon stimulation, the adrenal α_1 and α_2 receptors of the PVN respond with cAMP elevation which increases the synthesis of CRH and potentiates release of CRH via Ca^{2+} intake (Plotsky et al, 1989). The central effects of catecholamine are further enhanced by their systemic release into the periphery via CRH/ACTH where they act to raise blood sugar, mobilize free fatty acids, initiate proteolysis, increase cardiac output, enhance absorption of glucose into skeletal muscle, inhibit smooth muscle contraction, dilate airways, increase blood pressure, and attenuate nociceptive pathways (Wingfield and Romero, 2011; Wortsman, 2002). Furthermore, epinephrine and particularly norepinephrine have been shown to modulate the basolateral nucleus of the amygdala (BLA) via the β_2 and α_1 adrenergic receptors which synergistically increase cAMP and thus CREB, a process which has been shown to be

both essential to encode emotional memory in aversively-motivated tasks and beneficial to consolidating many other kinds of memory (Campolongo and Roozendaal, 2011; Tronsen et al, 2012; Poulace et al, 2011; Blass et al, 2012).

The amygdala is well known for its role in human emotion, a demonstrative case being the patient known as SP who presents with bilateral amygdalar calcification due to a rare genetic disorder, Urbach–Wiethe disease (Feinstein et al, 2011). The authors report that SP demonstrates intense curiosity and risk-taking in a manner not unlike Klüver-Bucy syndrome, demonstrating entirely normal emotions aside from the utter absence of fear. Interestingly, SP does not demonstrate profound issues of memory or emotional reporting despite that this has been shown in others with amygdalar damage accrued later in life (Squire and Zola-Morgan, 1991). It must be noted, however, that such comparisons are inherently fraught with difficulties due to confluent lesions. Lesions to the hippocampi of humans have also been shown to produce profound memory deficits (Corkin, 2002; Zola-Morgan et al, 1986) and some have suggested that the dynamic interplay between the amygdala and hippocampus permits recovery from amygdalar but not hippocampal atrophy (McGaugh et al, 1996). In another study utilizing SP's rare condition and 20 others with unilateral amygdalar lesions, Anderson and Phelps suggest that amygdalar damage does not alter emotional self-reporting on an acute or 30-day basis though the authors concur in a previous study that similar lesions do impair the perception of emotionality (2002; 2001).

Lesions to the central amygdaloid nucleus (CeA) produce profound deficits in CRH circulation and, consequently, diminish the release of circulating glucocorticoids (Gray, 2007). While most of the efferents from CRH neurons of the CeA converge with other tracts into the BST which in turn connects directly to the PVN, there are several second order means by which the

CeA modulates the PVN. Some of the projections from the CeA emit CRH directly onto the lateral hypothalamus, a center which when stimulated electrically elicits alertness mediated via orexin, increased appetite, and attenuated nociception (Hagan et al, 1999; Delgado and Anand,1952 ; Naleway, 2011). The action of catecholamine on the lateral hypothalamus has been shown induce anorexia (Leibowitz, 1975; Berridge and Valenstein, 1991). Other projections of the CRF-positive CeA include the medial and lateral parabrachial nuclei (mPBN and lPBN), mesencephalic nuclei of the trigeminal, the dorsal vagal complex, mesencaphalic reticular formation, and the periaqueductal gray (Gray, 2007); functionally, these areas represent the transduction of taste, the coordination of mastication, the parasympathetic innervation for the thoracic cavity, a center essential for maintaining general arousal, and a complex center for regulating nociception and stereotyped defensive behaviors. The CeA receives afferents from CRH neurons in the hypothalamus and throughout the midbrain, dopamine (DA) tracts serotonin (5HT) from their respective nuclei, and substance P innervation from the brainstem (Gray, 2007), suggesting roles for HPA feedback loops, satiety regulation, environmental monitoring, and nociception.

Most authors are careful to delineate at least three differentiable sources of HPA/HPI activation:

1) stressors which elicit pain/discomfort,
2) those which alter directly physiological conditions, and
3) those which necessitate the assembly of multiple sensory experiences and rely on memory to associate the stimulus as a threat.

The first pathway is necessarily interconnected by the input from the anterolateral system and, indeed, the PVN receives afferents from the spinothalamic and spinoreticular tracts,

also sending analgesic signals to the dorsal column upon electrical or glutamate stimulation (Palkovitz et al, 1995; Rojas-Piloni et al, 2008). This signal may be further modulated by the amygdalar connections to the trigeminal ganglion-CGRP neurons (essential in the transmission of nociception from the facial nerves) as well as extensive innervations with dynoprhinergic neurons (an endogenous source of opioids), but is important to note that experimental pain still effects sympathetic changes in rats with ablated CeA (Gray, 2007). Thus limbic input is not necessary for basic allostatic adaption to nociceptive stimuli, but instead merely modifies it (Gray, 2007; Herman and Cullinan, 2007).

The second source of activation comprises brainstem tracts to the hypothalamus sufficient to create a full-fledged neuroendocrine response, as evidenced by various lesion studies (Herman and Cullinan, 1997; Buijs and Eden, 2000; Van de Kar and Blair, 1999). Regardless, it has been shown that limbic regions, such as the amygdala and neocortex play a significant role in modulating this response, especially in attenuating HPA/HPI activation over time in accordance with stressor magnitude (Gray, 2006; Snyder et al, 2011). The primordial necessity for the regulation of the internal physiological state is intimately connected to the HPA/HPI axes, as is evidenced by the architecture of this ancient pathway.

The third source of activation is due to the integration of sensory signals and the distribution of integrated to retention centers for storage and subsequent retrieval. This may lead to responses to complex stressors such as psychosocial interactions, changes in environmental conditions, perceived prospects, and prediction of future outcomes. The production of such complex stressors relies on telencephalic limbic structures such as the hippocampus and amygdala in order to integrate various inputs, often associating the noxious and benign through pattern recognition as is accomplished in instrumental conditioning (Van de Kar and Blair, 1999).

Lesion studies reveal that the BLA of the amygdala and CA1 area of the hippocampus are essential for successful avoidance conditioning as well as a special reliance on the prefrontal cortex (PFC) in mammals (Herman and Cullinan, 1997; Kim et al, 2006). Saha and Datta further affirm the roles of these limbic structures in learning by demonstrating a profound increase in CREB in the hippocampus, amygdala, and hypothalamus upon successful avoidance training (2007). Similarly there is significant evidence that acute HPA activation is reciprocally engaged with hippocampal neurogenesis (Brown et al, 2007; Jacobsen and Sapolsky, 1991; Snyder et al, 2007), though there is conflicting evidence about the role of adult neurogenesis in learning (Leuner et al, 2006). Altogether, the CeA clearly plays a paramount role in the assimilation of various sensory data as well as the recall of past experiences, whereas the BLA proves essential for consolidating such memories for later reference and preventing inappropriate excitation of the CeA via GABAergic inhibtion (Poulos et al, 2009). In turn, the hippocampus dually serves to mitigate HPA/HPI as well as other specific functions of memory and recall. It is apparent that these limbic organs are critical for both acquisition and extinction of the allostatic state due to complex stressors (Tronson et al, 2002; Feinstein et al, 2011).

The effect of acute corticosteroid elevation on cognition is complex, but appears to be strongly dependent upon dose and duration (Lupien and McEwen, 1996). The two receptors of glucocorticoids present in the CNS of mammals, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), have different affinities and different distributions. The MR, structurally similar to the MR present in the kidneys that binds aldosterone, has an approximately tenfold higher propensity to bind cortisol/corticosterone than the GR and, in the CNS, is limited to the hippocampus, amygdala, and lateral septum, with smaller distributions throughout. The GR is predominantly located in the hippocampus but finds residence fairly ubiquitously in the CNS of all vertebrates (Denver, 2009). The effects of binding these receptors are both immediate and

long-lasting, leading to genomic changes after a GR is brought into the nucleus and interacts (typically promoting) genes proximal to a glucocorticoid response element (GRE). (Herbert et al, 2006)

Glucocorticoids, in addition to all other neurosteroids (Majewska, 1992), allosterically modulate $GABA_A$Rs in both a positive or negative manner, dependent on their sulfation and concentration (Majewska, 1985). During positive modulation, this is essentially the same mechanism of action as benzodiazepines and an immediate anxiolytic phenotype does indeed occur with the exogenous administration of various corticosteroids in fish and mammals (Cerda-Reverter and Canosa, 2009; Denver, 2009; Maninger et al, 2009; Lambert et al, 2009; Mellon and Griffin, 2002). This topic is covered in greater depth in the coming section regarding the acute actions of allopregnanolone in the CNS.

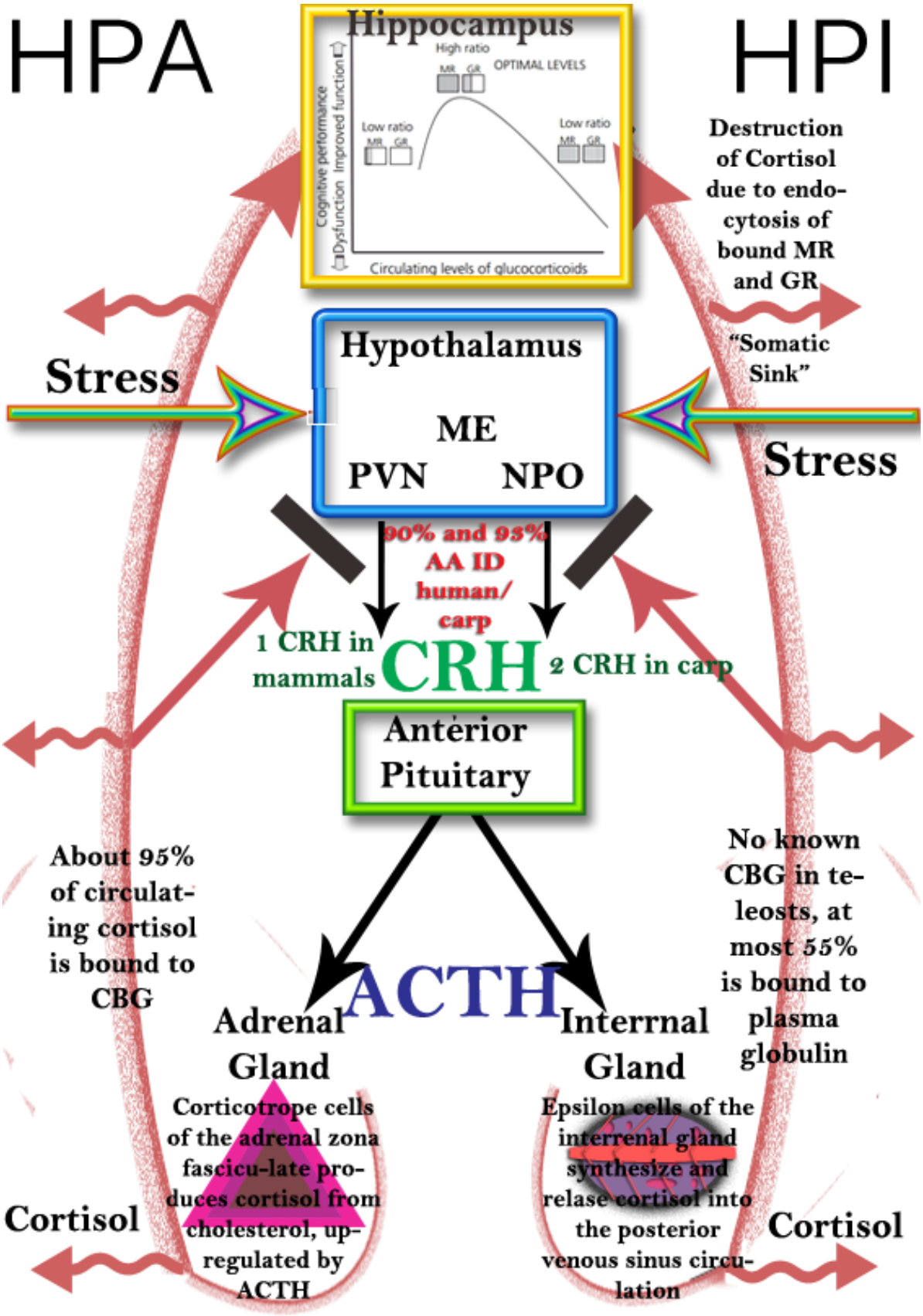


FIGURE 2: THE HPA/HPI AXES. ME: MEDIAN EMINENCE, NPO: NUCLEUS PREOPTICUS, PVN: PARAVENTRICULAR NUCLEUS, CBG: CORTISOL-BINDING-GLOBULIN

HPA/HPI Regulation and Dysregulation

During periods of homeostasis there are multitudinous regulators that hold tight rein over the HPA/HPI/SAM systems given their functional overlap with daily activities of both behavior and metabolism. Circadian rhythms operate both independently of and are modified by HPA/HPI diel cycles (Buckley and Schatzberg, 2005; Azpeleta, 2010). It has been shown in rats that corticosteroid regulation via the suprachiasmatic nucleus (SCN), the central "biological clock" of the brain, is mediated via ACTH secretion due to direct input to the hypothalamus, though the SCN itself lacks glucocorticoid receptors (Herbert et al, 2006). Feeding has also been shown to raise glucocorticoid levels in many animals, especially fish, and corticosteroids in synergistally increase insulin secretion and potency (Van der Boon et al, 1991; Dallman et al, 1993).

Various disorders can modulate HPA activity as well. Sleep disorders and obesity are the central points for modern researchers (Buckley and Schatzberg, 2005; Madden and Morrison, 2009). It has also been demonstrated that epigenetic factors, such as early maternal care, can produce lifelong alterations to the typical MR/GR profile and lead to predispositions toward depression and anxiety disorders (Herbert et al, 2006; Lemaire et al, 2000). It has been repeatedly shown that many individuals suffering from major depression and dementia exhibit an enduringly suboptimal elimination of corticosteroids from circulation, especially when challenged by an acute stressor such as dexamethasone (Bhagwagar et al, 2002; Barden et al, 1995; O'Brien et al, 1993; Pariante and Lightman, 2008)

Indeed, even gender is complicit in corticosteroid regulation (Elakovic et al, 2011; Kumsta et al, 2007), with estrogen at the forefront of negative feedback antagonism through the ER_{α} of the hypothalamus (Weiser and Handa, 2009). This reveals the innate importance of sex steroid regulation of the HPA/HPI axes and elucidates part of the role of metabolic syndrome in producing persistent feed-forward derailment given the strong correlation between hypere-

strogenism and obesity (Mattson et al, 2009; Cooper and Steward, 2009; Madden and Morrison, 2009).

Bruce McEwen considers prolonged periods of allostasis to be at the root of various psychiatric conditions, particularly those of chronic anxiety and depression, citing the hypertrophic pattern in the amygdala and trend toward smaller hippocampal and, to a lesser extent, prefrontal cortical volumes of those suffering post-traumatic stress disorder (PTSD), major unipolar depression (MD), and chronic anxiety (McEwen, 2004). McEwen, originally famous for his contributions to the Glucocorticoid Cascade hypothesis of accelerated aging (Sapolsky et al, 1986), posits glucocorticoid excess to significantly contribute to cellular damage and a specific pattern of metabolic influence that is defined by the upregulation of inflammatory cytokines, oxidative stress, and SAM activation leading to a manifestation of a portrait of chronic inflammation, metabolic syndrome, hypertension, immunosuppression, failure to thrive, and loss of fertility (McEwen, 2009). These changes are very similar to a Cushingoid state, a preclinical constellation of findings related to chronic hypercorticosteroidemia, though rarely a precursor to the actual Cushing's syndrome which is typically precipitated by a ACTH-hypersecretory adenoma (Wolkowitz et al, 2009).

The idea of corticosteroids' involvement in psychiatric disorders is long-standing as many HPA axis abnormalities are observed in these patients, but the data is imprecise and psychiatry is far from employing biomarkers for diagnosis (Kraemer et al, 2002; O'Brien et al, 2004). While HPA axis abnormalities have been consistently found to be present in Alzheimer's disease, dementia, MD, anorexia nervosa, and PTSD, there is no clear correlation for any specific biomarker and the prevalence or severity of these disorders (Kaye et al, 1987; Gold and Chrousos, 2002; O'Brien et al, 1993). It is proposed by Schuder and his colleagues that varying degrees of

adrenal insufficiency affect many psychiatric patients, particularly those who have suffered traumatic episodes, some of whom he has treated effectively with regular hydrocortisone treatments (2005). At least some subsets of PTSD and MD have been shown to respond to replacement hydrocortisone therapy (Holsboer, 2001), but it must be noted that hypercortisolemia is not always comorbid with these disorders (Penninx et al, 2007; Pariante et Lightman, 2008) nor that cortisol is necessarily the best candidate measure for hyperactivity of the HPA axis given its inhibitory effect on the release of CRH and ACTH in a normal organism and its physiological role as a blunting effect to the acute stress response (Herbert et al, 2006).

Others suggest that CRH is a better indicator of HPA impairment. The exogenous administration of CRH percipitates depressive symptoms and there has been some success in the use of CRH_1R receptor antagonists in pilot studies on rats (Holsboer et al, 2008). Others advocate the use of the dexamethasone suppression test (DST) relative to plasma CRH as a useful predictor of modern SSRI response (Ising et al, 2005). Florian Holsboer is the leading proponent behind the development of antidepressive and anxiolytic drugs modeled after modulation of the HPA axis hormones in the CNS as well as the among the first to utilize the DST in depressive patients (Holsboer, 2001). He postulates that a combination of impaired CRH_1R sensitivity, mineralcorticoid- (MR) and glucocorticoid (GR)-mediated HPA inhibition, and generalized inability to attenuate HPA activity is central to the etiology of many diverse psychiatric disorders. The sharpened edge of his argument lies on the diverse nature of antidepressant drugs that are only united by a fairly consistent clinical profile rather than receptor specificity or biological activity; he posits HPA dysfunction as the underlying cause in many diverse psychiatric conditions and HPA modulation to be the overarching modus operandi of modern antidepressants.

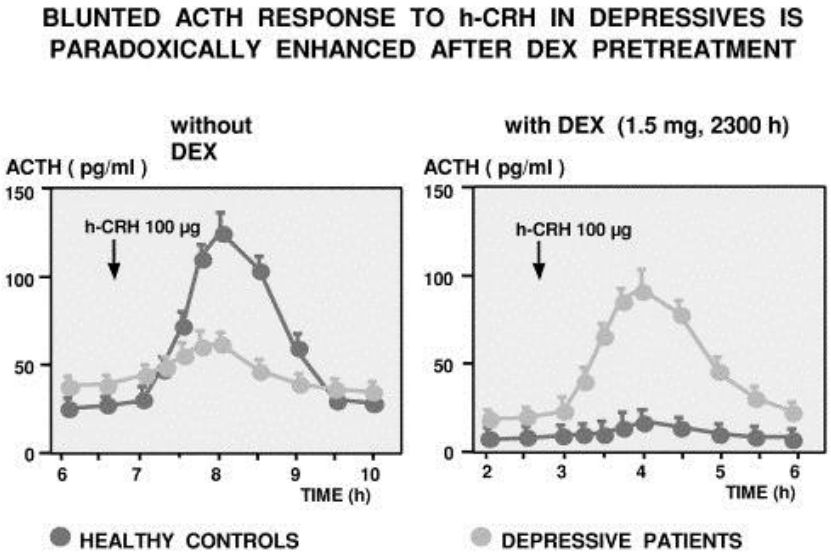


FIGURE 3: DEX/ACTH CHALLENGE DIAGNOSTIC. THE ADMINISTRATION OF DEX, A POTENT GLUCOCORTICOID WITH HIGHER MR/GR AFFINITY THAN CORTISOL, BLUNTS THE RESPONSE TO CRH IN HEALTHY VOLUNTEERS BUT DEPRESSED PATIENTS EXHIBIT EXAGGERATED ACTH RELEASE, LIKELY DUE TO EITHER CRH_1R DENSENSITIZATION AND/OR DYSFUNCTIONAL REGULATION OF THE NEGATIVE CORTICOSTEROID FEEDBACK EFFECS OF DEX. FURTHER FINDINGS DO IN FACT REVEAL AN IMPARIMENT OF DEX AND EXOGENOUS CORTISOL RESPONSE ONLY IN DEPRESSED PATIENTS. (ADAPTED FROM HOLSBOER, 2001)

While the results of these DST are fascinating, their conclusions are complicated by the facilitation of multi-drug resistance (mdr) by p-glycoprotein (Pgp), an exporter of exogenous and some lipophilic endogenous compounds back into central circulation from the rather selective blood brain barrier (BBB), which rapidly removes DEX, other steroids such as DHEA, and even endogenous cortisol in the human and rodent brain (Karssen et al, 2001; Asaba et al, 2000). Since brain concentrations of cortisone and 11-dehydrocorticosterone, considered to be the inactive 11-keto steroids of cortisol and corticosterone respectively, are not affected by the presence of a Pgp inhibitor, this raises the possibility that the neural actions of glucocorticoids are regulated by the presence of the enzymatic conversion of these metabolites at the CNS loci of their activity (Karssen et al, 2001). This hypothesis is supported by the widespread CNS distribution of 11β-hydroxysteroid dehydrogenase (11β-HSD) type I, particularly in the hippocampus, cerebellum, and cortex of mammals (McEwen, 2007; Herbert et al, 2006). Other investigators have traced similar distrubtions of this enzyme in zebrafish, coupled with a similar system for the regulation for the BBB (Sakamoto et al, 2001; Miller et al, 2002; Eliceiri et al, 2011).

These findings are intriguing, but offer no hint toward the etiology of HPA dysfunction: does psychiatric disease precede or follow HPA dysregulation? The best evidence comes from

recent observations regarding the feedback mechanisms of the HPA/SAM system and their changes over time during prolonged states of allostasis.

Various changes to gross and ultrastructural anatomy of the hippocampus as well as electrophysiology have been observed: decreased hippocampal volume, significant loss of synaptic spines in all but the CA2 region of the hippocampus, substantial loss of hippocampal functions such as memory and spatial learning, potentiated connections to the lateral habenula from the ventral tegmental area (VTA), and the downregulation of GRs in the hippocampus and hypothalamus (Li et al, 2011; Hajszana et al, 2008; Song et al, 2006; Buwalda et al, 2005; Buwalda et al, 2000). Benedict and colleagues also observed persistent inhibition (averaging four weeks) of LTP in the hippocampus in a model of learned helplessness, inescapable foot shock (2009). This effect was not affected by genetic strains supposed to be predisposed to depression nor was the helpless behavior displayed correlated with the magnitude of the suppression of normal LTP (Ryan et al, 2010). Similar designs have shown that this effect is prevented via the administration of allopregnanolone, an endogenous neurosteroid that acts centrally as an anxiolytic and neurogenic agent, directly to the hippocampus or amygdala (Shirayama et al, 2010). Other experiments have shown the attenuation of hippocampal neurogenesis due to inescapable stress to be averted with a dosing schedule of fluoxetine (Malberg et al, 2003).

The potentiation of the VTA-lateral habenular connection has been correlated with increased helplessness, anxiety, stress, pain, depression, and acquisition of avoidance learning (Hikosaka et al, 2008; Thornton and Bradbury, 1983; Sutherland, 1980). The lateral habenula is thought to be the functional center of aversive motivation, though its ablation only produces significant deficits in fear-conditioning in scenarios of high stress (Thronton and Bradbury, 1983). The electrical stimulation of this region profoundly suppresses the raphe nuclei via a GA-

BAergic mechanism and leads to a startled (described as “disappointed” in primates) behavioral response (Wang and Aghajanian, 1977; Hong and Hikosaka, 2008). Lateral habenular activation also acts through GABAergic transmission to utterly (98%) and transiently (~85ms) terminate dopaminergic response at the loss of an expected reward (Huifang and Shepard, 2007; Hong and Hikosaka, 2008; Matsumoto and Hikosaka, 2007). The vast majority of lateral habenular afferents are from the VTA and approximately 50% of these neurons are dopaminergic, perhaps indicating a role in the temporary suppression of 5HT and NE (Gruber et al, 2007). Indeed, dopaminergic stimulation of the habenula proper transitively suppresses 5HT release in the cat (Reisine et al, 1982). The purported role of lateral habenular signalling in the recognition of “failure” to reach a forseen outcome is consistent with these findings: when the VTA activates the lateral habenula it inhibits 5HT, NE, and DA release, and thus limits plasticity and the establishment of the present behavioral paradigm. The inhibition of the dopaminergic lateral habenula via tryptophan depletion in rats and deep brain stimulation in humans has been shown to ameliorate the symptoms of treatment-resistant depression (Sartorius and Henn, 2007; Sartiorius et al, 2010). Two entrepeneuring scientists have even gone so far as to patent the use of zebrafish for screening cerebral drug efflux from the BBB due to the extensive similarities to the human system (Goldsmith and Fleming, 2007).

Since the mechanisms of inhibition are GABAergic in nature, this implies that negative modulators of the $GABA_AR$ may act as antidepressants. DHEA, one neurosteroid with such activity, has been demonstrated to be an effective therapy for some subsets of MD (Wolkowitz et al, 1997). It should be noted that the opposite effect of $GABA_AR$ allosteric potentiation may also improve depressive symptoms, as is evidenced by the effects of potent $GABA_AR$ modulators such as benzodiazepines and allopregnanolone (Zhao et al, 2011; Pinna, 2010). These paradoxical findings suggest that $GABA_AR$ modification does not contribute significantly to the antidepres-

sant activities of these drugs. Instead, it is possible that genomic modficiation mediated through neurosteroids that alter hippocampal dynamics may be responsible for recovery from the state of chronic stress. It is well known that many drugs known to improve depressive symptoms up-regulate the peripheral benzodiazepine receptor (TSPO) which in turn increases the synthesis of neurosteroids (Pinna, 2010). Both allopregnanolone and DHEA have been demonstrated to have genomic effects in the hippocampus altering axon guidance, neurite outgrowth, neuronal cell cycle, neurodegeneration, inflammation, steroid receptor synthesis, and neurogenesis (Rebala, 2011; Mo et al, 2009).

A recent hypothesis has been forwarded regarding the regulation of glucocorticoid receptors in the hippocampus during times of allostasis (Herbert et al, 2006). The hippocampus's extraordinarily high density of MRs and GRs has been calculated to be the primary "somatic sink" in at least mammals according to a recent *in situ* analysis (McAuley et al, 2009). In other words, all somatic cells express MR/GRs and their binding leads to the removal of circulating glucocorticoids but the hippocampus holds a unique place in this regulatory schema due to its presence in the CNS, its high metabolic rate, its preeminent density of corticosteroid receptors, and its capacity for adult neurogenesis. Since prolonged exposure to allostatically elevated glucocorticoids has been shown to prevent mitosis of neurogenic cells, halt maturation, and induce atrophy, the hippocampus seems unusually vulnerable to circulating glucocorticoids and this mechanism of atrophy is not ostensibly adaptive (Kassahn et al, 2009; McEwen, 2009),.

There is also increasing evidence that adult hippocampal neurogenesis plays less of a role in learning than was once thought (Kerr et al, 2010; Snyder et al, 2011; Leuner et al, 2006). This is concurrent with the finding that most antidepressants increase mRNA levels of both MRs and GR, increase neurogenesis, and upregulate the production of neurogenic/neuroprotective

steroids (Barden and Reul and Holsboer, 1995; Jacobs et al, 2000; Pinna et al, 2009). Santerelli and collegues have demonstrated that both the 5HT1A receptor and hippocampal neurogenesis are essential for antidepressant response (2003).

In a brilliant recent study by Snyder and his compatriots, glial fibrillary acidic protein (GFAP) promoter was used to produce herpes simplex thymidine kinase in the radial glia precursors to adult neurogenesis in mice who were then treated with the antiviral valganciclovir, an acyclic nucleotide analogue that destroys only mitotic cells expressing viral thymidine kinase (2011). These transfected mice were no different than controls in any measure unless they were treated with the antiviral, which was demonstrated to abolish adult neurogenesis in the hippocampus via doublecortin histology. A series of common tests for depressive behavior and anxiety were performed (novelty-suppressed feeding, forced swim, sucrose preference, elevated plus maze); those animals without neurogenic hippocampi exhibited greater anhedonia, depression, acute elevations of corticosteorids, but did not show increased anxiety as indicated by the elevated plus maze. Finally, a DST as well as exposure to a restraint stressor demonstrated that the neurogenically impaired mice were much slower to eliminate their plasma corticosterone. These results were repeated in another model of annihilation of hippocampal neurogenesis employing X-radiation over a limited portion of the telencephalon designed to only ablate the dentate gyrus; very similar methods in another radio-ablation study verify these results, strongly affirming that hippocampal neurogenesis plays a central role in attenuating the long-term effects of HPA activation through the endocytosis and of circulating glucocorticoid via endocytosis and degradation (Santarelli et al, 2003).

These findings corresponds well with the notion that hippocampal neurogenesis may be implicated as the modus operandi of antidepressant drugs. Since it is well established that signif-

icant HPA axis dysregulation occurs in depressive illness (Barden et al, 1995); that neurogenesis plays a central role as a negative feedback (Snyder et al, 2011); that hippocampal function is often impaired by depression , chronic stress, and glucocorticoid elevation (Bhagwagar et al, 2002; Burke et al, 2005; Het et al, 2005; Holsboer, 2001); it is unsurprising that the mice require both the 5HT1A receptor (which stimulates neuroproliferation) and intact hippocampi to experience the antidepressant effects of fluoxetine (Santarelli et al, 2003). Furthermore, the antidepressant response has been correlated with return to baseline neurogenesis across many different drugs (Malberg et al, 2000; Duman et al, 2001; Malberg, 2004).

Graziano Pinna began to recognize the importance of hormonal signals for antidepressant effects due to drugs (e.g., desipramine, lithium, paroxetine) and sleep deprivation, identifying increased glial prevalence of T_3 in treated rats and a strong upregulation brain-derived T_3 during HPA allostatic induction (Pinna et al, 2003; Baumgartner et al, 1998). Pinna was the first note that the clinical doses of fluoxetine almost certainly lead to plasma concentrations of the drug that are inadequate to modify the serotonnin reuptake transporter (SERT) but are sufficient to stereospecifically and positively modulate the 3α-steroid dehydrogenase (3α–HSD) to upregulate the production of allopregnanolone from progesterone (2005). Impressively, he shows that only the s-enantiomers have significant action on 3α–HSD, with s-norfluoxetine being the most effective at decreasing aggressive responses and increasing allopregnanolone, despite the non-stereospecific regulation of SERT modulation. Later work correlates the normalization of the allopregnanolone synthesis after excessive androgenic steroids use or models of PTSD is correlated with both behavioral recovery and the upregulation BDNF (Matrisciano et al, 2010; Nin et al, 2012; Pinna, 2010). Pinna has demonstrated that the infusion of s-norfluoxetine into the rat BLA is sufficient to increase local allopregnanolone synthesis and attenuate aggression in socially isolated mice, though this effect did not occur in microinfusions into the PFC or

nucleus accumbens (Nelson and Pinna, 2010). These results suggest the sustained GABAergic modulation via allopregnanolone in BLA may attenuate activation of CeA/VTA during recalled arousal due to complex stressors, a mechanism of anxiolysis independent of hippocampal function.

Waner-Schmidt and Duman have also found evidence for the importance of growth hormones in neurogenic-based antidepressant response, but instead identify VEGF as an essential signal in two different models of depression, learned helplessness and UCMS (2006). The authors conclude that these behavioral effects likely through the VEGF-Flk-1 cascade due to their complete prevention and/or extinction of fluoxetine or desipramine response by the Flk-1 antagonist SU5416. Furthermore, they indentify an *in vitro* response in subventricular zone (SVZ) neural prgenitors. The genomic actions of the steroids may explain the upregulation of growth signals like VEGF and BDNF (Rebala, 2011).

The ratio of MRs to GRs both within the hippocampus has been shown to be correlated with stress coping styles, depressive/anxiety symptomology, early childhood experiences, and impaired glucocorticoid disposal (Veenema et al, 2003; Lopez et al, 1997; Rozeboom et al, 2007; Oitzl et al, 2010; Herman et al, 2006). Sapolsky proposes in the classical Glucocorticoid Cascade hypothesis that the downregulation of MRs in the hippocampus following prolonged or excessive glucocorticoid exposure leads to the degenerative changes and thus a higher MR/GR ratio is protective against stress (Sapolsky et al, 2002). It has since been shown that mice transgenically modified to express a mild upregulation of MRs in the forebrain do indeed exhibit less anxiety-like behavior, express fewer GRs in the hippocampus, and display additional limbic $5HT_{1A}$Rs (Rozeboom et al, 2007). In rats exposed to unpredictable CMS and in the post-mortem brains of suicide victims, the opposite pattern is expressed: the decreased binding and expression of

$5HT_{1A}$Rs, higher basal corticosterone and the downregulation of MRs in the hippocampus (Lopez et al, 1997). While imipramine, the first antidepressant drug, prevented these effects in rats, fluoxetine was shown to actually increase corticosterone levels over that of the control while simultanously improving $5HT_{1A}$ response (Lopez et al, 1997). It has also been observed that the MR and GR can heterodimerize intracellularly, with the effect of inhibiting the transpritional activity of GR; this represents a direct method for the suppression of GR-induced transcriptional modification while MRs are not fully saturated, though this is only possible in cells which express both MRs and GRs (Liu et al, 1995). In the common carp, the downregulation of the MR due to chronic hypercortisolemia is concomitant with the equivalent downregulation of GR mRNA ().

The dynamics between neurogenesis, the MR/GR ratio, and $5HT_{1A}$ receptors in the hippocampus all participated in common negative feedback loop for the HPA/HPI axis, though it is also clear that this inhibition becomes overridden over times of prolonged allostasis.

Cortisol in Humans and Fish

Cortisol represents a unique point of convergence for most teleosts and most mammals: while there are some exceptions (e.g., rodentia), both employ cortisol as the principle regulator of glucocorticoid-mediated HPA/HPI activation (Mommsen et al, 1999). These functions do overlap substantially, but the additional importance of cortisol to as an osmoregulator akin to aldosterone in mammals is still poorly understood, notably because it is a less attractive theory to hypothesize about than to pander to the strong motivations to employ fish as experimental models of neuroimmunoendocrinology (Blaser et al, 2010; Steenbergen et al, 2011; Green et al, 2011; Popesku et al, 2008). This review will inevitably reflect this bias in research volume.

Cortisol is the glucocorticoid secreted by the adrenal glands of mammals and the interrenal glands of teleosts in response to stress, of any of the previous three varieties

discussed. The effects are pronounced and universal: every somatic cell in the body expresses MRs/GRs and the resulting interactions result in increased energy mobilization (e.g., gluconeogensis and lipolysis) and increased metabolic rate (favoring catabolism) as part of overall sympathetic activation. It is the culmination of sympathetic response and direct activation of MRs/GRs in the amygdala and hippocampus that causes a complex profile of effects on memory, learning, and cognition (Buijs and Van Eden, 2000). These changes, which have been shown to have to act as pronounced modulators of cognitive function, are mediated by variables including the length of cortisol exposure, titer of free-circulating cortisol versus bound-cortisol, and the measure of cognition; this section will tease apart the abundance of data on cortisol in both humans and teleosts with a special focus on the fear-conditioning or avoidance paradigm.

Cortisol is the often considered the utmost hormone responsible for stress adaptation and, less exclusively, wakefulness and attention. The free-circulating end-product of the HPA/HPI axis, cortisol is elevated as the result of a cascade of interactions stemming from either a stressful event or as a diel process which climaxes during peak wakefulness and postprandially. When stress is tranduced by either the limbic system, in cases of social or emotional distress, or brain-stem nuclei, when the stress is of immediate threat to homeostasis such as thermoregulatory challenges, an afferent arrives at the PVN of the hypothalamus which stimulates the secretion of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) into the pituitary portal in mammals or portal vein fish. The cells of the anterior pituitary then secrete adrenocorticotrophic hormone (ACTH) in response, which circulates until it acts on the adrenal glands to stimulate cortisol release in a pulsile manner. Cortisol may then act on any somatic cell in the body when it not bound to a corticosterone binding protein (CBP), as previously described, and provides its own negative feedback to the hypothalamus and pituitary

gland, eventually terminating its elevation and returning to homeostasis after the stress has been resolved. Other negative feedback is provided by the hippocampus, the BST, and the preoptic area, which innervate the PVN with GABAergic synapses. (Herman et Cullinan, 1997)

In medicine, this steroid has been of particular interest because of its gross malfunctioning, its involvement in aging or stress-related general changes, and its role in mental health. Chronic hypercortisolemia, also known as Cushing's disease, is indicated by hypertension, fatigue, hyperglycemia, increased liquid intake, irritability, loss of libido, depression, and severe memory deficits. In over 70% of the cases, this extreme malignancy manifests itself after the formation of a pituitary adenoma, a neoplasm derived from the cells of the anterior pituitary that secrete ACTH (Loughlin et al, 2005). The opposing syndrome, hypocortisolemia, adrenal insufficiency, or Addison's disease, is less common and less studied.

Even subclinical chronic hypercortisolemia is associated with poor prognoses: severe deficits to memory, increased oxidative stress, and widespread atrophy of muscle are some of the principle elements of stress-induced aging (Liu et Mori, 1999; McEwen, 2009). The discussion of cortisol as an aging accelerant has been ongoing since the inception of the free-radical theory of aging and has in fact generated its own unique theory of aging that relates the decreased capacity of geriatrics to recover homeostasis from challenging events (Holliwell et Gutteridge, 1986; Sapolsky et al, 2002). Studies in the elderly suggest that lower cortisol is not only correlated with longer life, but healthier neurological outlooks: aerobic fitness, the exertion of which is well-established as cortisol depleting, was shown to correspond with significantly larger bilateral hippocampal volume and increased spatial skills (Erickson et al, 2009). Later, it was found that higher levels of BDNF, a neurotrophic factor with a diel cycle similar to cortisol but with significant decline as one ages, correlates to a higher preservation of hippocampal

volume (Erickson et al, 2010). Another longitudinal study with elderly participants concluded that higher cortisol salivary titers predicted decreased declarative memory function, which is intimately linked to the hippocampus, and poorer executive abilities after three years (Li et al, 2006). The study of hippocampal volume does not necessarily correlate to function, however, and a recent study does well to expound the high rate of variability in both young and old populations, casting a shadow of doubt on the often accepted theory of corticosteroid-induced aging (Lupien et al, 2007).

No doubt, much of the research on cortisol has been focused on the degenerative effects its chronic elevation on the hippocampus, a brain structure essential for spatial and temporal cognition, declarative memory, and normal affect (Kandel et al, 2000). Aside from the integral role of the hypothalamus and pituitary gland in the glucocorticoid cascade, the hippocampus was the first brain region with observed phenotype variation due to stress as it contains the highest concentration of adrenoglucocorticoid receptors of the high-affinity variety (MR) in the entire CNS in higher vertebrates (McEwen, 2007). The hippocampus is thus the most significant “sink” for neural cortisol and, more importantly, its activity acts to attenuate the release of CRH and AVP in the PVN (Herman et Cullinan, 1997). Interestingly, the decline in hippocampal function serves as a feed-forward mechanism in cases of chronic hypercortisolemia, often considered to render the diseased state. The mechanism responsible for this has been dually implicated as direct neurotoxicity to pyramidal cells during periods of high concentrations and chronic inhibition due to milder doses leading to gradual apoptosis of inactive cells (Wolkowitz et al, 2009). Recent interdisciplinary work has produced a mathematical model of the HPA axis and its probable effects on the hippocampal CA1 region, which is particularly prone to atrophy when inhibited by cortisol; the model ultimately concluded an approximate four-fold decrease in hippocampal activity due to chronic stress

compared to an identical aging profile with non-elevated cortisol (McAuley et al, 2009). The decline in hippocampal size and function observed in many cases of chronic hypercortisolemia has important implications for mood, memory consolidation, and cognition as the hippocampus plays an essential role in all of these disparate processes.

The relationship between psychiatric disorders, chronic stress, and hippocampal function has been a burgeoning field in human subjects given the relative ease of assigning tasks that noninvasively measure spatial and/or temporal learning, yielding an abundance of evidence on the long-term ramifications of stressful events and the potential mechanisms via which such complex psychological trauma can alter physiological systems. In a study of 24 police officers who had all undergone similar trauma but only 50% of which developed symptoms of post-traumatic stress disorder (PTSD) it was found that the PTSD group had significantly higher salivary cortisol upon waking and billaterally smaller hippocampi according to fMRI imaging (Lindauer et al, 2006). Furthermore, the PTSD sufferers performed significantly worse on verbal recall (traditionally correlated with hippocampal function) while this did not necessarily associate with the volume of their hippocampi, suggesting another factor in their impairment which the authors' believe may be attributed to the selective inhibition of the BLA. Cortisol is known to exert some effect on memory recall, manifesting at its most severe as retrograde amnesia, through the inhibition of the BLA, though this effect has not been directly reproduced in humans undergoing a chronic stress-state such as the members of the PTSD trial (Wolkowitz et al, 2009; Het et al, 2005). It has been shown that even in non-clinical conditions such as personality is relatable to hippocampal volume, with those possessing lower self-esteem and external loci of control having significantly smaller hippocampi (Pruessner et al, 2005).

Because of the hippocampus's role in cognition, it is possible to study its functionality indirectly through behavioral outcomes such as skill acquisition (O'Keefe et Nadel, 1978). The shuttle-box paradigm, in which an aversive stimulus (the unconditioned stimulus or UCS) is paired with an otherwise neutral stimulus (the conditioned stimulus or CS), presents as a well-accepted task which most experimental animals readily learn under normal circumstances. When rats have been treated with cortisol and subjected to this test in the past, however, results have been inconclusive but suggestive of a non-linear, dose-dependent curve that presents the poorest cognitive outcomes at very low and very high doses (Lupien et McEwen, 1996). In a task in young, healthy humans who were pretreated with metyrapone, an 11β-HSD inhibitor, or given tested later in the afternoon when cortisol is naturally very low, the administration of cortisol improved the delayed recall (hippocampal-dependent) task performance; when cortisol was given during a time of circadian-regulated peak concentration (i.e., the morning), exogenous significantly impaired performance on this same task (2001). This is consistent with the multitude of effects cortisol has on the telencephalon, notably the improvement of memory consolidation due to BLA interactions and the inhibition of retrieval via the hypopolarization of the hippocampus, both effects relying heavily on dose (Het et al, 2005). If this conclusion were true, it would cement the portrayal of cortisol as an adaptive compound to improve learning during times of relative stress.

The use of teleost models, specifically the goldfish and zebrafish, has become increasingly popular for studies involving steroids and even psychopharmeceuticals (Popesku et al, 2008; Egan et al, 2009). The strong conservation of the steroidal system and homologies in the telencephalon make teleosts desirable models for preliminary drug trials, as these systems are in some ways more related than that of mouse and human (Salas et al, 2006): specifically, cortisol takes on the same role in fish but not rodents (Bernier et Peter, 2001). Additionally, the

teleost forebrain presents two regions akin morphologically as well as functionally similar to the hippocampus and the amygdala in higher vertebrates: the lateral and medial pallia, respectively (Flood et al, 1976; Wullimann et Rink, 2002; Portavella et al, 2004; Salas et al, 2006; Northcutt, 2006). Such homologies have also benefited research due in part to the availability of teleost subjects and several established paradigms, including shuttle-box avoidance.

The goldfish model also benefits from a vast body of literature regarding cortisol and stress. Since cortisol circulation raises during peak activity in most animals in a diel cycle, the concentrations in goldfish, a notable exception, have been of interest (Bernier et al, 2001; Butler, 2005). It has shown that goldfish in fact have two near-equivalent peaks and an intermediate, lesser trough in their maximal serum cortisol (Singley et Chaven, 1973). These endogenous levels reveal incredible range in extreme conditions: from 4nlμg/g to up to 400μg/g (Noeske et Spieler, 1983). This is further complicated by the many feedback regulators of cortisol: melatonin decreases cortisol, various hormones released while feeding increase it while starvation has no effect, infections paradoxically increase cortisol release, and sexual cycles also have dichotomous, drastic effects (Azpeleta et al, 2010; Bernier, 1988; Garina et al, 2007; Li et al, 2008; Butler et al, 2005; Fryer et al, 1992). Thus, like cortisol experiments in humans and rodents, it is important to control for variables such as the time of day, the time of feeding, and the temperature.

Allopregnanlone as a Mediator of the HPA/HPI

For a brief time in the hey-days of steroid syntheses and mammalian sterol research, allopregnanolone was dismissed as a “physiologically inactive” metabolite of pregnenolone found (at first) only in the corpus luteum of ovulating females (Jensen, 1935). By 1938, allopregnanolone was isolated from the adrenal cortex and suggestions were made regarding its associations with progesterone, assertions of a possible role in gestational activity given its metabolic

and physiologic proximity to progesterone (Beall et Reichstein, 1938). It has since been demonstrated that allopregnanolone increases its concentration linearly with increases in serum progesterone and that hypothalamic concentrations significantly decrease gonadotrophin production (Frye et al, 2011; Laconi et al, 2001). Another study found that rats injected with an allopregnanolone antiserum showed more pronounced lordosis behavior and increased the number of oocytes released upon ovulation (Genazzani, 1995). The role of allopregnanolone in the estrous cycle is still mildly controversial, but most agree that allopregnanolone plays an important role in attenuating the psychoactive effects of other "luteal surge" hormones, perhaps ameliorating the depressive effects of progesterone withdrawal (Lambert et al, 2009). The principal support for this theory arises from the study of premenstrual dysphoric disorder (PMDD), an affective disorder characterized by a unipolar depressive symptomatology and progesterone withdrawl, the morbidity of which is consistently exhibited with lower serum and cerebrospinal fluid concentrations of allopregnanolone (Girdler et al, 2001; Rapkin et al, 1997). The hypothesized justification for these differences bridges two disparate theories of depressive affective dysregulation: the neurogenesis theory of depression and the progesterone-withdrawal model of post-partum depression (Stoffel and Craft, 2004; Jacobs et al, 2000).

Allopregnanolone has been described as the most potent endogenous facilitator of the $GABA_A$ receptor, the ligand-gated ion channel which composes the vast majority of the brain's inhibitory circuits (Brot et al, 1997; Bitran et al, 1999; Majewska et al, 1992). There have been at least two binding sites for allopregnanolone identified thus far, with binding to either site eliciting a full potentiation of chloride influx (Bracamontes et al, 2011); this is somewhat debatable, however, as some argue that the steroid is highly lipophilic and prefers a transmembrane site for $GABA_AR$ modulation (Chisari et al, 2010). Allopregnanolone and its related neurosteroids are synthesized in both glial and neuronal cells, with recent research implicating neurons as its pri-

mary source in the CNS (Agis-Balboa, 2006; Tsutsui et al, 2006). It has been shown that allopregnanolone is regularly secreted from these cells, acting on the $GABA_A$ receptors in both a tonic (i.e., synaptic) and phasic (i.e., extrasynaptic) manner, with different subunit compositions rendering different sensitivities to this effect (Mukai, 2007; Turkman et al, 2011). The actions of allopregnanolone on the $GABA_AR$, while the most potent endogenous allosteric modulator of this promiscuous receptor, not unique; all neurosteroids enact allosteric effects upon $GABA_ARs$ (Majewska, 1986).

The hippocampus is perhaps the central organ of allopregnanolone's action, where it has been shown to have neuroprotective effects in response to acute glucocorticoid release as well as exhibit the greatest neuroprotective activity (Lennartsson et al, 2012). Work in ovariectomized rats reveals that allopregnenolone improves memory in inhibitory avoidance retention but impairs acquisition when microinfused into the dorsal hippocampus, whereas oestradiol plus progesterone significantly impaired learning and retention (Escudero et al, 2011). The amygdala also responds to and produces allopregananolone and it has been shown that the antidepressant response triggerd by the neurosteroid is partly attributable to amygdalar effect, likely through a GABAergic mechanism (Shirayama et al, 2010; Nelson and Pinna, 2010).

Understanding the phenotypic shift from the a baseline state to stress-induced allostasis necessitates understanding not only the direct effects of allopregnanolone but also the role of the $GABA_A$ receptors, which are extensively versatile and ubiquitous inhibitory forces in the CNS of all vertebrates.

$GABA_A$ Receptors: Structure and Function

GABA has long been considered the primary inhibitory neurotransmitter of the CNS (Kandel et al, 2000). Its actions in neurons and some glia are regulated by interactions with the ionotropic $GABA_A$ receptor ($GABA_AR$) family or the retina's $GABA_C$ receptor family; slower metabotropic responses to GABA are the result of $GABA_B$ receptor activation (Jacob et al, 2008).

Subtype	Relative abundance in rat brain (%)	Location and putative function
α1β2γ2	43	Present in most brain areas. Localized to interneurones in hippocampus and cortex, and cerebral Purkinje cells
α2β2/3γ2	18	Present on spinal cord motoneurones and hippocampal pyramidal cells
α3βnγ2/γ3	17	Present on cholinergic and monoaminergic neurones where they regulate ACh and monoamine turnover
α2βnγ1	8	Present on Bergmann glia, nuclei of the limbic systems, and in pancreas
α5β3γ2/γ3	4	Predominantly present on hippocampal pyramidal cells
α6βγ2	2	Present on cerebellar granule cells
α6ββδ	2	Present on cerebellar granule cells
α4ββδ	3	Present in thalamus and hippocampal dentate gyrus
Other minor subtypes	3	Present throughout brain

Location and function are listed where these have been investigated, and are not comprehensive. Other minor subtypes include α1α6βγ2, α1α3βγ2, α2α3βγ2 and α5βγ2δ subtypes and are represented together as a small population.

TABLE 2:$GABA_AR$ ISOFORM DISTRIBUTION IN THE RAT BRAIN (MCKERNAN AND WHITING, 1996)

While there are other fast-acting inhibitory receptors, specifically the glycine receptors, GABAergic systems outnumber these other receptors by at least two to one in the rat (Kandel et al, 2000). Modern predictions claim that GABAergic synapses account for approximately 30% of the total number of synapses in the human brain (Möhler, 2006).

Given GABA's ubiquity and essentiality in the nervous system, its receptors have been isolated from virtually every part of the mammalian brain, albeit in different isoforms. $GABA_AR$s, the most abundant and best studied of the three classes of GABA receptors, have a total of 19 presently discovered subunits: 6α, 3β, 3γ, δ, ε, π, θ, 3ρ. Since the $GABA_AR$ belongs to the superfamily of ligand-gated ionopores it shares a common structure with other receptors such as the $5HT_3R$, nAChR, and GlyR: it exists as a pentamer with subunits of 4 transmembrane domains (TMs), with a loose, intracellular peptide "loop" between the TM3 and TM4 segments,

and the TM2 portion likely lining the membrane. The structure of the nAChR is canonical of this superfamily and, unfortunately, much of what is inferred about the structure of $GABA_AR$ is derived from data on this relative, while no direct characterization has yet been performed using imaging techniques. (Jacob et al, 2008; Olsen et Sieghart, 2008)

Despite the number of theoretical combinations (totaling 1889568, assuming that order is significant, that repeated receptors are permissible, and not taking into account the several known posttranscriptional modifications), certain $GABA_AR$ isoforms are more prevalent, often in definable anatomical regions. These populations are summarized in Table 2 (McKernan et Whiting, 1996).

It is immediately apparent that the α1β2γ2 subtype predominates; more recent evidence suggests that this single subtype might even reflect over 60% of the total $GABA_AR$ population in the human brain (Möhler, 2006). This particular combination is composed of two α1 units, two β2 units, and one γ2 unit and is found at especially high densities in the hippocampus. It has been shown through synthesis and implementation/combination of subunit trimers and dimers in the membrane of Xenopus oocytes that the functional configuration of these subunits is in the order γ2β2α1β2α1 (Baumann et al, 2002).

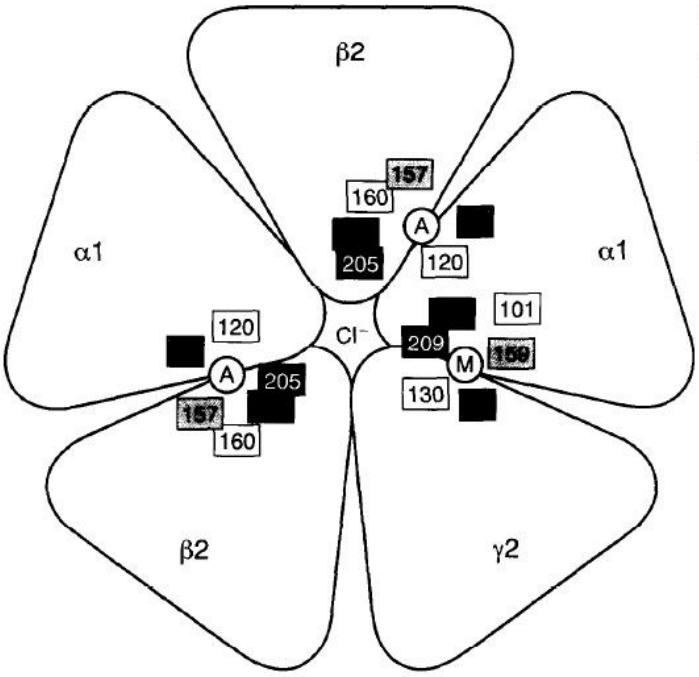


Fig. 2. Hypothetical model of the $GABA_A$ receptor with the agonist and modulatory sites at subunit interfaces. Five subunits are arranged around a central Cl^- ion-selective pore, that opens as a consequence of agonist (GABA) binding. The numbers indicate amino acid residues of the corresponding mature rat subunit isoform. Amino acid residues of agonist and modulatory sites identical or directly homologous to each other are shown in the same colour. A, agonist (GABA) binding site; M, modulatory site for benzodiazepine-type ligands. As two subunits contribute to each, the agonist and to the modulatory binding site, they must be located at subunit interfaces. If the modulatory site is occupied, this fact is allosterically communicated to the agonist binding site whose function is altered as a consequence.

FIGURE 4. $GABA_AR$ STRUCTURE (FROM MCKERNAN ET WHITING, 1997)

This receptor, as do most α1, α2, α3, and α5 subunit expressing receptors, possesses a benzodiazepine binding site that allosterically enhances activation to strengthen the flow of chloride ions intracellularly. This site has been the topic of much speculation as it is of clinical importance and not always present in receptors with the appropriate subunits. Recent theory concludes that the site is in fact interfacial, formed at the junction of certain α and γ subunits (Sigel and Buhr, 2005). Recent evidence has shown that a single DNA base, γ2F77I, encodes a critical site that, when altered, eliminates benzodiazepine activity completely in nearly all subtypes (Ramerstorfer et al, 2010).

Many other endogenous ligands have been discovered to modulate $GABA_ARs$. Zinc has also been shown to bind interfacially attenuate Cl^- current as long as zinc concentrations are constant, the biochemical mechanism of which is now fully elucidated (Trudell et al, 2008); it has also been shown that zinc ligands play an important role in regulating cortical plasticity, not in small part due to their effect on the $GABA_AR$ which participates in the phasic control of pyramidal output (Nakashima, 2009). There are binding sites for protons, which respond to changes in intracellular pH to maintain homeostasis, and protein kinases, which play an

important role in receptor regulation (McKernan et Whiting, 1996). New ligands for $GABA_A$Rs are constantly being tested, given their incredible pharmacological value; for example, valerenic acid (a constituent of valerian, a common herbal sleep aid) has been shown at low doses to modulate nonspecific $GABA_A$Rs likely at the loreclezole (an anticonvulsant) binding site; ultimately, valerenate increases chloride currents into the cell by five- to tenfold and synthesized analogs have maximally increased this activation to a staggering 100-fold (Sascha et al, 2010).

Categorizing $GABA_A$Rs based on subunit motifs has become a useful means of characterizing the behavioral effects of different subclasses. After an extraordinary review of pharmacology, Hanns Möhler concludes that α subunits best predict the actions of the drugs that target any one of the 28 known wildtype receptors (Möhler, 2006). He states that activation of α1-containing receptors tend toward hypnotic, sedative effects; that α2 subunits typically lead to anxiolytic effects; that α3, while more enigmatic, have an important role in catalepsy; that α4 helps maintain tonic integrity in thalamic signals; and that α5 subunits, which confer sensitivity and respond predominantly to neurosteroidal modulation. They feature prominently in extrasynaptic parts of pyramidal cells in the hippocampus, facilitating LTP.

The differences between receptors include not only different binding sites, but also operate via distinct kinetics. A comparison between rat α1β2γ2 and α3β2γ2 subtypes revealed that the former receptors are both faster to inactivate and exhibit stronger desensitation of their post-activation effects (Barberis et al, 2007). A specialized form of GABAergic neuron, the neurogliaform cell, is present in the cortex and hippocampus and emits GABA at an extremely slow (10-30ms) rate to extrasynaptic targets along with THDOC (a neurosteroid closely related to allopregnanolone) to highly sensitive $GABA_A$Rs with α5 and δ subunits to elicit a powerful, paracrine, neuromodulatory IPSPs (Szabadics et al, 2007).

Vertebrate Neurosteroids

The modulation of $GABA_AR$s with neurosteroids is of particular interest given both compounds':

1. Role in regulating stress homeostasis
2. Protective role in preventing excitotoxic episodes
3. Enhancement of hippocampal and cortical plasticity

These effects are accomplished by both the immediate actions of the neurosteroids as allosteric modulators of the $GABA_AR$s as well as the genomic modifications due to their actions as steroids. Since the $GABA_AR$ is ubiquitous in the CNS and its structure has been strongly conserved in vertebrates, this receptor will be the predominant topic in the coming section. Unfortunately, the transcriptional activities of neurosteroids remain to be classified in-depth and this publication bias will define the review.

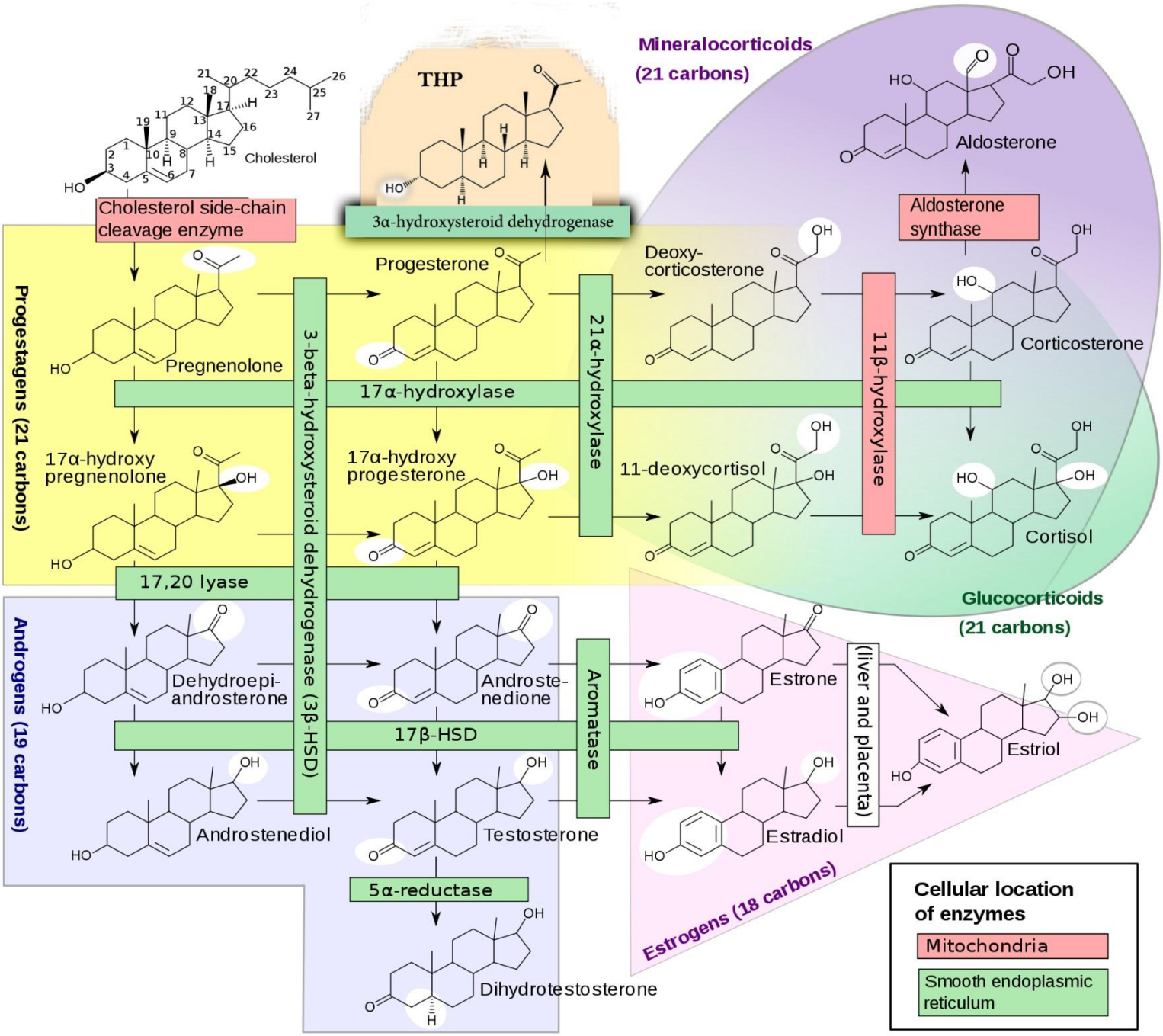


FIGURE 5. STEROIDOGENESIS: THE PRODUCTION OF MAJOR STEROIDS AND THEIR CATEGORIES (COURTESY OF WIKIPEDIA: HTTP://EN.WIKIPEDIA.ORG/WIKI/FILE:STEROIDOGENESIS.SVG); NOTE: THP = ALLOPREGNANOLONE

The synthesis of neurosteroids is, by definition, from within the central nervous system, typically from nonspecialized (e.g., oligodendrocytes) glial and neuronal cells, though it is important to note that many other steroids also influence brain function. All neurosteroids originate from pregnenolone, which in turn is converted from cholesterol, the origin of all steroids. Pregnenolone and progesterone, which is a single enzymatic step away, are converted into an array of different neurosteroids which all fall under the three categories: the glucocorticoids/mineralglucocorticoids, which are responsible for regulating stress, glucose mobilization, and ionic homeostasis; androgens, the male sex hormones, often act as selective growth factors in the CNS; and estrogens, the female sex hormones, have anxiolytic and glucose-consolidating effects. The neurosteroids discussed in the work known to positively modulate the $GABA_AR$ are allopregnanolone (also known as tetrahydoprogesterone or THP), tetrahydrodeoxycorticosterone (THDOC), and androsterone; these steroids particularly act bimodally to enhance Cl^- burst duration at low doses (20-30 µM quantities in rats) as well as behaving as a direct agonist at only slightly higher doses. Neurosteroid antagonists include pregnenolone sulfate (PS) and dihydroepiandrosterone sulfate (DHEAS). Cortisol, a glucocorticoid produced predominantly in the adrenal cortex but also locally in the hippocampus and cortex via 11β-hydroxysteroid dehydrogenase (11βHSD), also contributes a unique role in the modulation of the $GABA_AR$ according to the same hormetic pattern. (Majewska et al, 1991)

As noted before, many important steroids that act on the brain originate from other sources, typically the adrenal cortex or primary sex organs, and act on the brain or undergo further processing. This is a minor pathway for allopregnanlone, but much more significant for cortisol despite circulating cortisol being actively exported from the brain, favoring the glucocorticoid produced locally from serum cortisone (Auchus, 2009). The sulfated forms of the

antagonistic hormones as well as cortisol are hydrophilic, produced in the adrenal cortex, and transported throughout the body, but the blood-brain-barrier effectively eliminates their transport into the brain; while this may occur to a marginal extent, it is with the aid of organic anion transporting peptides which have been demonstrated, for at least DHEAS, to efflux at a rate more than ten times higher than influx (Asaba et al, 2000). For the non-sulfated neurosteroids, transport into the brain is less inhibited due to their lipophilic nature (Majewska et al, 1991). The enzyme responsible for their synthesis, 3α-steroid dehydrogenase (3α-HSD), is in high concentrations in the CNS and their precursors are in fact the $GABA_AR$ antagonistic modulators, implying that the local production of these steroids is essential (Celotti et al, 1992).

The binding site for all of these neurosteroids is considered to be one and the same, or at least very similar in localization and effect (Belelli and Lambert, 2005). Research done on allopregnanolone has revealed that a single asparagine residue present on M1 of the α subunits 1-5 determines the ability for the neurosteroid to achieve modulation of the $GABA_ARs$' chloride current. Given that replacing this residue with another, shorter-chain nucleophile (serine or threonine) leads to somewhat compromised potentiation, it has been shown that this residue, which occurs twice in two α-subunit receptor subtypes, need bind only one molecule of neurosteroid to bring about its maximal effect. (Hosie et al, 2008)

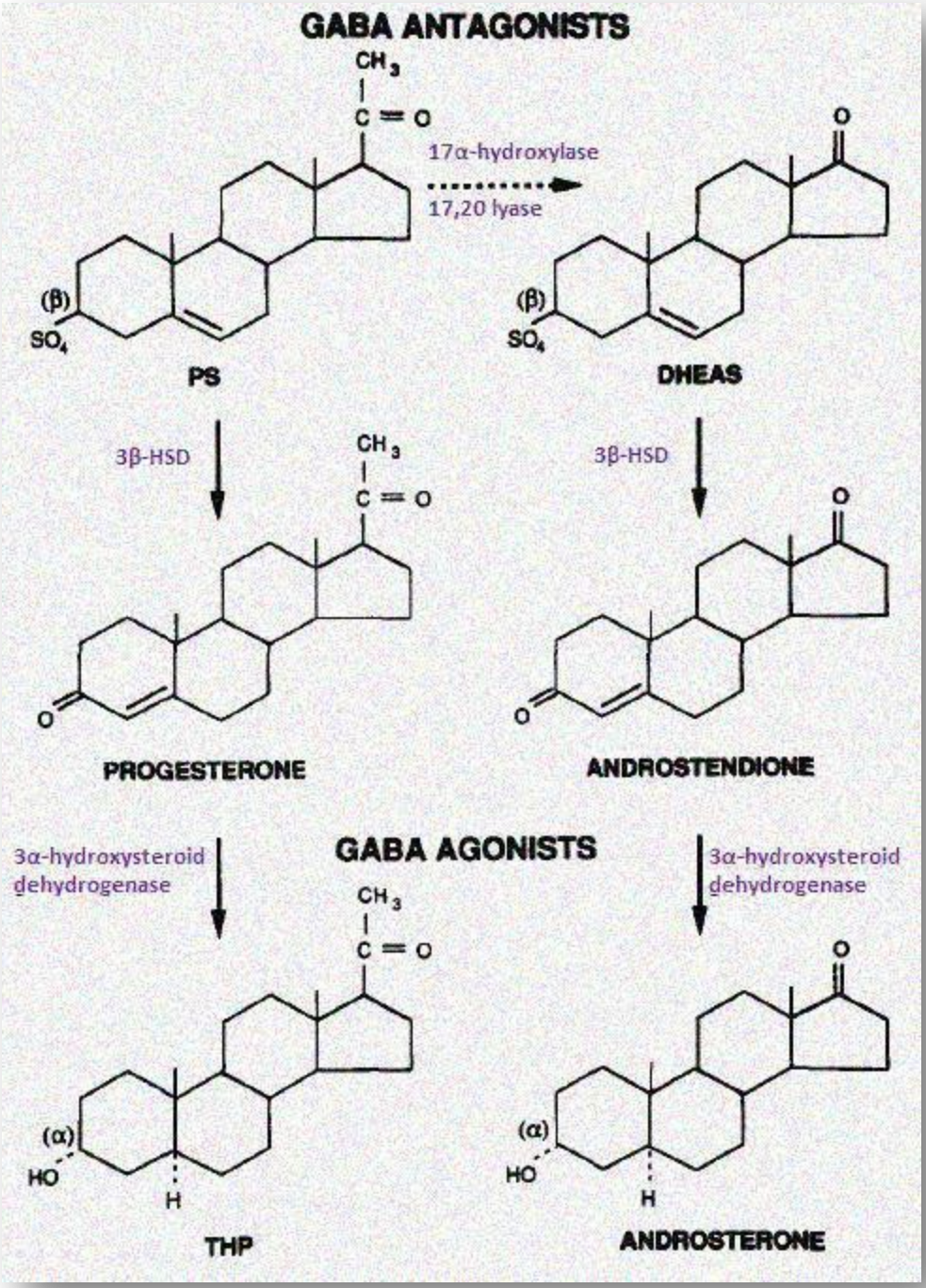


FIGURE 7: COMMON NEUROSTEROIDS ORIGINATING FROM 3A-HSD. (ADAPTED FROM MEJEWSK ET AL, 1991)

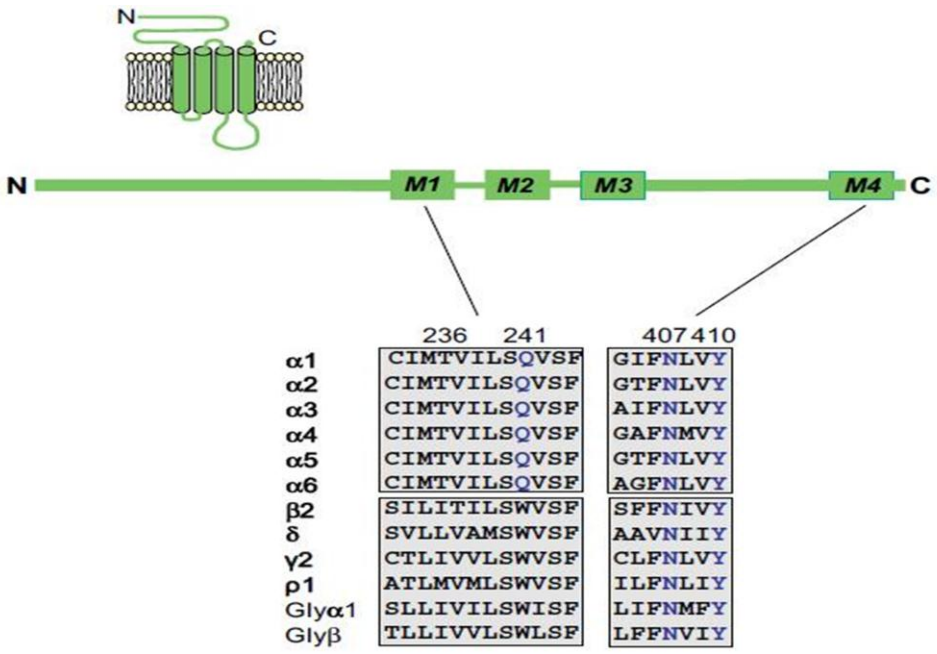


Fig. 1. Primary sequences for the neurosteroid potentiation binding site. The schematic depicts the generic topology of a single $GABA_A$ receptor subunit across the membrane and when linearised. The primary amino acid sequences below show two segments from M1 and M4, containing the residues (blue) that are important for the neurosteroid potentiation site and their relative conservation across $GABA_A$ receptor subunit families; the $GABA_C$ subunit, ρ1 and the glycine (Gly) subunits, α and β. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

FIGURE 8. THE CONSERVATION OF THE ACTIVE BINDING RESIDUES IN THE GABAAR SUBUNITS (FROM HOSIE ET AL, 2008)

While the binding site has been fully elucidated and neurosteroids have been shown to directly act on the α-subunit of the $GABA_AR$, different subunit isotypes confer different characteristics. Among the α subtypes, α5 has been demonstrated to be about 50% as effective as its relatives and this insensitivity has rendered the extrasynaptic (tonic) inhibition in the CA1 region of the hippocampus significantly less responsive to neurosteroids than other nearby regions, such as the dentate gyrus' granule cells. The neurons of the dentate gyrus, among other brain areas considered very sensitive to neurosteroidal modulation, tend to express extrasynaptic $GABA_ARs$ containing δ subunits, which have been long known to further enhance the Cl^- influx due to neurosteroid potentiation. This effect has been determined to be a matter

of enhanced transduction rather than alternative/further binding, indicating that the inclusion of the δ subunit's smaller size and different shape lends itself to allosteric sliding due to interstitial binding (Ooishi et al, 2007). Another contributing factor to neurosteroidal insensitivity is the activity of protein kinase C (PKC), which has been shown to inhibit allopregnanolone modulation when active and permit it when inhibited. PKC_{ε} particularly has shown to produce greater susceptibility to ethanol, barbiturates, and benzodiazepines and a less anxious phenotype upon genetic knock-out, matching the effects of high concentrations of allopregnanolone in the WT (Vergnano et al, 2009). (Belelli et Lambert, 2005)

The modulation of these receptors *in vivo* occurs in when local neurosteroids are upregulated due either to: 1) stress, 2) global hormonal changes, 3) drug-induced upregulation of the 18kDa translocator protein (TSPO[1]). It has been demonstrated in many species that social isolation, defeat, and cortisol exposure lead to enhanced levels of brain allopregnanolone (Zimmerberg et al, 1994). Pregnancy, pubescence, menopause, andropause, development, and menstruation are but a few of the recognized hormonal paradigms of the body in which neurosteroids are well represented; particularly, serum increases in progesterone can be imported to local sites of neurosteroidal synthesis and this increases output substantially (Maguire et al, 2007). Allopregnanolone has been shown to protect against ischemia and contusive injury in rat models (Djeballi et al, 2005; Morali et al, 2011; Sayeed et al, 2009) and the TSPO has also been shown to be upregulated during traumatic brain injury, oxidative stress, and neurotoxins (Papadopoulos and Lecanu, 2009; Martin et al, 2009).

The activation of TSPO, a translocator protein responsible for transporting cholesterol into the mitochondria, is considered the rate limiting step in neurosteroid synthesis. While new ligands for this receptor are only recently being developed, the primary endogenous ligands are

[1]18kD TSPO is the more modern name of the protein formerly named the periperhal benzodiaezipine receptor (TSPO) and the mitochondrial benzodiazepine receptor (TSPO)

not yet fully understood (Corsi et al, 2008). Many drugs, such as ethanol, caffeine, barbiturates, and fluoxetine, have been shown to activate PBE and increase allopregnanolone concentrations through increased availability of pregnenolone to the subsequent enzymes, 3β-HSD (pregnenolone to progesterone) and 3α-HSD (progesterone to allopregnanolone) (Asaba et al, 2000). Natural elevations of TSPO are especially common the hippocampus, piriform cortex, cerebellum, and cortex when mice are exposed to neurotoxins and inflammatory cytokines on an acute or chronic basis (Chen and Guilarter, 2008). Drugs that agonize the TSPO prove to be potent anxiolytics with very favorable side-effect profiles compared to modern benzodiazepines (Nothdrufter et al, 2012). Others have suggested these ligands as candidates for the treatment for affective disorders (Rupprecht et al, 2010).

Wehrenberg, Prange-Kiel, and Rune have identified extensive colocalization of 3β-HSD, TSPO, and Aromatase in the hippocampi of marmosets and rats, indicating a central role of TSPO in local neurosteroid synthesis (2001). Researchers have also shown the widespread distribution of these enzymes in the CNS of zebrafish, particularly correlating with zones of higher neurogenesis (Sakamoto et al, 2001).

There are multiple effects of neurosteroids independent of the $GABA_AR$. In the short term affected neurons display increased inhibition and ,on a greater timescale, trophic and neuroplastic changes are rendered to the cellular architecture (Tsutsui et al, 2011; Chisari et al, 2010; Ioannis et al, 2008; Ooishi et al, 2007; Mellon et al, 2002). The mechanism for either of these effects is not fully elaborated. Some speculate that the interplay between the $GABA_AR$ and VDAC2 channel, a voltage-gated anion channel, which when colocalized via β-tubulin may play a role in instigating long-term effects (Belilli et Lambert, 2005). Others seek more conventional explanations for plasticity through the effects of PKC, which frequently but contextually render

the $GABA_A$Rs insensitive to neurosteroids (Mukai et al, 2007). For example, in the supraoptic nucleus, magnocellular oxytocin-secreting cells are inhibited by progesterone and allopregnanolone; during parturition, allopregnanolone unavailability coupled with the phosphorylation of $GABA_A$Rs via PKC render them suddenly uninhibited and capable of releasing oxytocin during birth and indefinitely after, as long as circulating progesterone remains low (Belilli et Lambert, 2005; Frye et al, 2011).

Both cortisol and corticosterone are glucocorticoids implicated in the modulation of the $GABA_A$R in a biphasic manner: at low concentrations they do not potentiate nor attenuate the actions of the receptor until a threshold, then increase the chloride influx in a positive manner, and then, as concentrations continue to increase, the effects drop off again, ultimately inhibiting the current slightly. This parabolic response is common to many drugs and plays an important part in the equilibrium of alertness and anxiolysis that is controlled dynamically by the steroidal system during times of chronic stress. (Majewska, 1985)

Allopregnanolone's ability to promote hyperpolarization through the $GABA_A$R is especially valuable during periods of allostasis mediated by elevated glucocorticoids. Given cortisol's propensity toward excitotoxic apoptosis in hippocampal cells at high concentrations and allopregnanolone's upregulation by inflammation, these two effects act concordantly to spare hippocampal cells during acute stress (Gravanis and Mellon, 2010). Allopregnanolone and THDOC particularly have been implicated in this compensatory neuroprotective effect (Schumaker et al, 2004). Slightly modified versions of allopregnanolone have also been demonstrated to potently antagonize 11β–HSD type II, suggesting a direct effect on cortisol production as well (Latif et al, 2005). The net effect of progesterone (and its metabolites) during

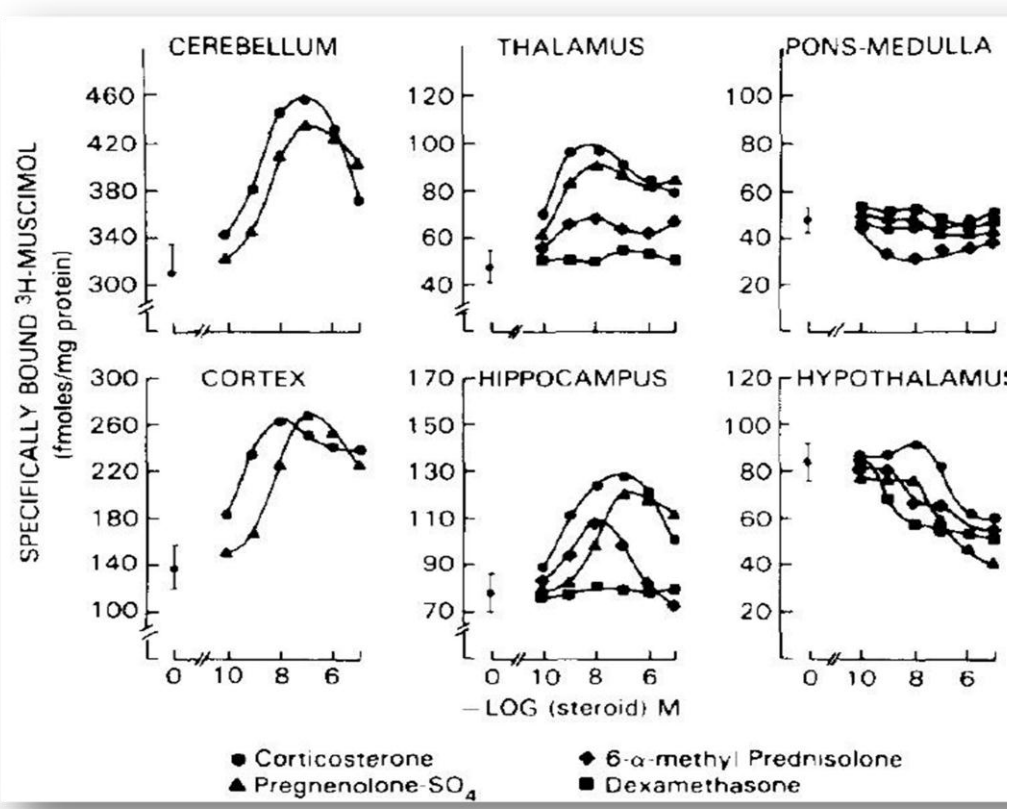


FIGURE 9. THE INVERTED-J SHAPED EFFECTS OF GLUCOCORTICOID MODULATION OF THE $GABA_AR$ ON MUSCIMOL (GABA AGONIST) BINDING (FROM MAJEWSKA, 1985)

homeostasis leads to slight neuronal inhibition, the secretion of myelinating signals from juxtacrinic neurons and astroglia, and long-term neuroplastic inhibition (Jacob et al, 2008).

One last important role for the actions of the paracrinic neurosteroids is their participation in development. There are distinct changes in neurosteroidal sensitivity (controlled partly via PKC phosphorylation, described above) as an organism ages; a mouse at 10-days, for example, is roughly threefold more sensitive to positive modulation of the $GABA_AR$ than at 20-days (Belilli et Lamber, 2005). Furthermore, GABA is essential in development as a neurotrophic and chemotaxic signal, where it may either depolarize or hyperpolarize depending on the cell type and maturity (Manent et al, 2005; Behar et al, 1998). It participates in the developmental processes of cell migration, patterning, and fate determination and is a principal regulator of

early migratory signals by means of its ability to influence calcium transients (Komuro et al, 2005).

Fetal alcohol syndrome can be partly explained by the disruption of the $GABA_AR$ in embryogenesis with the exogenous agonist ethanol (Kandel et al, 2000). Many other psychiatric and neurologic disorders are associated with altered levels of neurosteroids in a similar way, due to long-term shifts in neuronal plasticity and function. Other symptomatic displays of neurosteroidal abnormalities include epilepsy, schizophrenia, unipolar and bipolar depression, anxiety disorders, mental retardation, substance abuse, Parkinson's disease, Addison's disease, Cushing's syndrome, and Alzheimer's disease (Jacobs et al, 2008).

Conservation of Vertebrate Neurosteroids between Teleosts and Humans

It has been shown previously that goldfish are well-quipped with the enzymes necessary to produce neurosteroids such as cortisol and allopregnanolone (Popesku, 2008; see above review). Functionally, these neurosteroids may have divergent yet overlapping roles in both teleosts and humans.

Cortisol is in fact an essential hormone as it doubles as both a mineralcorticoid and glucocorticoid in teleosts, whom lack aldosterone. The dual control by cortisol as the most selecteive ligand for MRs and GRs in teleosts is indicative of an overlapping function of osmoregulation and stress regulation; indeed, increased cortisol is produces copious urine extretion (Mommsen et al, 1999). The transition to more hypertonic waters, easily definable as a challenge to physiologic homeostasis, represents an

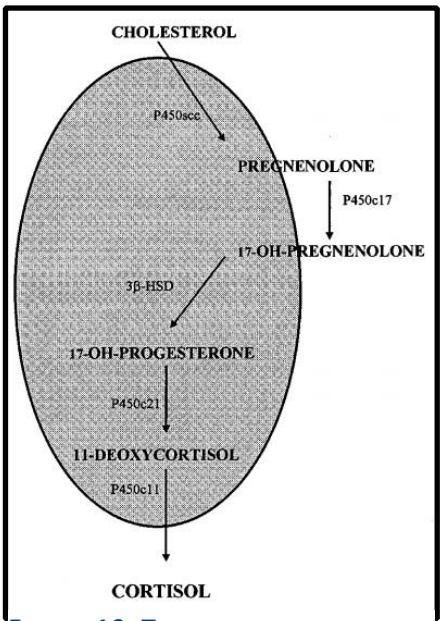


FIGURE 10: THE ENZYMATIC PATHWAY FOR CORTISOL PRODUCTION IN TELEOSTS. THOSE ENZYMES WITH THE GREY ELLIPSE ARE MITOCHONDIRAL (ADAPTED FROM MOMMSEN ET AL, 1999)

essential regulation of stress in fish which increases circulating cortisol (Porthe-Nibelle and Lahlou, 1974). Fish have been shown to possess an MR despite previous doubts (Asterbery et al, 2011). The teleost MR displays greater affinity for 11-deoxycorticosterone than cortisol, though both GRs and and the MR of the carp have equivalent affinity for cortisol (Stolte et al, 2005). MR transcription is significantly higher in the CNS (especially in the Dl, CRH-cells, and the NPO, pituitary pars distalis) over the peripheral organs (e.g., the kidneys, gills, skin) in at least the rainbow trout and carp. This implies either a greater role of the carp MR in HPI regulation over osmoregulatory functions. During periods of chronic hypercortisolemia, both GRs and MR mRNA is downregulated at the same ratio; chronically hypocortisolemic fish express one variant of the GR about twice as much as the other in Dl region of the dorsal pallium, a region analogous to the hippocampus of tetrapods (Sturm et al, 2005; Stiolte et al, 2008). This theory of MR/GR_1/GR_2 ratio regulation of HPI allostasis has not yet mounted sufficient evidence to rival the human equivalent, but is suggestive of a similar mechanism of CNS cortisol regulation as has been previously described in the human condition.

It has been shown that the lone GR in most teleosts operates genomically through intranuclear homodimer formation which binds GREs, identical to the human condition (Aluru and Vijayan, 2008). The conservation of GREs in vertebrates is well documented (Denver, 2009; Chang and Hsu, 2004), though it is important to note teleosts alone appeared to have a whole genome duplication event some 230 mya, leading to both new ligand/receptor types and GRE sequences (Santini et al, 2008; Alsop and Vijayan, 2009; Asterbery et al, 2011). The full implication of this is far from characterized, though the preservation of HPA/HPI axis consituents is strong and many such duplicated genes have been deleted in different species (Stolte et al, 2008; Alsop and Vijayan, 2008).For example, while the common carp possesses two slightly different (95% AA identity) CRH hormones, the zebrafish has been shown to have only a single CRH

sequence (Stolte et al, 2008; Flik et al, 2005). The conservation of HPI/HPA function is largely supported by the similar effects of exogenous cortisol on teleosts as in mammals (Van der Boon et al, 2003; Alsop and Vijayan, 2008; Aluru and Vijayan, 2009). There are accumulating differences between human and teleost cortisol function as the topic becomes better researched (Lin et al, 2011; Asterbery et al, 2011; Flores, 2011). It must also be noted that cortisol plays a major in development of teleost fishes, with the formation of the function HPI system within hours of hatching (Roussea et al, 1999; Flik et al, 2005; Alsop and Vijayan, 2008).

Goldfish possess a bimodal crest of heightened cortisol activity during their diel regulation which is intimately tied to their time of feeding (Noeske and Spieler, 2006; Singley and Chavin, 1973). An interaction between sex steroids and cortisol has also been suggested due to a potent pulse of cortisol four to eight hours prior to ovulation (Cook and Peter, 1979). While teleosts have not been shown to possess a CBG protein that regulates available cortisol in the blood, the CRH-binding protein (CRH-BP) is fairly ubiquitous and has been shown to be regulated through stressor exposure and steroid effects (Flik et al, 2005). DHEAS has similarly been shown to be produced in the head kidneys of fish, a neurosteroid known to modulate glucocorticoid systems in other vertebrats (Bentley, 2001).

The effects of cortisol or other neurosteroids on the $GABA_AR$ have not been documented in fish, though this thesis predicts an acute GABAergic function of the neurosteroid allopregnanolone due to the remarkable conservation of the $GABA_AR$. Its predecessor has even been localized even in Porifera (*Chondrilla nuclea)*, a species which has been shown to have all of the workings of a GABAergic nervous system: glutamate decarboxylase, GABA transporters, and the metabotropic $GABA_B$ receptor (Ramoino et al, 2007). The first appearance of the actual receptor is in cnidarians (including Nematostella vectensis) who share a modern version of the $GABA_AR$ β2-subunit with organisms as distant as humans (Hemmrich et Bosch, 2008). This extensive con-

servation is likely due to the essentiality of these genes and the receptor in development, as evidenced by the high rate of retention of all LIM homeobox genes (Srivastata et al, 2010). The complexity of the $GABA_AR$ and its many facets of modulation are made sensible in light of the 9 million years of evolution that it took to achieve (Ramoino et al, 2007).

The neurogenic, neuroprotective, and neuroplastic effects of allopregnanolone through genomic action may conserved, but there is no research into this topic to date. Allopregnanolone has been shown to play important roles in development in taxa as diverse as amphibians and African lungfish (Hollis et al, 2004; Mathieu et al, 2001; Rebali, 2011), though its role in species prior to the tetrapodian-actinopterygian split is presently unknown. It is well documented that fish experience life-long neurogenesis in many parts of the brain (Zupanc, 2008; Cayre et al, 2002;). Social stress or hierachal status has been shown to significantly attenuate overall neurogenesis in the teleostean brain (Maruska et al, 2012; Sorensen et al, 2007). Interestingly, socialization via electrocommunication in the electric fish *Apteronotus leptorhynchus* has been shown to be sufficient to stimulate neurogenesis, not unlike the effects of ECT in humans (Dunlap et al, 2008; Scott et al, 2000).

The Case for Telencephalic Homologies from Teleosts to Tetrapods

The conservation of functional and structural components of the HPI and HPA are sufficient to draw many interesting parallels from studies on stress in fish to other vertebrates, but the targets of their action on cognition is complicated by intricate differences in comparative neuroanatomy (Rodriguez et al, 2007). The regions of interest in humans, the hippocampus and amygdala, likely predate the split from the actinopterygii-tetrapods but the morphology of the Actinopterygian dorsal telencephalon has proven to be exceptionally deviant from the preceding or following taxa, making assertions of homology particularly difficult (Nieuwenhuys, 2009)

Eversion vs. Evagination

To appreciate the distinctiveness of the everted forebrain organization, it becomes necessary to elucidate the how the actual mechanism unfurls in contrast to the more typical process of evagination. Prior to either of these processes, the neural tube bulges at the future sites of the three primary divisions (forebrain, midbrain, and hindbrain) with an additional point lateral thickening to begin the formation of the diencephalon. In evagination, the telencephalon then proceeds to form the lateral ventricles through ventromedial growth followed by dorsocaudal movement, ultimately resolved in animals with especially large telencephalons (e.g., cetaceans and hominidae) with a caudolateral shift that forms the temporal lobes. In eversion this process is initially reversed: the common ventricle, covered only by a thin tela chorioidea, expands dorsolaterally while the telencephalon grows ventromedially, becoming largely enveloped by the ventricle and narrowly separated from the contralateral hemisphere, joined by a pronounced anterior commissure. These processes are juxtaposed in transverse illustrations in Figures 13 and 15. Recent hodological evidence indicated that there is possibly an additional caudolateral migration with a following partial rostral component in the periventricular zones in teleosts with large telencephalons, though this is controverted by more recent evidence (Yamamoto et al, 2007; Mueller et al, 2011). Altogether, even a simple eversion completely reverses the typical layout of the four-unit pallium, jumbling the ventral pallium (VP), medial palli-

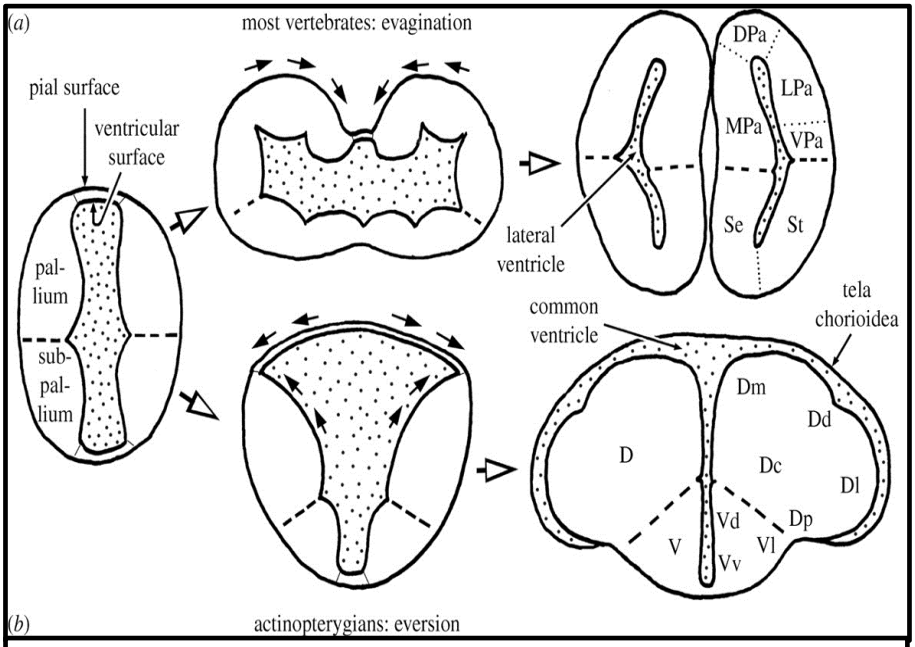


Figure 11. Contrast of Teleost Eversion vs Mammalian Evagination. All four pallial regions are well-defined in typical vertebrates, whereas there are only hypotheses regarding their location in the Actinopterygian associated with a new nomenclature representing the anatomical relativity of histologically similar regions. (Adapted from from Ito et al, 2009)

um (MP), dorsal pallium (DP), and lateral pallium (LP) into positions where their names no longer anatomically represent them. (Northcutt, 2008)

The question of why the Actinopterygii have such unique forebrains has not gone unnoticed. According to Georg Striedter and Glenn Northcutt, both prominent evolutionary neuroscientists and the latter an expert in this field specifically, it seems likely that eversion was a solution to a morphological problem posed by selective pressures:

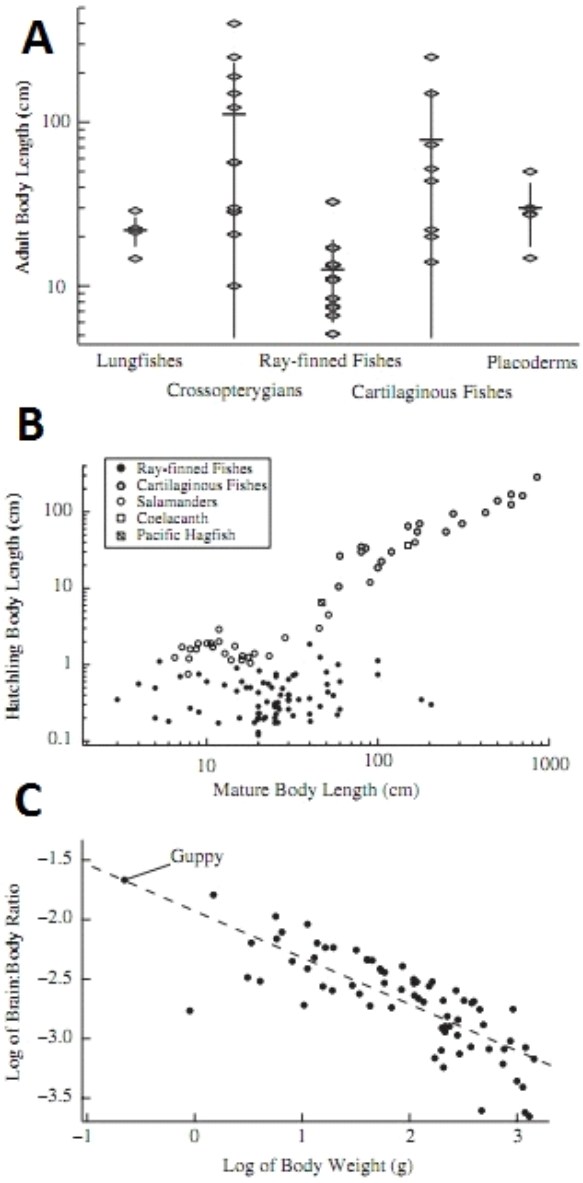


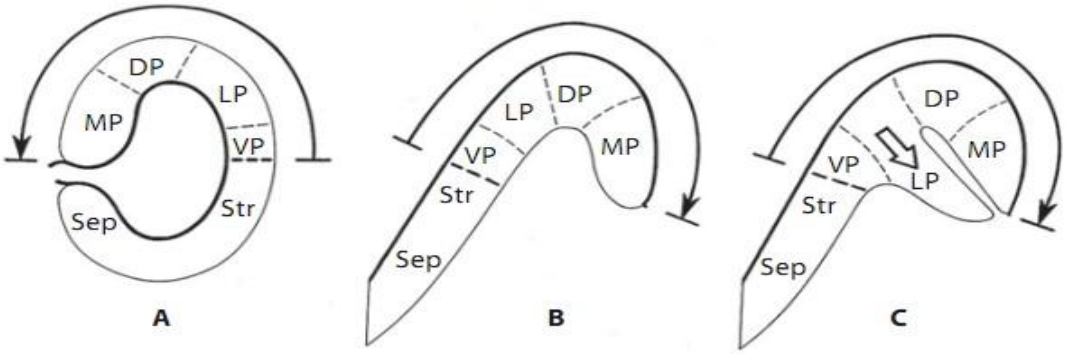
FIGURE 12. A) THE RELATIVE BODY LENGTHS (BL) OF A REPRESENTATIVE SAMPLE OF EACH TAXON; B) MATURE BL VS LARVAL BL, NOTE THAT ONLY ACTINOPTERYGII TEND TO HAVE SIMILARLY SMALL LARVAL BL REGARDLESS OF ADULT BL; C) A SAMPLE OF EXCLUSIVELY ACTINOPTERYGII REVEALS A STARK NEGATIVE CORRELATION OF INCREASING BRAIN AND BODY RATIO AND INCREASING BODY WEIGHT (ADAPTED FORM STREIDTER ET NORTHCUTT, 2006)

[No one has] proposed any adult functional significance of telencephalic eversion versus evagination. Therefore, we propose that telencephalic eversion in the ray-finned fishes is most probably an evolutionary "spandrel" (Gould and Lewontin 1979) or "forced move" (Dennett 1995), a feature that evolved not because of its own functional significance but because it was a mechanically expedient solution to constraints imposed by other changes in the species.

Northcutt and Striedter survey a number of reasons for this hypothesis: the Actinopterygii underwent a dramatic decrease in body size from previous groups, approximately five times smaller on average compared to the massive placoderms and even tinier than the more modest Sarcoptergyii; a paradigm shift in reproductive strategy, focusing on the expulsion of high quantities of larvae without parental support, contemporaneous with the novel trend toward a consistently small size of juvenile stages that were no longer correlated to adult body size; lastly, it is well-known that vertebrate brain-body mass ratios generally increase as body size decreases and the hasty shrinking of Actinopterygii body size may have resulted in a cramped neurocranium. In an analysis of 64 catfish and 21 shark embryos as well as a qualitative analysis of another five species, the investigators determined that the catfishes' brains occupied more than twice as much cranial volume as the sharks' on average and this was not correlated with significant changes in the volume of the olfactory or optic organs. While this does not prove their hypothesis directly, it does provide substantial support for this theory. (2006)

Given the relationship of this unique morphology to its predecessors and the substantial evidence indicating conservation of major developmental systems with better studied animals, it is tempting to assert homologous structures. This has proven to be exceptionally difficult. The Teleostei have recently been of particular interest to modern researchers for their potential as model animals for pharmacology and endocrine analysis due to the remarkable similarity of their neuroendocrine system to higher vertebrates (Popesku et al, 2008; Hammerschmidt, 2011; Steenberger et al, 2010; Renshaw and Trede, 2012; Gestri et al, 2011) and this has sparked debate over the relevancy of such a comparison, especially in preliminary drug trials with behavioral measures (Maximino, 2010). The truth is that the exact nature of these homologies remains uncertain, but the long-disputed identity of the dorsal pallia of teleosts is advancing rapid-

FIGURE 13. COMPARING SIMPLE EVAGINATION (INVERSION), A, TO SIMPLE EVERSION, B, TO COMPLEX EVERSION, C



ly over the past year with the aid of new molecular markers after a stalemate of theorizing spanning over five decades.

Teleostean Dorsal Pallium

In 1963, Nieuwenhuys proposed the common neutral nomenclature for the basic nissl-stain contrasting cell groups, an array of terms still in use today. The regions of common histology were named purely by anatomical location: D represents the area dorsalis telencephali (the entire pallium) with its total of (at most five) major subdivisions, pars centralis (Dc), pars ventralis (Dv), pars dorsalis (Dd), pars medialis (Dm), and pars posterioralis (Dp). Further analysis has led to the christening of many additional cellular regions, all of which are much more controversial and none pertain to the interpretation of the primary pallial divisions.

The controversy over the placement of these regions has arisen from various disparate attempts to characterize this region from phylogenetic, hodological, embryogological, topological, behavioral, and genetic approaches: all of these lines of inquiry have resulted in unique positions regarding the correspondence to homologous pallial divisions (the MP, LP, DP, and VP).

This paper does not presume homology to tetrapods, but acknowledges the possibility of their existence as do the preeminent scientists in this field (Nieuwenhuys, 2009; Northcutt, 2006). The most recent evidence of four distinct pallial divisions has been found in the zebrafish, by far the most common teleost model (Mueller et al, 2011). By infusing markers of NOS, GABA signaling, and mitosis into the mature zebrafish telencephalon, Mueller has confirmed that Dp is derived from migratory neurons from Dl, that Dc in fact originates from a histogenic site near the sulcus ypsiloniformi that migrates caudoventrally, and surmises that Dd likely does not exist at all in the zebrafish, instead representing the heterogenous distribution of Dc cells that cling to the paraventricular space prior to migration.

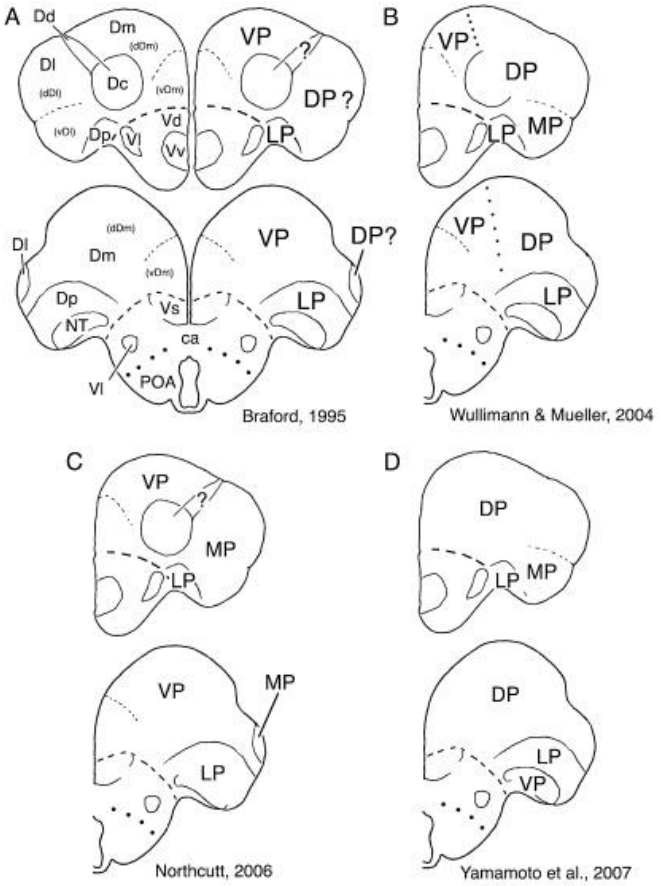


FIGURE 14: THE FOUR MOST INFLUENTIAL THEORIES FOR HOMOLOGOUS PALLIAL DIVISIONS STAND IN DIRECT CONTRADICTION. A) PROVIDES LEFT-SIDED REFERENCE TO THE NIEUWENHUYS' NOMENCLATURE. ALL OF THESE ILLUSTRATIONS REPRESENT THE MOST RECENT PROPOSALS FROM EACH GROUP EXCEPTING B (WULLIMANN ET MUELLER, 2004), WHICH WAS DRAMATICALLY ALTERED IN THE MORE RECENT PAPER BY MUELLER AND A GERMAN COLLABORATIVE TEAM IN MARCH 2011. (ADAPTED FROM YAMAMOTO, 2009)

The first finding confirms long-held hypothesis by Northcutt, Nieuwenhuys, and Gans based on the mutual connectivity of the lateral olfactory tract to the Dp and Dl of basal ray-finned fishes. The latter findings are encouraging, but Northcutt expresses concern over generalizing from the zebrafish to the teleost forebrain in general and Mueller subsequently agrees that there is work to due in establishing these regions in more basal actinopterygians in order to con-

fidently assert such as homologies (Northcutt, 2011; Mueller, 2011). There is still significant doubt as to whether or not teleosts possess a region equivalent to the mammalian neocortex, ultimately derived from the DP.

These discoveries suggest the likelihood of a simple eversion process in which DP is pushed ventrally by the overgrown VP and MP. Indeed, this suggestion is supported by the analogous functions of these regions to the amygdala and hippocampus of other vertebrates, respectively. (Northcutt, 2011). (Appendix II further characterizes the controversy and history of this topic essential to the establishment of fish as relevant model organisms for psychoactive compounds.)

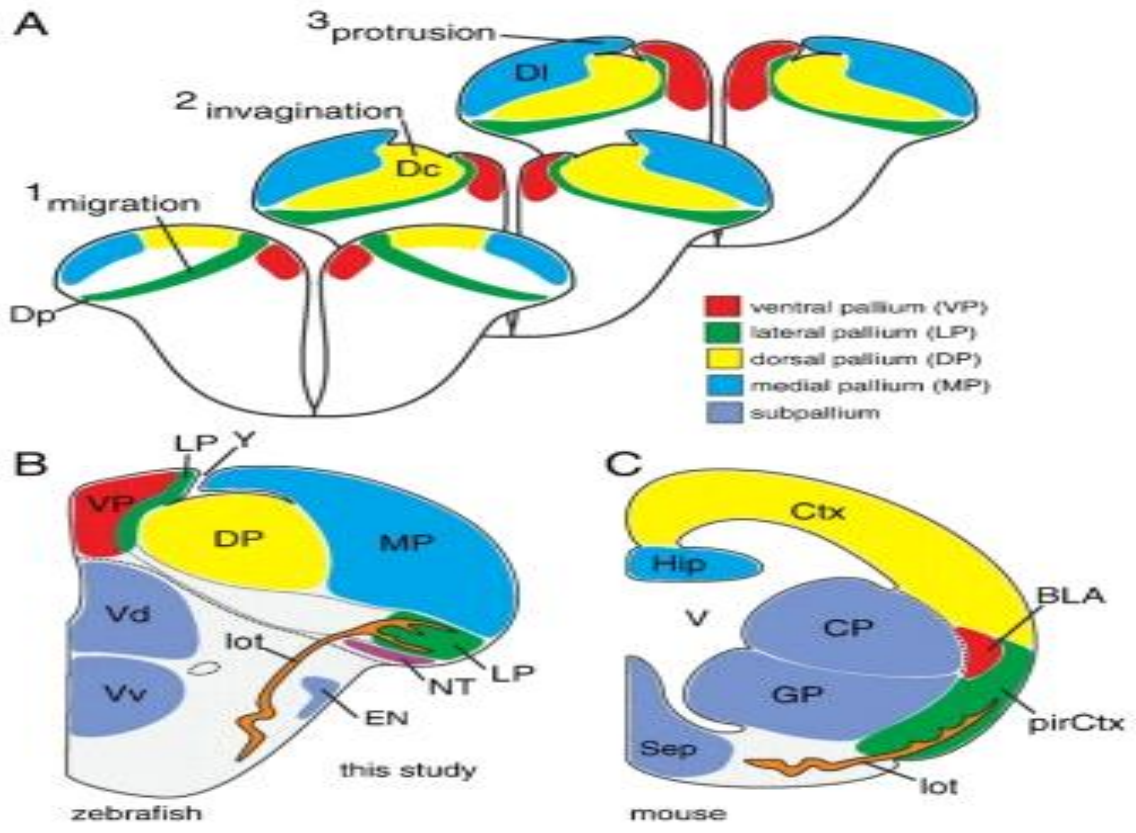
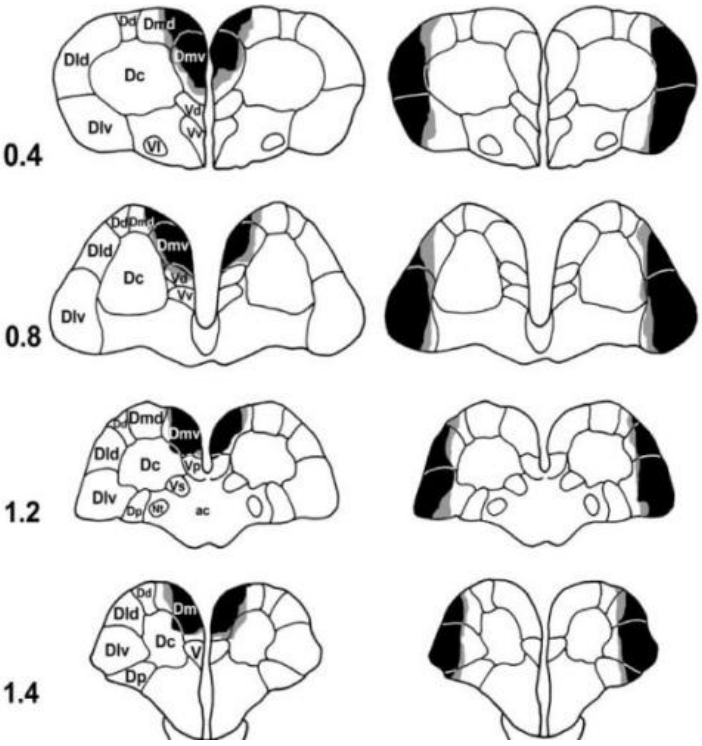


Figure 15. A new model of partial eversion based on embryonic and genetic evidence. A) Three ontogenetic stages of the teleostean forebrain; B) Adult zebrafish brain at the level of the nucleus ementia; C) Adult mouse brain at the level of the basolateral nucleus of the amygdala (From Mueller et al, 2011)

Behavioral and Lesion Studies

A group from the University of Seville, Spain have been working to supplement the hodological evidence with behavioral studies of ablation of certain regions of the pallium. Setting out to prove the analogous functions of the teleostean Dl and Dm with the tetrapodian hippocampus and amygdala, the work of these individuals has contributed greatly to the modern enthusiasm for the use of zebrafish as model animals in behavioral studies. A contrasted summary of their work to date in zebrafish and goldfish compared to various studies in rodents are illustrated below.



(B)

	Teleosts		Mammals	
	LP	MP	H	A
Spatial memory	+	-	+	-
Cue learning	-	-	-	-
Reversal learning	+	-	+	-
Trace motor classical conditioning	+	-	+	-
Delay motor classical conditioning	-	-	-	-
Heart rate conditioning	-	+	-	+
Delay avoidance conditioning	-	+	-	+
Trace avoidance conditioning	+	+	+	+

FIGURE 16. LEFT: LOCALIZATION OF LESIONS IN PORTAVELLA AND VARGAS, 2005; THE BLACK ZONE REPRESENTS THE SMALLEST LESION WHEREAS THE GREY DEMONSTRATES THE BOUNDARIES OF THE FURTHEST LESIONS. RIGHT: REVIEW OF LESION STUDIES IN TELEOSTEI COMPARED TO SIMILAR OUTCOMES IN RODENTIA; H = HIPPOCAMPUS, A = AMYGDALA (ADAPTED FROM PORTAVELLA ET AL, 2004; BROGLIO ET AL, 2006)

A representative experiment performed by Portavella and colleagues tested the effect of “medial pallial” and “lateral pallial” lesions in a two-way active avoidance paradigm, a task which is better known as fear-conditioning in part due to the necessary activation of the amygdala in mammals (Portavella, Torres, and Salas, 2004). They demonstrated that all groups performed equally well upon making a criterion level of accuracy and sustained at least 75% avoidance of the noxious stimuli (electric shock) for at least six sessions before undergoing surgery. Given that the homologous medial and lateral pallial divisions are not yet resolved, the scientists took the terms as anatomically representative: in the lateral lesion they ablated all of Dp and Dl, marginally nicking the side Dc in the process and in the medial surgery they removed about 50% of the most medial portion of Dm. Altogether, “medial pallium” ablation (n = 8), “lateral pallium” ablation (n = 8), total telencephalon ablation (sparing the anterior commissure; n = 8), sham-operated (n = 8), and control (n = 8) groups were subjected to an avoidance paradigm during which they participated in 10 trials a day with an intertrial interval averaging about 90 seconds with a 15-second presentation of the CS, an overlapping 5 second exposure to the UCS, and a 10 second rest in the event of a failure; a measure of behavior very akin to the Delay condition utilized in this experiment (see Methods). In a second experiment, the training procedure was identical with the exception of the CS terminating 5 seconds prior to the onset of the UCS, termed a “Trace” condition, replicating exactly the design of the Trace condition in this thesis. Only the complete telencephalon and “medial pallial” lesions significantly decreased in performance, dropping to about 10% in avoidance that gradually increased over six sessions to about 30% less than the other groups, strongly performing at 80%. In the Trace condition, it was shown that the “lateral pallial” lesion group performed just as poorly as the other two groups in the previous experiment, with all three damaged groups performing avoidance behaviors about 20% of the time. The author concludes that this is further grounds for homology between these

structures, but the criticism stands that behavioral evidence such as this allows only for assertions of *analogy*, or the similarity in function across taxa, and that the lesions employed in this study were not precise for deliberate statements regarding the function of such structures.

In another study the Spanish group demonstrated that Dd of goldfish may be selectively responsible for the same kind of "trace retention" behavior implicated by lesions to Dl (Vargas et al, 2009). Rodriguez and colleagues determined that very similar learning reversal of trained turtles and goldfish in a closed-cross maze can be effected by damage to the medial cortex and Dl, respectively; furthermore, this is consistent with previous experiments of rodents with hippocampal damage (2002). In an open field task, modeled after the rodent standard, it was found that lesions to Dl and Dp, as compared to lesions to Dm or Dm and Dd, produced significant deficits in performance in finding the way to an open gate, an effect further potentiated when the position of the goal was reversed (Broglio et al, 2010). Altogether, coupled with many more studies of a similar nature, the Spanish group has strongly elucidated the function of the Dm and Dl regions of the teleostean brain as functionally analogous to the amygdalar and hippocampal formations in other taxa, respectively.

Rationale for the Present Study

Goldfish provide an excellent model for studies on neurogenesis and its effects on cognition as the majority of the fish brain undergoes extensive neural turnover throughout adulthood (Zupanc, 2009) and the majority of trophic and hormonal factors are shared across vertebrates (Popesku et al, 2008).

Since the HPI axis is regulated via a similar hormonal and anatomical system as the HPA loop, chronic cortisol treatment should render a state akin to Cushing's syndrome with severe memory and cognitive deficits largely related to hippocampal atrophy and dysfunction. The ef-

fect of antidepressants has been shown to be dependent upon the presence of the hippocampus and improved neurogenesis.

It is known that fluoxetine increases neurogenesis through the $5HT_{1A}R$ and the upregulation of allopregnanolone, but still has a negative effect on fear-conditioning aquisition in goldfish. This may be due to the inadequate experience of fear or other memory impairments caused by fluoxetine.

Allopregnanolone has been shown to acutely attenuate the acquisition of certain tasks, including those that are hippocampal-dependent, likely through $GABA_AR$ modulation. The long-term effects of allopregnanolone are poorly characterized, but known to effect transcription to increase synaptic plasticity, escalate neurogenesis, and promote neuron survival. There is substantial evidence that allopregnanolone is produced in abundance in the hippocampus when a functional HPA axis encounters a stressor or the CNS is exposed to a toxic agent.

Previous studies on goldfish demonstrate the hippocampal-dependency of Trace avoidance conditioning (Portavella, Torres, and Salas, 2004) and others demonstrate the utility of scototaxis as an anxiety-like behavior (Maximino et al, 2010; Maximino et al, 2010). By testing the acute effects of both fluoxetine and allopregnanolone on affect it can be established which has the greater acute anxiolytic profile, potentially implicating or ruling out this effect as a means of cognitive impairment. Through the use of both Delay and Trace operant learning paradigms, it is possible to localize general effects of cognition necessary for avoidance training compared to the specific effects imposed on the hippocampus.

Hypotheses

Scototaxis

1. Allopregnanolone will decrease scototaxis
2. Fluoxetine will decrease scototaxis
3. Cortisol will not effect on scototaxis
4. Those fish treated with cortisol and an anxiolytic will display decreased scototaxis over those who received cortisol alone

Delay Conditioning

5. Cortisol treatment will effect fewer avoidances, greater escapes and failures
6. Fluoxetine will produce a deficit in avoidance acquisition
7. Allopregnanolone will delay avoidance acquisition
8. Cortisol plus anxiolytic groups will have avoidance behavior on par with the control

Trace Conditioning

9. Cortisol treatment will produce fewer avoidances, more escapes and failures
10. Fluoxetine will attenuate avoidance behavior
11. Allopregnanolone will delay avoidance acquisition
12. Cortisol plus anxiolytic groups will achieve control levels of avoidance
13. Allopregnanolone+cortisol will improve avoidance learning over fluoxetine+cortisol

Methods

This experiment was carried out under IUCUC protocol # R 3893 in Pritzker Marine Laboratory at New College of Florida under the supervision of Dr. Alfred Beulig.

Subjects

Ninety-six subjects of the species *Carassius auratus auratus* were acquired from Seascape Aquaria, a local retailer. The goldfish were weighed, measured, and acclimated to laboratory conditions for five days prior to testing. The "Comet" variety was chosen.

All subjects were treated in accordance with IUCUC protocol #R3893, which, among other mandates, requires that all subjects receive appropriate medical care for any distress. There were several days in which the fish were not tested but instead allowed to rest and individually treated with either antibiotics (Erythromycin + Muracin), copper (CupriSol©), or sea-salt to manage infections such as Saprolegnia fungus, Ichthyophthirius multifiliis, and Flexibacter columnaris. These days were instrumental in maintaining the health of these immunocompromised animals and many exhibited increased performance on the subsequent testing days. During all trials a small dose of antibiotics was used in the shuttle box itself to help control infection,

prevent cross-contamination between tanks, and prevent the annihilation of the bacterial biofilter in their home tanks.

Husbandry and Aquaria

Paramount to the success of this experiment was the maintenance of the health of the subjects in experimental conditions, particularly in the treatment groups in which all subjects were administered a high dose of cortisol and thus immunocompromised. Immediately upon receiving the goldfish they were housed in two large communal tanks and treated aggressively with a combination of erythromycin, sea salt, chloride, and heat. While many of the fish had no apparent symptoms of bacterial or parasitic infection and this treatment was thus exercised prophylactically, most of the animals were ostensibly affected by a combination of "mouth/body fungus" (Flexibacter columnaris, ≈10%), "gray mould" (Saprolegnia fungus, ≈10%), and "ick" (Ichthyophthirius multifiliis, ≈50%). The doses for erythromycin were as directed by the manufacturer, a full four-day treatment, and salt was administered at a dose of one teaspoon per 10 gallons per day for four days. Heat was applied conservatively during water changes by replacing about 40% of the holding tanks' water and replacing it with heated, chlorinated tap water, gently bringing the tanks to a temperature of approximately 33°C for less than half an hour. This combination of treatments effectively removed all signs of disease in every fish, which were individually examined prior to injections to ensure their health.

When not participating in experimental trials, the fish were housed in eighteen 75-liter (≈20-gallon) tanks with two partitions in each, making for 48 separate compartments. The partitions are perforated enough to allow visual and chemical contact between the fish, preventing stress due to social isolation. Their feedings were scheduled daily and, on a testing day, 30 minutes before trials. The tanks were kept at 25^{o}C, heating as needed provided by a single aquarium heater placed in the central compartment of each tank. All natural lighting was barred

from the laboratory housing the fish and three full-spectrum, high-wattage bulbs were automatically timed to set a 10:14 hour light-dark photoperiod. Regular water changes were mandated to maintain the health of the fish given limited filtration, with a 25% exchange with lukewarm chlorinated tap water every eight days and a 50% exchange every eight days on a schedule so that there was a water change every four days of testing (see the experimental log).

Subject group assignment was accomplished with a pseudorandom number generator inherent to behavioral tracking software. The groups were generally housed together based on drug assignment, as is necessary due to the water-solubility of fluoxetine. In order to avoid housing other groups in a tank with fluoxetine it was necessary to fill one additional 7-gallon tank with a single goldfish on the same dosing schedule as the other members of its group; while this subject did not have chemosensory communications with his compatriots, his tank was placed within inches of a non-experimental holding tank, the inhabitants of which he ostensibly interacted with visually.

Location

The experiment was conducted at Pritzker Marine Laboratory at New College of Florida. Permission for the use of Dr. Beulig's lab was attained. The mailing address is 5800 Bay Shore Rd., Sarasota, FL, 34243.

Apparatus

Eight aquatic shuttle-boxes were enclosed within isolation chambers ("Skinner-boxes"). The shuttle-box has two chambers of equal size, separated by a hurdle that comes up to six centimeters below the top edge of the box. Both sides provide AC current and the fish cannot remain on the hurdle. Two 28V lights were suspended a few inches above the top of the tank over each compartment and the electric shock is delivered by a metal-mesh electrode connected to a stimulator set to discharge 3.8V for 500ms per second during the appropriate stage. With two

exceptions, the device is as described in Brush and Knaff's 1959 publication for an automated shuttle-box: two lights were placed overhead, rather than on each side, and the central hurdle contained no plateau and only one sensor.

Coulbourn Graphic State interfacing software (Coulbourn Instruments, Allentown, PA) was used to regulate the stimuli and their timing. It was also employed for data collection from the photosensor. G*Power 3 was employed for the a priori power tests; SAS 9.3 was used to generate statistical analyses.

Hydrocortisone, allopregnanolone, and fluoxetine were acquired from Sigma-Aldrich.

Data Collection

The order by which the fish are nominated for their trials on test days was determined with a “random subject” feature provided by the Coulborn Graphic State software. The Scoto-taxis trials were performed the day before dosing and two days after. The Trace and Non-trace Cognitive trials were contemporaneously carried out over a course of sixteen days at twenty trials per day immediately after the Scototaxis trials concluded.

The water was changed after each trial for each shuttle-box in order to renew oxygen saturation and drain-off pheromonal signals of fear (“Shrekstoff”). This water was then filtered by a carbon filtration system before returning to the stock used to fill the shuttle-boxes.

Scototaxis

The scototaxis (“movement to darkness”) paradigm was carried out as described in a recent protocol publication in Nature by Maximino, et al (2010). Fish were placed in a half-dark and half-light tank with the central hurdle allowing passage between these chambers. After five minutes of habituation, the number of passes through from one chamber to the other is record-ed as well as the total duration in each side. Goldfish have been previously demonstrated to

exhibit a strong dark-sided preference during the day to permit crypsis and the cumulative time spent in the light side as well as moving from one side to the other are considered indicators of fearlessness, explorative tendencies, and positive affect. Crypsis, simply a term for hiding behavior, plays a significant role in the defensive repertoire of goldfish in their native ecology as a means of avoiding predation.

Fish were placed in the modified boxes with one light bulb removed and an opaque plastic sheet divding the chamber in half, one side light and the other dark. The fish were all observed to be on the light-side as the boxes were closed, allowing one to discern the position of the fish based on the number of photocell breaks.

This protocol was employed before any other in two trials (pre- and post-treatment administration). In order to avoid the obvious confounds that would result from the use of the same skinner-box and exposure to the same stimuli (light), scototaxic trials were only performed before the intiation of avoidance training.

Delay Learning Task

This protocol is designed to test an association between an unconditioned stimulus (UCS) and a conditioned stimulus (CS) as is typical in classical conditioning. The UCS is a mild electric shock that is always preceded and accompanied by the presentation of light, the CS. Nearly all subjects learn this association over time, but their performance in avoiding the UCS is affected by a confluence of factors including affect, recall and learning.

A fish was placed in an unmodified shuttle box and the door shut, sealing off all light and most noise. The experimental protocol proceeds in four stages: in S1, there is no stimulus for an interval of 20 seconds with a 52% chance of proceeding to S2; in S2, the lights turn on for 10 seconds without any shock at which point the fish may cross the hurdle to register an Avoid and

return to S1, otherwise proceeding to S3; in S3 the light and a 3.5V shock alternate for half a second at a time for 5 seconds, during which the fish may cross the hurdle to complete an Escape or fail to do so and proceed to S4; in S4 there is no stimulus and that state counts as a Failure, with 3 seconds elapsing before the return to S1.

This routine was repeated 20 times per testing day per subject, which occurred for 16 consecutive days with several interruptions as described previously.

Trace Learning Task

The trace cognitive task is designed to measure the ability of the subjects to associate the CS and UCS across a small degree of temporal separation, employing a process presumably similar to working memory to retain the "trace" of stimuli that is quickly fading from experience. Since the CS is ostensibly uncoupled from the UCS in this experiment (no light is present during shocks nor does it immediately precede S3), successful mastery of this task requires temporal awareness that has been shown in other studies to be dependent upon the lateral aspect of the dorsal pallium, a region not essential to the Delay paradigm. All of the state conditions are identical to the non-trace task with the addition of a 5 second stage (S5) without light or shock between the CS (S2) and the UCS (S3). The order thus proceeds in a "failed" trial: S1, S2, S5, S3, and then S4. These trials ran concurrently with the delay learning task on the same 16 days at the same times at twenty trials a day.

Experimental Design

The ninety-six fish were divided into twelve groups of eight individuals each. All groups received injections of either vehicle (coconut oil) or one of five solutions of experimental compounds. The fish were randomly selected from a large holding tank and assigned to a group ("all", "flu", "con", "allocort", "flucort", "cort"), and condition (delay or trace) for the avoidance

trials; an example of such a name would be CON3T representing control #3 in the trace condition.

The fish were first tested via the scototaxis paradigm once, injected after completing the task, given one day to rest, and then tested identically again the next day with no further injections. The scototaxis test was thus only sensitive to the acute anxiolytic effects of drugs, such as the benzodiazepine-like activity of allopregnanolone or the actions of SERT inhibition by fluoxetine. It is notable that the antidepressive effects of fluoxetine at clinical and experimental doses onset only after a latency period in the order of weeks, due to unknown factors that may be attributed to hippocampal neurogenesis (Tome et al, 1997; Jacobs et al, 2000). The same day that the scototaxis trials concluded the fish began avoidance training.

Treatment Preparation

All experimental compounds, excluding fluoxetine, are lipid soluble and relatively immiscible in water, thus oil-based preparations were necessitated. According to previous work with various teleosts, coconut oil provides an excellent lipid substrate for the i.p. delivery of

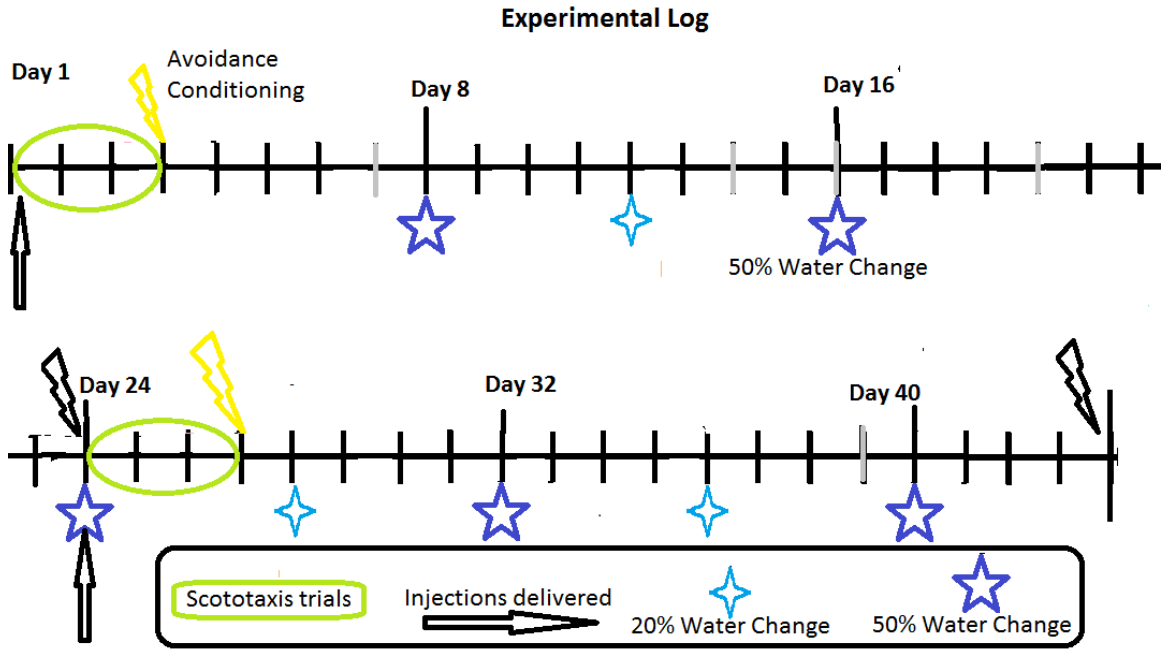


FIGURE 17: THE EXPERIMENTAL SCHEDULE. EACH SOLID BLACK LINE INDICATES A TESTING DAY WHEREAS EACH GRAY LINE IS DAY OF TREATMENT AND REST.

drugs, extending their metabolism and prolonging duration from a few days to several weeks (Vijayan et al, 1994; Vijayan et al, 1996; Vijayan et al, 1991; Reddy et al, 1995). Since Vijayan and colleagues assert that they find intact coconut oil implants in their cold-water fish up to three weeks after injection, the release of the drugs may be dependent on the melting of the vehicle which would place the expected time to be significantly shorter considering the higher holding temperatures of the goldfish in this study (18°C vs 23°C) and virgin coconut oil's total melting point at 24°C. The solutions were heated until the coconut oil was sufficiently liquefied and vortexed until solution was ostensibly homogenous with no visible solute. The fish were weighed prior to injection and then allowed to rest for 36 hours prior to further experimentation.

The treatment groups received the following compounds, providing 16 subjects per treatment: allopregnanolone, fluoxetine, vehicle-only, allopregnanolone plus cortisol, fluoxetine plus cortisol, and cortisol. The fish were further divided by condition (Delay or Trace) into groups of eight each.

The dose of cortisol, 250 µg/g, was chosen in light of a pilot study which revealed initial diminishing returns in avoidance conditioning level and enhancing effects at lower doses. The intention was to create deficits in avoidance conditioning due to chronic HPI activation that may in turn be treated with antidepressive and/or anxiolytic effects.

The dose of allopregnanolone was chosen based on previous studies with rats (e.g., Turkman et al, 2004) at 8 mg/kg. This dose proved insufficient to produce lasting anesthetic effects. No prior data on the use of allopregnanolone in teleosts exists.

Fluoxetine was dissolved in the water of the home aquaria at a concentration of 81µg/L, consistent with previous work (Beulig and Fowler, 2009). The metabolism of fluoxetine in teleosts has shown to produce the same active metabolite (norfluoxetine) as it does in human albeit

in lesser concentrations (Smith et al, 2010), assuring that the effects of fluoxetine continued to be experienced even after initial metabolism. Fluoxetine was replaced during each water change at a rate to maintain an equivalent concentration to the initial dose.

Results

Scototaxis

The scototaxic data is presented with asterisks indicating significant differences from the control and black lines which overlap those groups which displayed no significant differences between them.

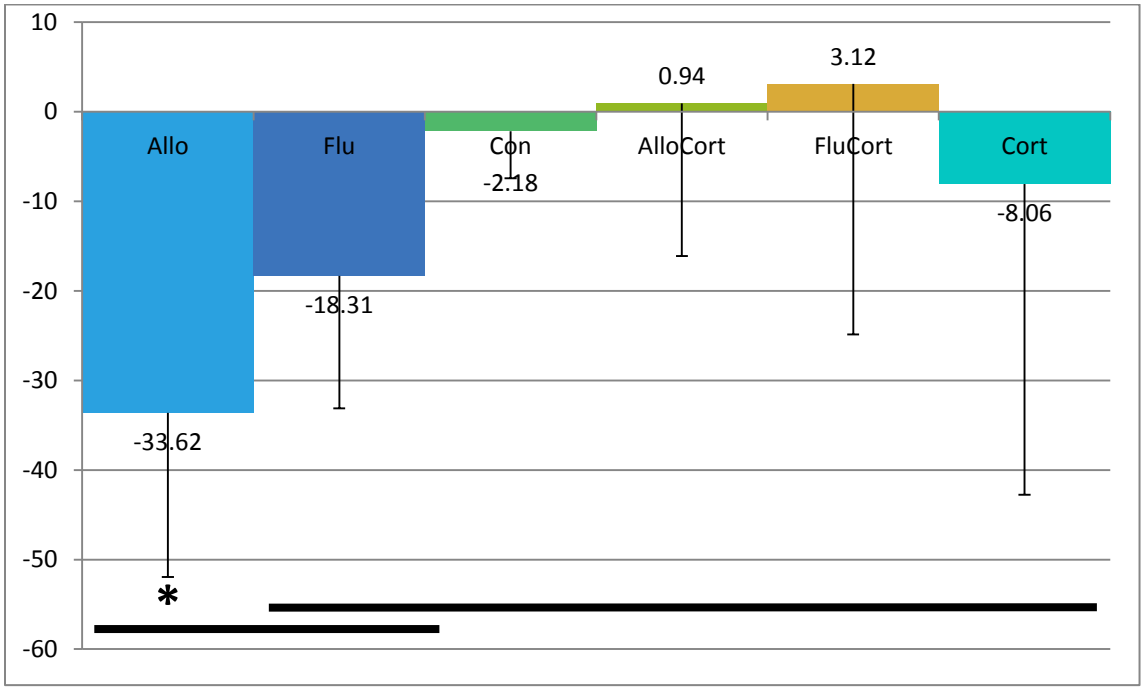


FIGURE 18: NUMBER OF TRANSITIONS, PRE-TREATMENT MEANS MINUS POST-TREATMENT MEANS. ERROR BARS INDICATE STANDARD DEVIATIONS, BOTTOM LINES DEMONSTRATE TUKEY'S GROUPING.

The data was checked for normality, equality, and homogeneity of variance via Mauchly's and Levine's tests. ANOVA was then employed to compare the means for both the number of transitions and the duration of time spent in the light. The main effect of treatment was significant in the difference (pre-injection minus post-injection) of transitions, $F(5, 96) = 6.60$, $p < .0001$. Tukey's Honest Signifcance Difference (HSD) shows that allopregnanolone ($M = -33.62$, SD

= 18.33) alone exhibited significance over the control (*M* = -2.18, SD = 5.23) and all other groups excluding fluoxetine (*M* = -18.31, SD = 14.80).

Given that the allopregnanolone-treated fish exhibited greater explorative behavior, which is repressed in more fearful fish, it is concluded that allopregnanolone produces anxiolytic in goldfish whereas no other compound achieved significance over the control.

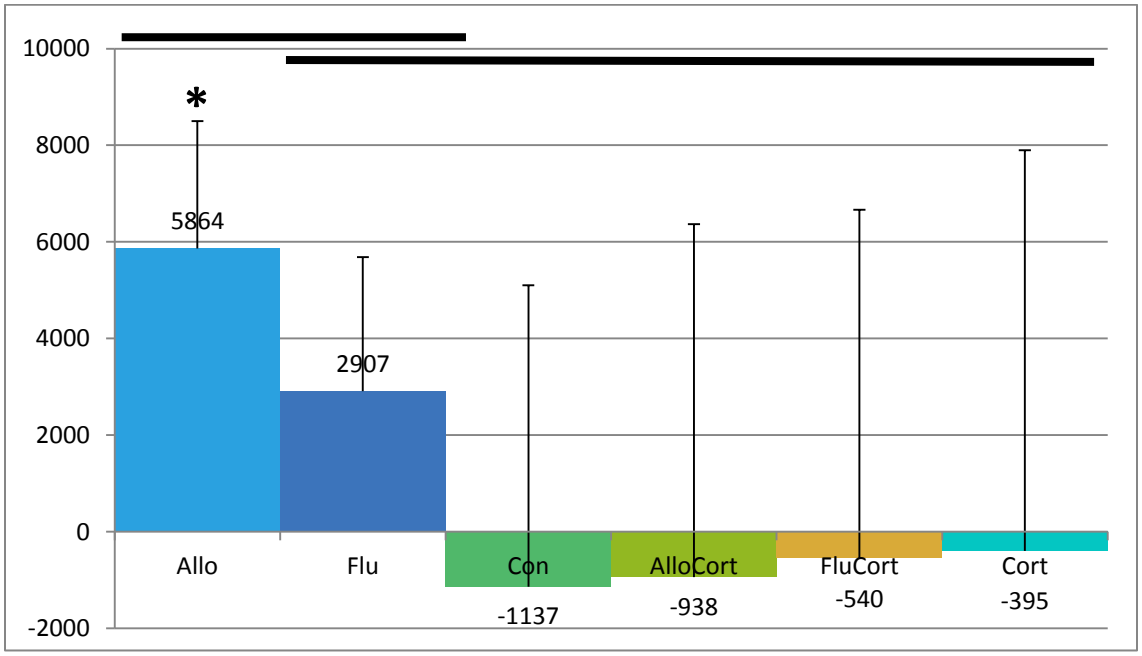


FIGURE 19: TIME SPENT IN THE LIGHT, PRE-TREATMENT MEANS MINUS POST-TREATMENT MEANS. ERROR BARS INDICATE STANDARD DEVIATIONS, TOP LINES DEMONSTRATE TUKEY'S GROUPING.

The main effect of treatment was significant when evaluated in terms of the mean difference of the duration of time spent in the lighted portion of the tank, $F(5, 96) = 6.88$, $p < .0001$. Tukey's HSD reveals that allopregnanolone (*M* = 5864, SD = 2636) alone exhibited significantly greater time in the lighted portion of the tank than the control (*M* = -1137, SD = 6237) and all other groups excluding fluoxetine (*M* = 2907, SD = 2775). High variability in all but the allopregnanolone groups is revealed in the above graph.

Since allopregnanolone-treated fish spent significantly more time in the light chamber than the control group they displayed a lesser tendency to hide in the dark, indicating decreased "anxiety" over controls.

Cognition

The data was checked for normality, equality, and homogeneity of variance via Mauchly's and Levine's tests. ANOVA utilizing repeated measures was then used to compare the means for the avoidances, escapes, and failures in each trial according their corresponding "block", comprising four consecutive days. The procedure for analysis was identical for both the Delay and Trace data.

All data on tests of cognition are presented in bar graphs with color indicating group, error bars displaying the standard deviation, asterisks revealing significant difference from controls, and lettering to demonstrate the groups of nonsignificance produced by Tukey's pairwise comparison. Those groups with significant differences between them but not over the control do not have asterisks but are instead grouped exclusively. For example, those grouped as "A" are significantly different than those in "B" and "C", but not significantly different than those also in "A", or those groups inclusive of the same group such as "AB" or "ABC". Each group is abbreviated so that allopregnanolone is "All", fluoxetine is "Flu", control is "Con", allopregnanolone-cortisol is "AllC", fluoxetine-cortisol is "FluC", and cortisol is "Cort".

Delay Learning

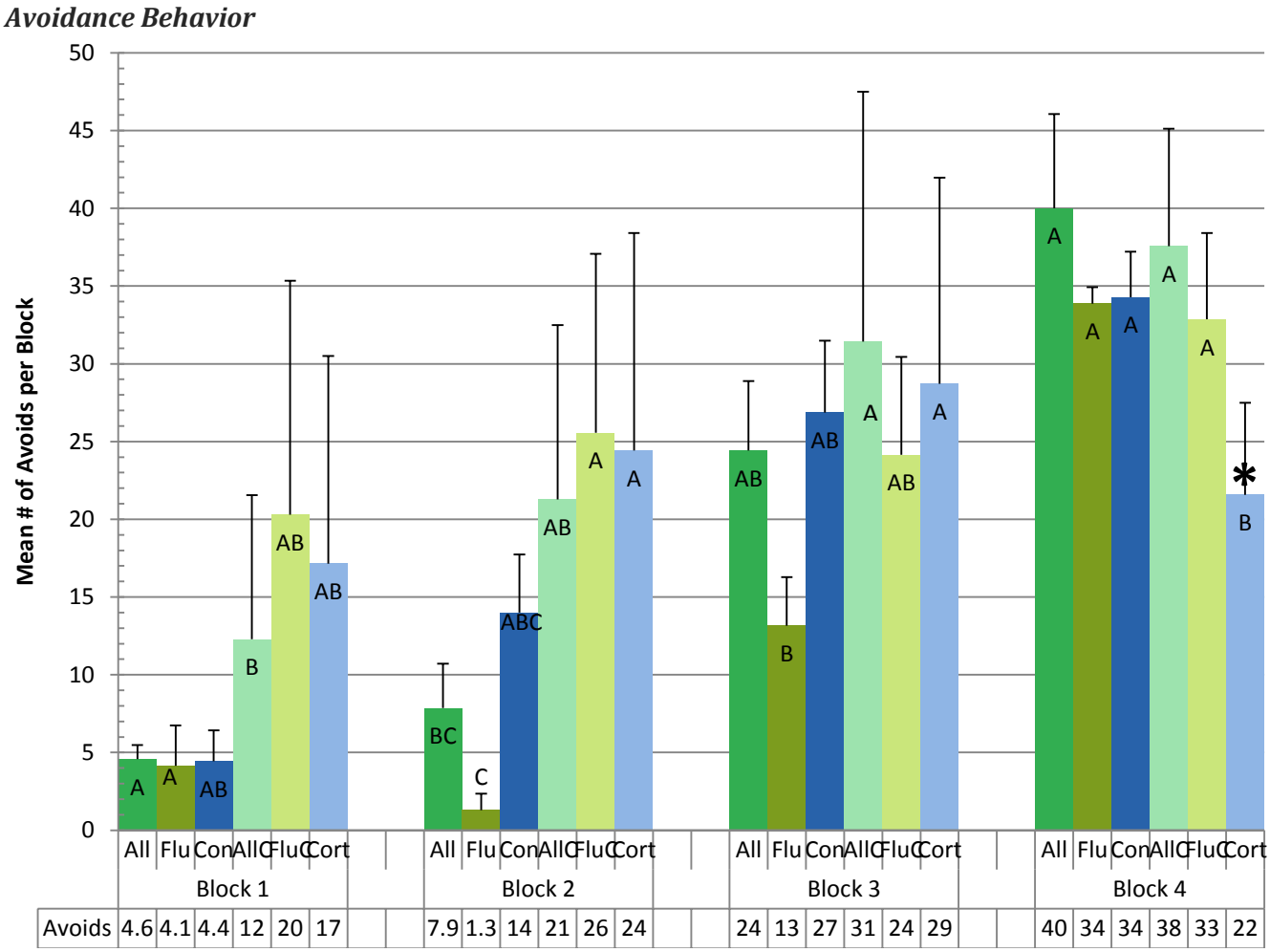


FIGURE 20. AVOIDANCE MEANS, DELAY CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was a significant overall effect for treatment for avoidance frequency, $F(5, 42) = 6.72$, $p = .0002$; block, $F(3, 42) = 72.19$, $p < .0001$; and an interaction between block and treatment, $F(15, 42) = 5.27$, $p < .0001$. Testing with repeated measures reveals that block 1 exhibits significance when evaluated by treatment, $F(5, 42) = 4.28$, $p = .0037$; block 2 is significant, $F(5, 42) = 8.50$, $p < .0001$; block three is significant, $F(5, 42) = 3.20$, $p = .0171$; and block 4 is also significant, $F(5, 42) = 10.51$, $p < .0001$.

Significant Pairwise Comparisons

Tukey's HSD shows that none of the results proved significant compared to the sham group excluding cortisol in the fourth block (*M* = 21.57, SD = 5.91) versus control (*M* = 36.71, SD = 2.93). Again, no further significance was found between the positive control, the cortisol group, excluding the comparison between cortisol and all other groups in block four.

Nonsignificant Pairwise Comparisons

While certain trends evade significance against the control groups, there are various groupings depicted by the lettering schema on the above graph. For example, the fluoxetine group performed consistently lower than all others prior to block four: there is a very modest mean in block one (*M* = 4.14, SD = 2.59), the lowest mean of all avoidances in block two (*M* = 1.28, SD = 1.07), and a relatively very small average in block three (*M* = 13.14, SD = 3.13). While none of these effects reach significance over the control, the block one fluoxetine group M = 4.14, SD = 2.59) is significantly worse than the fluoxetine-cortisol group of the same block (*M* = 20.29, SD = 15.06), the highest block one mean, which was also significant compared to allo-pregnanolone and all other groups that did not receive cortisol. In block two, fluoxetine (*M* = 1.28, SD = 1.07) was also significantly worse than all of the groups which received cortisol. In block three, fluoxetine (*M* = 13.14, SD = 3.13) subjects performed significantly worse than corti-sol (*M* = 28.71, SD = 13.25) and allopregnanolone-cortisol (*M* = 31.43, SD = 16.06). Fluoxetine (*M* = 33.86, SD = 1.069) performed as well as the other groups in the final block.

Trends

Despite a strong trend toward improvement of avoidance behavior over time, the Delay condition presents two decreases in mean avoidance: fluoxetine from block one to block two (*M* = 4.14, SD = 2.59 vs *M* = 1.29, SD = 1.06) and cortisol from block three to four (*M* = 28.714, SD = 13.25 vs *M* = 21.57, SD = 5.91). Additionally, there is an abrupt rise in the average performance

of the fluoxetine group from block three to four (*M* = 13.14, SD = 3.13 vs *M* = 33.857, SD = 3.132).

Fluoxetine in block two presents with the overall lowest avoidance mean (*M* = 1.28, SD = 1.07) and allopregnanolone in block four (*M* = 40, SD = 6.06) exhibits the highest overall avoidance mean in both Delay and Trace experiments.

Conclusions

It may be stated that that no treatment produced effects in Delay avoidance learning over the control, with the exception of cortisol over a period of 12 days. Since those fish treated with cortisol exhibited decreased avoidance but those who received an anxiolytic in addition to the cortisol-implant were on par with the control, it is concluded that anxiolytic treatment had a significant role in ameliorating the cognitive deficit produced by chronic hypercortisolemia.

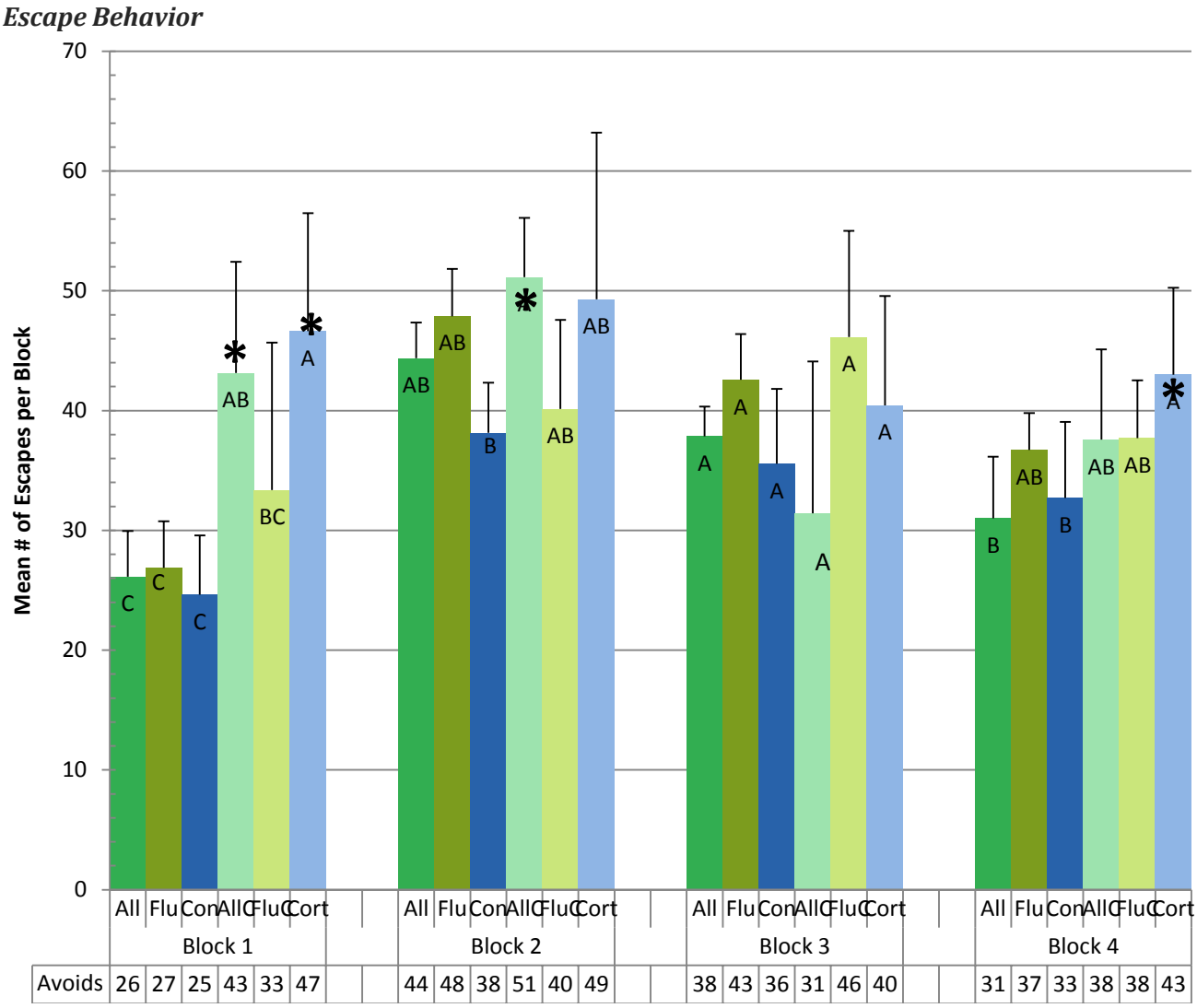


FIGURE 21. ESCAPE MEANS, DELAY CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was also a significant overall effect for treatment on escape frequency, $F(5, 42) = 12.20$, $p < .0001$; block, $F(3, 42) = 21.69$, $p < .0001$; and an interaction between block and treatment, $F(15, 42) = 2.70$, $p = .0016$. Testing with repeated measures reveals that block 1 exhibits significance when evaluated by treatment, $F(5, 42) = 9.56$, $p < .0001$; block 2 is significant, $F(5, 42) = 3.65$, $p = .0009$; block three is not significant, $F(5, 42) = 1.58$, $p = .1904$; and block 4 is significant, $F(5, 42) = 3.86$, $p = .0066$.

Significant Pairwise Comparisons

Tukey's HSD shows that, in block one, allopregnanolone-cortisol (*M* = 43.14, SD = 9.27) and cortisol (*M* = 46.63, SD =9.86) both significantly differed from the control (*M* = 24.63, SD = 4.95). In block two, only allopregnanolone-cortisol (*M* = 51.14, SD = 4.94) showed a significant deviation from the control (*M* = 38.14, SD = 4.18). In block four, cortisol (*M* = 43.00, SD = 7.26) proved to be significantly higher than the control (*M* = 32.71, SD = 6.34).

Nonsignificant Pairwise Comparisons

There is an increase in all groups' mean escapes from block one to two that is especially pronounced in the non-cortisol groups. Allopregnanolone, fluoxetine, and the control groups steadily decline in the number of escapes from blocks two to four. Cortisol shows a slight drop in mean escapes with a concomitant decrease in variability from blocks two to three (*M* = 49.29, SD = 13.93 to *M* = 40.43, SD = 9.14), followed by a marginal increase in mean and decrease in standard deviation from blocks three to four (*M* = 40.43, SD = 9.14 to *M* = 43.00, SD = 7.26). Allopregnanolone-cortisol and fluoxetine-cortisol show similar nonsignificant fluctuations from blocks two to four, defying the consistent reduction of escapes observed in the anxiolytic and control groups.

Trends

The escape data presents much higher variability than the cortisol-implanted groups exhibiting consistently higher variance than the control or the anxiolytic-only groups. While block-one escapes are especially low in the control, allopregnanolone, and fluoxetine cohorts compared to the cortisol-containing groups, this effect did not prove significant and these values largely normalize by block two.

Conclusions

Since cortisol in block four produced significantly more escape behavior than the control, it is concluded that the deficit to Delay avoidance learning is likely not due to motor system complications associated with the drug.

Failure Behavior

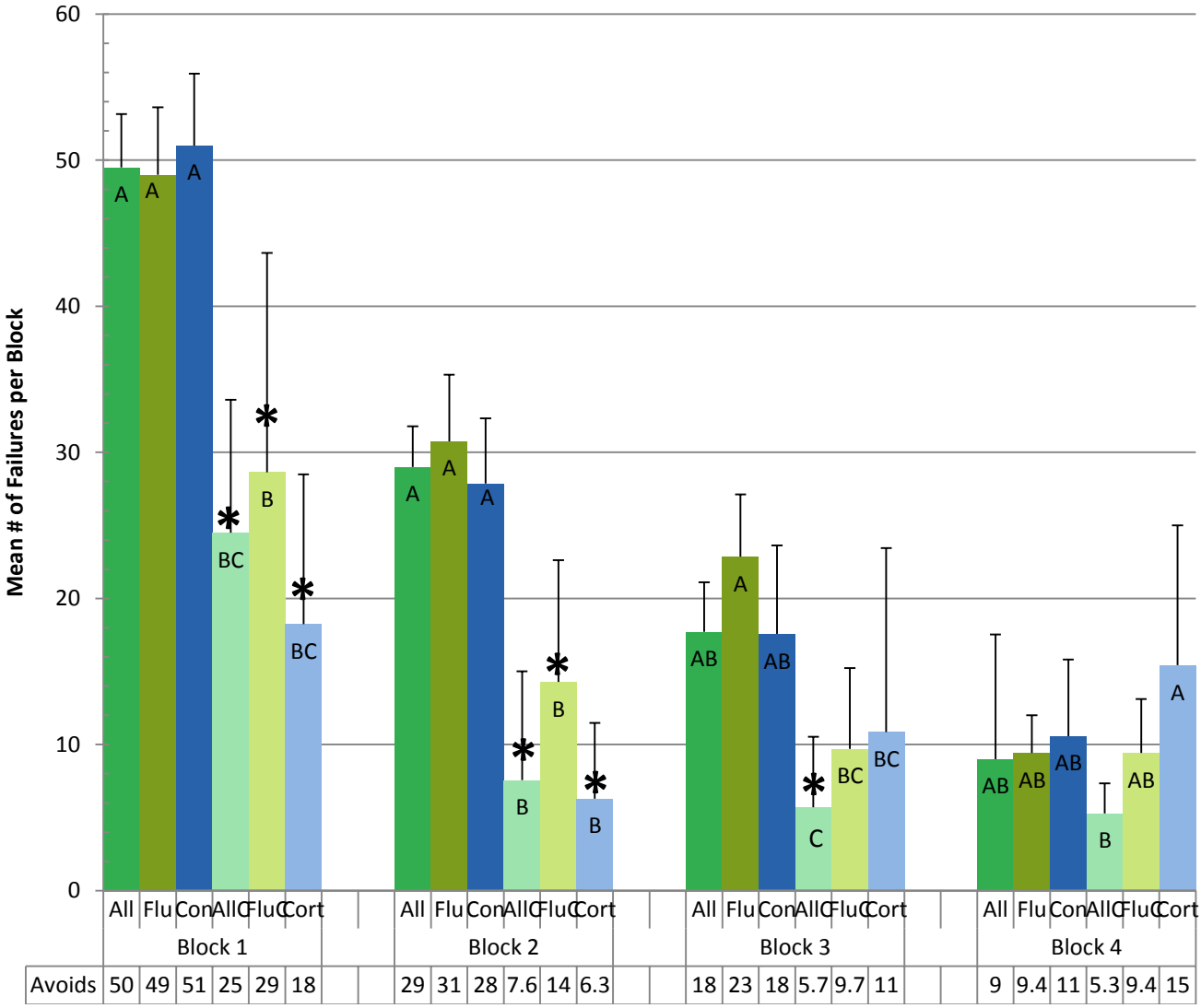


FIGURE 22. FAILURE MEANS, DELAY CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was a significant overall effect for treatment on failure frequency, $F(5, 42) = 27.21$, $p < .0001$; block, $F(3, 42) = 138.53$, $p < .0001$; and an interaction between block and

treatment, F(15, 42) = 8.78, $p < .0001$. Testing with repeated measures reveals that block 1 exhibits significance when evaluated by treatment, F(5, 42) = 21.80, $p < .0001$; block 2 is significant, F(5, 42) = 25.94, $p < .0001$; block three is significant, F(5, 42) = 6.09, $p = .0004$; and block 4 is not significant, F(5, 42) = 2.08, $p = .0912$.

Significant Pairwise Comparisons

Tukey's HSD reveals significance between the control (*M* = 51.00, SD = 4.93) and allopregnanolone-cortisol (*M* = 24.50, SD = 9.10), fluoxetine-cortisol (*M* = 28.15, SD = 15.02), and cortisol (*M* = 18.25, SD = 10.25) in block one. Again, allopregnanolone-cortisol (*M* = 7.57, SD = 7.44), fluoxetine-cortisol (*M* = 14.29, SD = 8.34), and cortisol (*M* = 6.29, SD = 5.19) proved to be significantly lower than the control (*M* = 27.88, SD = 4.45) in block two. By block three, only allopregnanolone-cortisol (*M* = 5.71, SD = 4.82) presents as significantly lower than the control (*M* = 17.57, SD = 6.05) but not cortisol (*M* = 10.86, SD = 12.59).

Trends

There is a strong trend in all groups to decline in failures from block one to four at a decelerating rate suggestive of a floor effect. The only exception is the increasing rate of failures and oscilating variability in the cortisol group from block two (*M* = 6.29, SD = 5.187) to block three (*M* = 10.86, SD = 12.59) to block four (*M* = 15.43, SD = 9.57). It should also be noted that the last group to lose significance over control is the allopregnanolone-cortisol cohort, ultimately presenting with the fewest failures by block four (*M* = 5.29, SD = 2.06).

Conclusions

While all of the cortisol-treated groups exhibited significantly more failures in blocks one and two, they did not display significantly less avoidance nor escape behavior in the same blocks. This suggests perhaps helpless behavior in these earlier blocks that was not due to motor

inhibition, since many of the fish demonstrated significantly higher escapes over the same period.

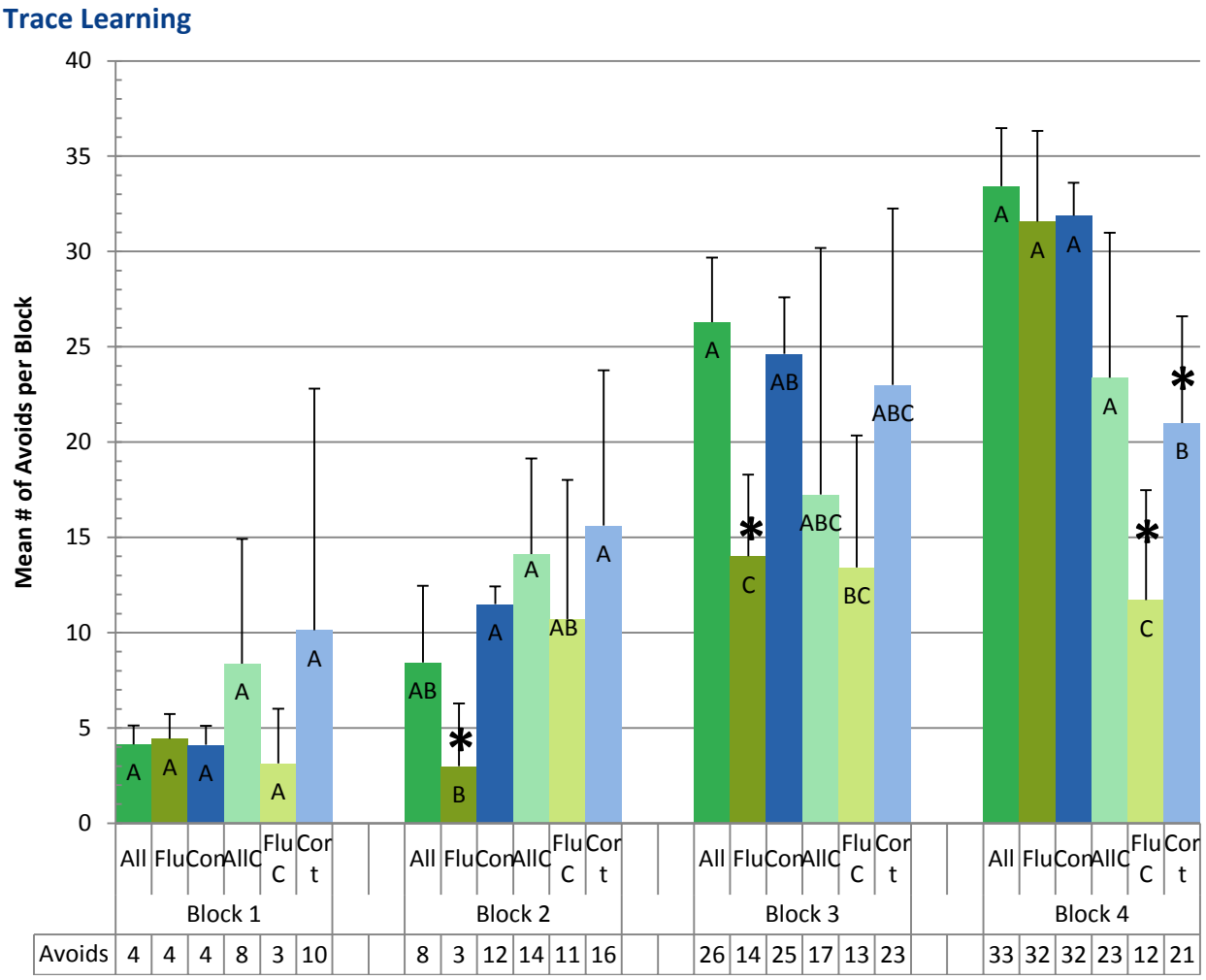


FIGURE 23. AVOIDANCE MEANS, TRACE CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was a significant overall effect of treatment on avoidance in the Trace condition, $F(5, 45) = 9.05$, $p < .0001$; block, $F(3, 45) = 118.12$, $p < .0001$; and an interaction between block and treatment, $F(15, 45) = 5.90$, $p < .0001$. Testing with repeated measures reveals that block 1 does not show significance when evaluated by treatment, $F(5, 45) = 1.59$, $p = .1892$; block 2 is

significant, F(5, 45) = 5.48, p = .0007; block three is significant, F(5, 45) = 4.48, p = .0025; and block 4 is also significant, F(5, 45) = 21.23, p < .0001.

Significant Pairwise Comparisons

Tukey's HSD reveals significant differences from the control in blocks two, three, and four. In block two, fluoxetine (M = 3.00, SD = 3.28) was significantly lower than the sham treatment (M = 11.5, SD = .92). In block three, fluoxetine (M = 14.00, = SD 4.29) was again lower than the control (M = 24.62, SD = 3.00). In block four, fluoxetine-cortisol (M = 31.57, SD = 4.76) and cortisol (M = 21, SD = 5.606) were both lower than the control and cortisol also exhibited a significantly lower mean than fluoxetine-cortisol. No comparisons reveal significant differences between the positive control and any other groups aside from those in block four, described already.

Nonsignificant Pairwise Comparisons

Cortisol exhibits a curious profile over time. Initially presenting the highest mean in block one and the greatest variation of all groups throughout in the Trace condition (M = 1.28, SD = 12.68), cortisol also overtakes the other groups in block two while maintaining the most variance in this block (M = 15.63, SD = 8.14). In block three, cortisol (M = 23.00, SD = 9.26) ranks above the other two cortisol-containing treatments and comparable to allopregnanolone (M = 26.63, SD = 3.40) and the control (M = 24.63, SD = 2.97) but, by block four, falls significantly below the allopregnanolone-cortisol (M = 17.25, SD = 12.94) group and all other non-cortisol groups.

Trends

Interestingly, from block three to block four, cortisol (M = 23.00, SD = 9.26 vs M = 21, SD = 5.61) and fluoxetine-cortisol (M = 13.43, SD = 6.92 vs M = 11.71, SD = 5.77) both decrease slightly despite a strong trend in all other groups towards improvement over all blocks. The only

other violation of this trend occurs from block one to block two with fluoxetine-treated fish (*M* = 4.43, SD = 1.31 vs *M* = 3.00, SD = 3.28). Another notable change is the abrupt improvement of the fluoxetine group from block three to four (*M* = 14.00, SD = 4.29 vs *M* = 31.57, SD = 4.76), resolving the significantly lower performance compared to control from blocks two and three.

Conclusions

Allopreganolone-cortisol displays significant improvement in block four over both fluoxetine-cortisol and cortisol, indicating its superior role in the retention of Trace avoidance learning in conditions of chronic hypercortisolemia. The significantly lower performance of fluoxetine-cortisol over the positive control suggests that fish who were treated with fluoxetine-cortisol exhibit impairments to hippocampal function that are greater than treatment with cortisol alone, though fluoxetine treatment did rescure avoidance learning in the Delay condition. The significant deficits produced by fluoxetine in blocks two and three are suggestive of a specific negative influence on temporal memory.

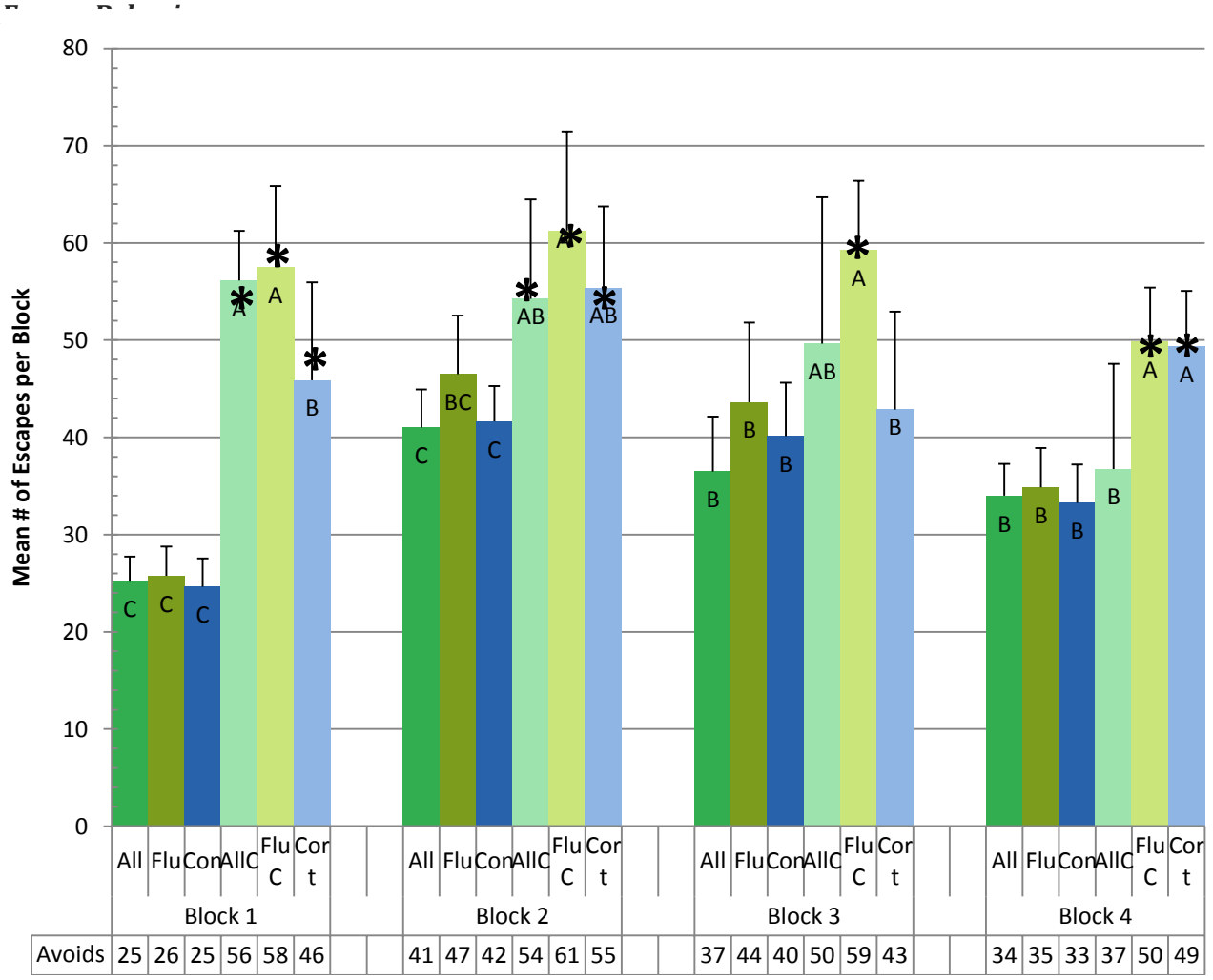


FIGURE 24. ESCAPE MEANS, TRACE CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was a significant overall effect of treatment on Trace escape performance, $F(5, 45) = 44.54$, $p < .0001$; block, $F(3, 45) = 21.87$, $p < .0001$; and an interaction between block and treatment, $F(15, 45) = 4.69$, $p < .0001$. Testing with repeated measures reveals that block one is significant when evaluated by treatment, $F(5, 45) = 49.30$, $p < .0001$; block two is significant, $F(5, 45) = 8.79$, $p < .0001$; block three is significant, $F(5, 45) = 5.28$, $p = .0008$; and block four is also significant, $F(5, 45) = 11.76$, $p < .0001$.

Significant Pairwise Comparisons

Tukey's HSD indicates a significant difference from control (*M* = 24.63, SD = 2.92) in the number of escapes in block one by allopregnanolone-cortisol (*M* = 56.13, SD = 5.11), fluoxetine-cortisol (*M* = 57.50, SD = 8.37), and cortisol (*M* = 45.88, SD = 10.08); cortisol is also significantly lower than both allopregnanolone-cortisol and fluoxetine-cortisol. In block two, allopregnanolone-cortisol (*M* = 54.25, SD = 10.22), fluoxetine-cortisol (*M* = 61.29, SD = 10.18), and cortisol (*M* = 55.38, SD = 8.37) remain significantly higher than the control, though cortisol is no longer statistically significant when compared to other cortisol-inclusive treatments. By block three only fluoxetine-cortisol (*M* = 59.29, SD = 7.11) possesses significantly greater escapes than the control (*M* = 40.13, SD = 5.49). In the final block, both cortisol (*M* = 49.38, SD = 5.71) and fluoxetine-cortisol (*M* = 49.86, SD = 5.55) produced significantly more escape behavior than the all other groups.

Significant Pairwise Comparisons

Initially all of the hypercortisolemic fish demonstrate significantly higher escapes and this trend is attenuated as the other treatment groups increase in escape behavior and all but fluoxetine-cortisol decline in escape reactions. By block four, allopregnanolone-cortisol (*M* = 36.75, SD = 10.81) is grouped with the control group (*M* = 33.25, SD = 3.96) and significantly lower than both fluoxetine-cortisol (*M* = 49.86, SD = 5.55) and cortisol (*M* = 49.375, SD = 5.71). It is also fascinating that cortisol (*M* = 42.88, SD = 10.06) is not significantly deviant from the control group (*M* = 40.13, SD = 5.49) in block three and significantly lower than fluoxetine-cortisol (*M* = 59.29, SD = 7.11) and then rises to significantly eclipse the control (*M* = 36.75, SD = 3.96) in block four. This increase is stark in light of the general trend toward decreased escape after block two, defied by both this increase and fluoxetine-cortisol's nearly constant escape performance which is both the highest in each block and significant over the control at all levels.

Conclusions

Since nearly all cortisol-treated groups displayed significantly higher avoidance over the control in nearly every block, this is strong evidence to rule out changes due to motor impairment or lethargy.

Failure Behavior

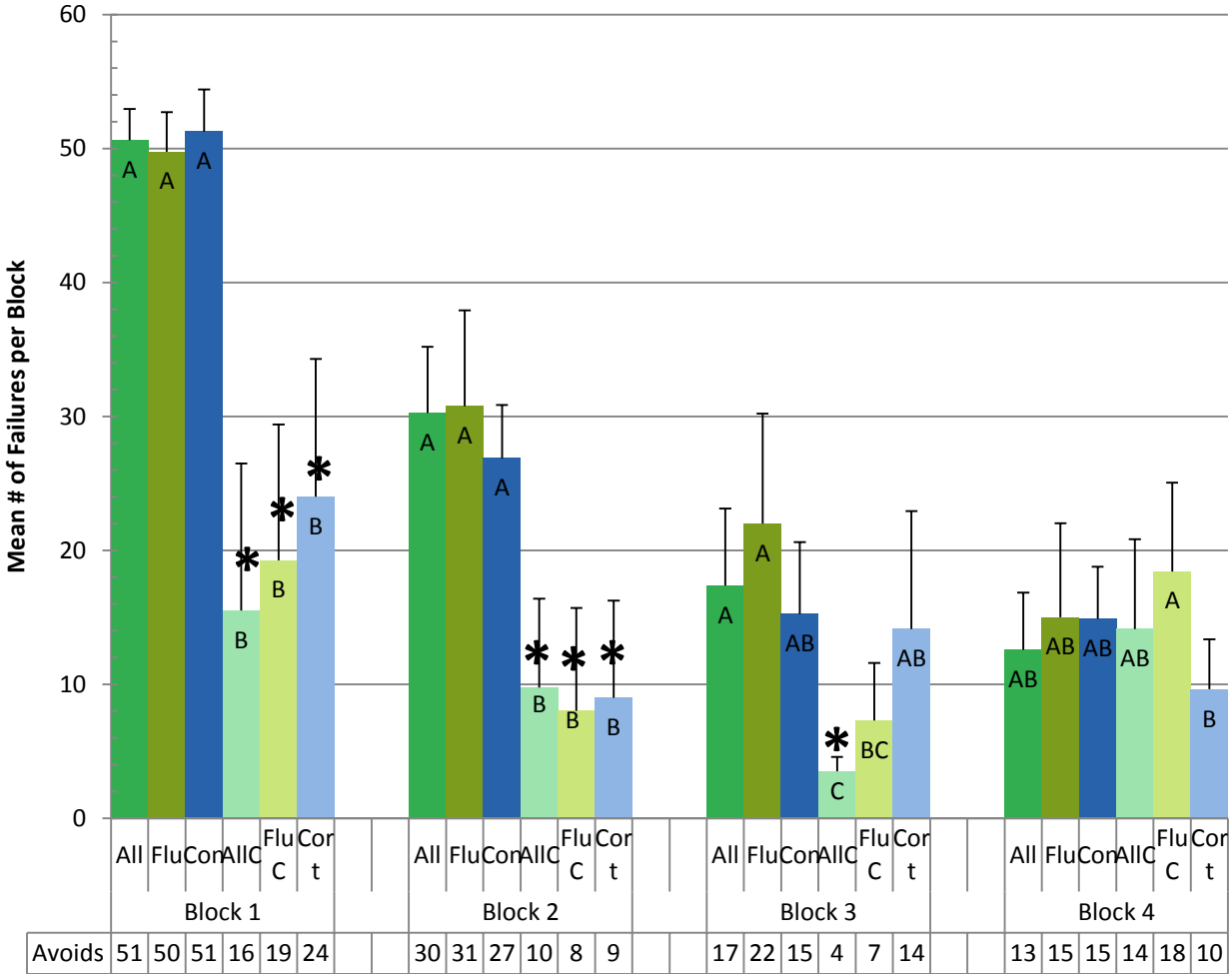


FIGURE 25. FAILURE MEANS, TRACE CONDITIONING. TREATMENT GROUP MEANS ARE DIVIDED BY BLOCK AND COMPARED TO THE CONTROL FOR SIGNIFICANCE WITHIN EACH BLOCK, INDICATED BY ASTERISK. PAIRWISE COMPARISONS ARE REPRESENTED VIA LETTERED GROUPINGS. ERROR BARS SIGNIFY STANDARD DEVIATION.

There was a significant overall effect of treatment on Trace failures, $F(5, 45) = 72.78$, $p < .0001$; block, $F(3, 45) = 98.05$, $p < .0001$; and an interaction between block and treatment, $F(15, 45) = 10.04$, $p < .0001$. Testing with repeated measures reveals that block one shows signifi-

cance, F(5, 45) = 36.22, $p < .0001$; block 2 is significant, F(5, 45) = 23.41, $p < .0001$; block three is significant, F(5, 45) = 9.24, $p < .0001$; and block 4 is not significant, F(5, 45) = 2.09, $p = .0879$.

Significant Pairwise Comparisons

Tukey's HSD deems allopregnanolone-cortisol (M = 15.50, SD = 10.98), fluoxetine-cortisol (M = 19.25, SD = 10.15), and cortisol (M = 24, SD = 10.28) statistically significant compared to the control in block one. In block two, allopregnanolone-cortisol (M = 9.75, SD = 6.65), fluoxetine-cortisol (M = 8.00, SD = 7.70), and cortisol (M = 9.00, SD = 7.25) remain significantly lower than the control (M = 26.88, SD = 3.98) and all groups that did not receive a glucocorticoid. By block three, allopregnanolone-cortisol (M = 3.50, SD = 1.07) drops to the minimum value for all mean failures in both conditions, significantly lower than the control (M = 15.25, SD = 5.37), cortisol (M = 14.13, SD = 8.81), and the anxiolytic groups. In block four no treatments significantly differed from the control (M = 14.88, SD = 2.91).

Nonsignificant Pairwise Comparisons

There is a very high rate of failure in the control and anxiolytic-only groups in block one that decreases steadily over time; a similar decline occurs from blocks one to three in all other treatments. By block four there is not only the absence of significant deviation from the control (M = 14.88, SD = 3.91), but all groups present equivovally excepting the significant difference between the greater rate of failure in fluoxetine-cortisol (M = 18.43, SD = 6.63) than cortisol (M = 9.63, SD = 3.74).

Conclusions

The Trace condition exhibits opposite effects of failures as observed from the Delay condition, with significanty fewer failures in those fish who were trated with cortisol versus those who were not. This is coupled with stronger findings indicating increased escape behavior over the other groups and relatively equivalent avoidance performance. This suggests that the

cortisol-implanted fish were energetic but incapable of establishing the CS-UCS association until later blocks.

Observations

Notably, all of the cortisol-only fish (16 of 16) remained chronically infected throughout all trials whereas a minority of fluoxetine-cortisol (5 of 16) and almost none of allopregnanolone-cortisol (2 of 16) contracted disease. While some of the 48 fish of the non-cortisol receiving groups presented with illness, the ailments were mild and most (45 of 48) required no further treatment than was given during daily testing. Regardless of group treatment, the rates of mortality throughout the entire experiment were nearly equivalent: one from each drug treatment group in the Delay condition and three from different treatments the Trace condition (in the Allo, Flu, and Allo+Cort schedules) expired before the completion of block 4.

Observation of the fish in their home tank revealed no evidence of enduring hyperactivity or hypoactivity. Immediately after injections there was a profound sedative action of allopregnanolone injection that bordered on anesthesia, which has been accomplished at much higher doses in rats (Singh et al, 2010), and a mild, inconsistent (five of sixteen subjects) increase in locomotive activity following cortisol-only injections. Both of these effects were transient and the latter may be attributable to injection/handling/restraint stress, but the former was consistent (fourteen of sixteen exhibited strong sedation) and persisted for approximately five minutes after injections, after which time the fish proceeded to swim and eat per baseline. Since the scototaxic procedure was carried out the day after injections, the fish were allowed ample time to rest before experimentation; it is worth considering that this possible locomotive impairment did not affect the animals during the procedure given the allopregnanolone group's significantly higher number of boundary crosses compared to all but the fluoxetine group.

Discussion

The results of the present experiment further support the findings of previous work in our laboratory indicating significant impairment of avoidance learning in goldfish by water-borne fluoxetine administration (Beulig and Fowler, 2006). Though these trends were present in both conditions, they only achieved significance in the Trace subset; this is likely due to the smaller group size used in this experiment compared to the previous investigation, rendering the present study less sensitive to this effect. No significant affective changes in fish treated with fluoxetine nor fluoxetine plus cortisol were observed, though fluoxetine was the only group to exhibit an effect similar to allopregnanolone in the tests for scototaxic behavior. This conflicts with previous studies in rats and humans, though the scototaxic trials only tested acute affective differences and there is a several week delay before antidepressant changes appear in other organisms (Zhang et al, 2000; Wilde and Benfield, 1998; Tome et al, 1997).

The learning impairment, spatial cognition deficit, and conditioned place aversion caused by allopregnanolone in rodents and memory dysfunction in humans was not observed in the goldfish (Silvers et al, 2003; Johansson et al, 2002; Beauchamp et al, 2000; Kask et al, 2008). In the present experiment, the allopregnanolone group alone exhibited no significant difference from the control nor did the allopregnanolone-cortisol cohort. In fact, the allopregnanolone group held the highest mean avoidances in both conditions by the end of the study and allo-pregnanolone-cortisol demonstrated significantly fewer failures in both conditions. The signifi-cant effect of allopregnanolone in both the difference in duration of time spent in the light chamber and the number of transitions made during the scototaxis procedure supports previous findings indicating its effeciveness as an acute anxiolytic (Engin and Treit, 2007). It may be sug-gested that the persistence of this effect from humans to rodents to teleosts supports its modus operandi via the highly conserved $GABA_AR$ (Brot et al, 1997).

Cortisol produced no significant effect in avoidance conditioning as is suggested by previous literature (Beatty and Beatty, 1970) despite the expectation that cortisol might enhance emotional memory consolidation via BLA activation (Buchanan and Lovallo, 2001) or that it may lead to deficits in declarative memory via hippocampal dysfunction (Kirschbaum et al, 1996). The net effects of cortisol on cognition are still not resolved in many taxa (Het et al, 2005). After a period of 16 days the fish which received cortisol or fluoxetine-cortisol performed significantly worse at Trace avoidance and the cortisol group proved to be the only significant difference from control in the Delay condition for avoidance behavior. Indeed, for cortisol explicitly, there was a significant decline in avoidance behavior in the final block of conditions that was almost exclusively associated with an increase in failures in the Delay condition and with escapes in the Trace paradigm. (These differential effects may be due to the greater innate stress of the Delay condition, which may require an action to avoid/escape as often as every 25 seconds, as compared to the Trace condition which permits additional five seconds per round, generally increasing the total run time from two to four minutes over the Delay condition. The additional stress of this paradigm is evidenced by the increased deaths in the Delay condition, in which six subjects died while only three expired during the Trace training.)

The results of the Trace condition support the hypothesis that the long-term effects of cortisol impair spatial/temporal learning possibly due to hippocampal dysfunction, as is well-represented in previous work in mammals (Lupien et al, 1998; Starkman et al, 1992). The decreased performance of in Trace avoidance in all cortisol regimens but the allopregnanolone-cortisol group implies that allopregnanolone successfully rescued avoidance aquisition in this condition.

While cortisol exhibited no significant difference in the scototaxis procedure, it exhibited the highest variance of all groups, implying differential effects which may be related to sex or age differences in the subjects (Wolf et al, 2001; Buchanan and Tranel, 2007).

Interpretations

Within the five days following injections, no significant effects are reported for any of the compounds. This is somewhat surprising given allopregnanolone's reputation for acute benzodiazepine-like effects (Brot et al, 1997) and fluoxetine's occasional association with hyperactivity at high doses (Riddle et al, 1990), but in line with behavioral observations which suggested no such effects except transiently after injections.

While it is worth noting that those groups which received cortisol performed better in general than those who did not in the first two blocks, there is also a startling degree of variability that precludes a stronger effect. Given that it is necessary that the formation of the UCS-CS association depends on the serendipitous crossing of the hurdle, hyperactivity or greater arousal might be considered advantageous (Carpenter and Summers, 2008; Brown, Laland, and Krause, 2007); this is very possibly the reason for the higher early performance of the cortisol groups as well as the higher variability, indicating perhaps that an association was not made but rather a greater likelihood of "accidental" avoidances.

This, in turn, could lead to sooner association of the stimuli, perhaps enhanced by the consolidating effects of HPI arousal via the BLA. This is supported in the Delay condition in which there are a higher number of initial avoidances than in the non-cortisol groups for blocks one and two, with all of the groups barring cortisol exhibiting nonsignificantly differences by block four. The final block revealed the only significance in the Delay condition in which cortisol's variance decreased while their mean also fell from the previous block. This is the predicted effect of long-term cortisol elevation: impaired memory and increased helpless behavior. Given that the

cortisol-anxiolytic groups rated very similarly to one another and the non-cortisol groups in the delay condition, it might be suggested that both allopregnanolone and fluoxetine proved effective in ameliorating the exogenous stress imposed by the cortisol implant.

The Trace condition, designed to demonstrate the coordination of hippocampal and amygdalar function in the fish, shows significant impairment of acquisition of the fluoxetine in blocks two and three compared to the control group. This is paradoxical given that fluoxetine has been shown to improve hippocampal function in patients suffering from depression as well as laboratory mammals: instead, the treatment including both fluoxetine and cortisol performed the worst in the final block when the compromising effects on the hippocampus should be most pronounced. The consistent and profound impairment of shuttle-box avoidance training through fluoxetine administration has been demonstrated previously several times in our laboratory with both goldfish and pinfish (Beulig and Fowler, 2008; others unpublished), though the Trace task had not yet been utilized for goldfish dosed with fluoxetine. The fluoxetine group's tremendous improvement from block three to four may be compared to the fluoxetine-cortisol group's contemporaneous failure to improve: perhaps the drug was thoroughly metabolized by the end of block three. If this were the case, the low-mean, low-variation fluoxetine group may have been liberated of cognitive impairment or affective helplessness allowing more optimal performance on the already acquired task; indeed, up to par with their conspecifics. The moderately-low mean, high-variance fluoxetine-cortisol group might have been benefitting from the hyperarousal provided by the cortisol without actually successfully establishing the UCS/CS connection due to the handicap posed by fluoxetine; as the active influence of the fluoxetine waned, the variance diminished but without a larger mean which in turn was dwarfed by the improvement of all other groups.

In the first ever analysis of fluoxetine metabolism in fish hepatocytes, Smith and colleagues document the clearance rates of four young goldfish hepatic microsomes when incubated with either 45 µg/L or 80 µg/L for either 30 or 60 minutes, respectively (2010). Using their limited data, it is calculated that would be possible for a single young goldfish liver to metabolize about 1092 µg/L/day, with substantial variance (SD = 1401) that limits the applicability of this data. While this *in vitro* measure reveals itself to be rather inconsistent, it does establish that Carassius is capable of digesting fluoxetine to a small extent to its active metabolite norfluoxetine, though this value is much lower than it is in mammals (about 2% total metabolism versus nearly 100% in clinical applications). The induction of the CYP family of hepatic enzymes was also observed, as is consistent in humans, and this may significantly increase the metabolism during chronic treatment. While fluoxetine has been found to be especially hepatotoxic to rainbow trout hepatocytes, the study in question employed what would be nearly lethal concentrations. This does not permit easy comparison due to the *in vitro* methodology, and employs a different teleost species (Laville et al, 2003); regardless, it is possible that hepatotoxicity hampered the ability of the fish to metabolize fluoxetine and possibly generated complicating factors that impaired avoidance performance.

Other explanations for the profound deficits in avoidance performance exhibited by the fluoxetine groups may arise from a mechanism of action specific to fish. Mennigen and colleagues have performed numerous studies to elucidate the effect of fluoxetine on fish due to the increasing ecological presence of the drug from wastewater effluent; indeed, some surveys have indicated that fluoxetine is present in rivers in the USA and Canada at levels up .54 µg/L Metcalfe et al, 2003; Brooks et al, 2003). What is more alarming is that fluoxetine bioaccumulates to a moderate extent in the nervous tissues, skeletomusculature, and liver (Nakamura et al, 2008; Mennigen et al, 2011); in a study utilizing only slightly less fluoxetine than the present,

.64 µg/L over seven days with 21 days of fresh water to detoxify, found a half-life of 9.4 ± 1.1 days with possibly longer accumulation of norfluoxetine in the Japanese medaka, *Oryzias latipes* (Paterson and Metcalfe, 2008). This indicates the potential for long-lasting effects in our experimental animals, though the cognitive effects may normalize over time or diminish due to declining environmental concentrations.

It has also been demonstrated that significant dysfunction of the male and female reproductive axes in goldfish is induced by low doses of fluoxetine (Mennigen et al, 2010), a fascinating finding considering the SSRI's potential role to promote GtH response via 5HT (Somoza et al, 1988). Fluoxetine may also effect nitrogen disposal in low doses to marine, the possibility of which suggests interaction with HPI axis, though the study was improperly designed to arrive at such a conclusion (Morando et al, 2009).

Fluoxetine has consistently deprecated avoidance performance in teleosts, but has been considered to improve cognitive function in rodents and people (Flood and Cherkin, 1987; Nowakowska et al, 1996). While older antidepressants deliver their benefits with significant impairments to memory, fluoxetine and other SSRIs were the first to ameliorate this side-effect which was thought to be associated with anticholinergic effects (Kumar and Kulkarni, 1996). Since it well known that fluoxetine increases synaptic plasticity in diverse areas of the brain in both neurons (Stewart and Reid, 1999; Vetencourt et al, 2008; Ampuero et al, 2010; Alme et al, 2007) and glia (Czeh et al, 2005), it is not surprising that fluoxetine would modulate certain forms of learning and those who find it reasonable that hippocampal neurogenesis is requisite for learning would find fluoxetine to be an attractive agent for improving hippocampal function (Li et al, 2009). Despite expectations, it has been found more consistently that fluoxetine hinders spatial and temporal tasks in rodents and that hippocampal neurogenesis does not provide

neural substrates for spatial learning (Satvat et al, 2011; Spankswick et al, 2007; Majlessi and Naghdi, 2002). Indeed, nearly all of the affirmative studies for fluoxetine's benefits to learning arise from models of traumatic injury, a very different circumstance than MD illness or chronic hypercortisolemia. Fluoxetine has also been shown to negatively affect spatial learning and increase anxiety irreparably in adolescent rats (Sass and Wortwein, 2011); in adolescent humans, fluoxetine treatment has been correlated with enduring memory problems (Bangs et al, 1994). Specific to avoidance conditioning, fluoxetine has been shown to hinder acquisition in rats independent of sensory alterations (Nelson et al, 1997).

Fluoxetine has also been shown to suppress circulating estrogen, perhaps through the redistribution of steroidogenic products toward neurosteroids such as allopregnanolone, which suppressed the recovery of ovariectomized rats with estrogen-replacement on a measure of spatial memory (Taylor et al, 2004). Both sex and age have are known to be important predictors for SSRI response, likely due to the variability of glucocorticoid receptor profiles (Joyce et al, 2003). Fluoxetine has also been shown to affect the binding of MRs but not GRs in the rat hippocampus, pituatry, hypothalamus, and prefrontal cortex, thus altering the MR/GR ratio (Elakovic et al, 2011); it is possible that these sexually dimorphic findings contributed to variable findings in the present subjects who were not sexed prior to the experiment.

Cortisol produced a deficit only over chronic exposure in both Delay and Trace conditions. All groups other exhibited a significant improvement in avoidance performance over both fluoxetine-cortisol and cortisol alone in the final block of the Trace condition. Fluoxetine in turn proved to be significantly worse than allopregnanolone in blocks two and three of the trace task, ultimately worse than no anxiolytic treatment to ameliorate the degenerative effects of cortisol on shuttle-box avoidance. This data, coupled with modern research on these compounds, is

highly suggestive of allopregnanolone's endogenous role in regulating the diverse organisms' response to allostatic states regulated by chronic hypercortisolemia.

Indeed, it has been shown that previous use of anxiolytic drugs (including SSRIs) is the greatest predictor of relapse in MD (Claxton et al, 2000). SSRI withdrawl, or discontinuation syndrome, is a challenging and well-known phenomenon with risks often greater than the initial depressive episode (Haddad, 1998). Some have even suggested fluoxetine as a treatment for SSRI withdrawal syndrome given the relatively mild severity of discontinuation compared to other SSRI-withdrawal symptoms (Bennzi, 2008), implying, perhaps, even more severe hippocampal dysfunction is associated with the use of other SSRIs during periods of chronic allostasis.

Possible Confounds

Goldfish have been shown via miRNA analysis to exhibit extensive neuroendocrine changes based on breeding status and seasonality (Zhang et al, 2009). While the issue of seasonality is controlled due to contemporaneous testing, the fish were not evaluated for maturity though the similar size affords some estimation of aging status. The former issue may lead to issues of replication whereas the latter was likely accounted for during randomization.

As noted above, the fish were not sexed prior to experimentation. The significance of sex in cortisol, allopregnanolone, and fluoxetine response has been noted already. These effects no doubt translate to the fish model as well as the utilization of these compounds corresponds to their mammalian counterparts, which raises concern over disproportionate response within groups which may have had an imbalance of one sex over the other. This is a legitimate concern given the small group sizes, though the differential response to cortisol and allopregnanolone are likely to be less significant at the high exogenous dosing schedule employed in this study. It has been shown that there is a sex-dependent effect of stress on MR/GR ratio in rats (Kitraki et al, 2004). This is at least partly mediated by changes in the concentration of circulating CBG due

to the the actions of estrogens and the glucocorticoid antagonizing effects of progesterone, which have been documented in both rats and humans (Young, 2008); the absence of an obvious CBG in teleosts implies that the mechanisms of sex-specific control may be regulated otherwise or to less an extent. Allopregnanolone's anxiolytic dose divergence across sex is likely not to be the product of $GABA_AR$ modification or regulation (Zimmerberg et al, 1999), indicating that the most likely conserved mode of action for fish to mammals is unaffected and implying similar acute effects as has been observed in this experiment.

Illness in the cortisol group remained a consistent problem throughout testing, leading to several "sick days" and adjunctive antibiotic therapy. While poor health or medical intervention may have hampered the fish's cognitive abilities, this is not obvious from the data. The cortisol group exhibited consistently high avoidances in both Delay and Trace paradigms until block four, by which the fish were in excellent relative condition. Furthermore, the cortisol group consistently had higher escapes and fewer failures: raking across conditions, the cortisol-infused fish scored significantly higher than the control in five blocks of escapes and significantly lower in four blocks of failures. The other cortisol-implanted groups were substantially more robust than their cortisol-only counterparts and exhibited similar trends.

As a last point, it is important to recognize that fluoxetine's antidepressant actions have come under recent controversy due to the analysis of previous unpublished negative findings.

Depression and SSRI Efficacy

In a groundbreaking meta-analysis by Kirsch and Sapirstein published in Public Library of Science in 2008, the authors concluded that the trials submitted to the FDA for the antidepressant drugs fluoxetine (a SSRI), venlafaxine (a SNRI), nefazodone (a weak SNDRI), and paroxetine (a SSRI) showed insignificant improvements in treating depression when the "unpublished" results are included. The FDA presently requires registration of all clinical trials

prior to their initiation, a practice in integrity that has yet to be implemented by the scientific community at large, though not all trials are required to be “published” in the sense of inclusion into the pending approval process despite all such registered trials remaining accessible to researchers. Not only did Kirsch and his contemporaries demonstrate that these drugs failed to produce significant decreases relative to baseline levels of depression in a within-subjects design, but that they also fail to improve Hamiliton Rating Score of Depression (HRSD) scores over placebo in all but the most extreme severity of baseline depression. Furthermore, these changes in severely depressed patients correlated more with declining response to placebo therapy than to changes in drug efficacy.

Fluoxetine (Prozac©) was once deemed “the miracle drug” for myriad psychiatric conditions, to date including six FDA-approved diagnoses and numerous off-the-label uses; it single-handedly reformed the cultural view of depression from a devastating illness that required similarly dramatic treatments (e.g., ECT, tricyclics, and insulin-induced comas) to a commonplace disease with excellent prognoses. Prozac quickly found itself embedded into the American lifestyle, and resulted in major depression disorder (MDD) diagnoses skyrocketing and encouraging psychiatrists to write scripts for a relatively benign pharmaceutical with “proven” effectiveness. Even nine years after Prozac's FDA approval in 1987, antidepressant use is still growing: from 1996 to 2005, total lifetime antidepressant use, the vast majority of which was with SSRIs, increased from 5.84% of the nation to 10.12% (Olfson et Marcus, 2009). Prozac specifically garnered approximately forty million patients worldwide by 2002 and sales grossed over 22 billion dollars in the United States alone before Eli Lilly, its father company, lost its patent exclusivity (Wong et al, 2005). These numbers have doubtlessly grown as the generic version of fluoxetine has made a cheap drug even cheaper, allowing for even greater accessibility.

The implications of any drug so widely used are obviously magnified. Resultantly, many controversial analyses of fluoxetine's efficacy and side-effects have arisen from the era even before its landmark release in 1987 (Wernicke et al, 1986; Benfielf et al, 1986). Research findings have been variable, but the most recent meta-analyses conclude that the majority of trials have produced either modestly significant advantage over placebo in cases of especially high severity or none at all; this, combined with selective publishing in favor of “positive” results, indicates an overall lack of efficacy over placebo (Kirsch et al, 2008; Turner et al, 2008; Barbui et al, 2011). Those meta-analyses which do not arrive at such conclusions are often very limited in scope and still maintain only detect advantages over other therapies (Arroll, 2005). Fluoxetine in particular, due in part to its pioneering role in the field, has been shown to be one of the most tested, yet least effective and most rejected of the modern SSRIs for MDD (Cipriani et al, 2009). Several scientists have even gone so far as to suggest unethical research practices and suppression of data as the cause of the the initial affirmative results and Phase III trials (Ioannidis, 2008). While this point is certainly disputable, it may be stated incontrovertibly that fluoxetine is not a particularly effective drug for MDD: at best, meta-analysis has found that there is a mere 8% difference over placebo and, at worst, there is no advantage to fluoxetine therapy at all (Kirsch et al, 2008).

The mechanism of action of fluoxetine in ameliorating depressive symptoms remains to be elucidated, it has historically been postulated that fluoxetine’s inhibition of SERT permits additional 5HT in the synaptic cleft (Fuller et al, 1991). While serotonin abnormalities have been observed in both humans and animal models of depression, there is no clear “deficiency” present in depressed brains (Tanti et Belzung, 2010).

Alternate mechanisms have been proposed, usually focusing on the modulation of trophic factors such as BDNF and allopregnanolone to provide anxioslysis or stimulate

neurogenesis (Schmidt, 2010; Alme et al, 2007; D'Aquila et al, 2010; Pinna, 2010; Shirayama et al, 2010; Rodriquez-Landa et al, 2009; Pibiri et al, 2008; Uzunova et al, 2004; Ugale et al, 2004; Akwa et al, 1999; Griffen and Mellon, 1999). The most conclusive evidence to date, however, reveals that only one of fluoxetine's enantiomers possesses the ability to improve symptoms of depression in rats, though both increase 5HT in the synaptic cleft; further research by Pinna and his colleagues also revealed that subclinical (i.e., too low to register a measurable change in synaptic 5HT) doses of fluoxetine can treat symptoms of depression in rats as well as clinical doses, prompting him to coin "SBSS" over the traditional SSRI for his alternate theory of fluoxetine action involving the stereospecific modulation of Selective Brain Steroids Stimulation (2009).

According to Pinna and his research team, brain steroids are responsible for the improvement of depression in Prozac treatment, as well as potentially other SSRIs. One particular steroid that has been identified, allopregnanolone as a neuroprotectant and potential therapeutic agent. Allopregnanolone is decreased in rats exposed to chronic stress (i.e., the depression model) and is likewise returned to baseline levels when these animals are treated with SSRIs. The model Pinna proposes is that allopregnenolone regulation is modified via fluoxetine's active enantiomer acting on 5α-steroid dehydrogenase, an enzyme that produces allopregnanolone in the brain. While this may eventually provide an explicit mechanism for fluoxetine therapies, it is emphasized that this would also explicate the indirect nature of the drug and account for its relative inefficacy compared to, for example, beta-blockers or any other non-empirically formulated drug-therapy. (Pinna et al, 2006)

The premise guiding the development of fluoxetine was based on the empirical findings supporting anxiolyitc qualities of adequate serotonin transmission and the, at that time, the presumed involvement of 5HT in mood disorders (Wong et al, 2005). While this is excellent

incentive for frontier research and, indeed, fluoxetine presents as an unprecedented tool for studying 5HT's role *in vivo,* this is a sticky situation for the clinician responsible for dispensing this drug without an adequate profile of its biological outcome. In pursuit of faster-acting pharmaceuticals, much of the research in MD is focued on means to expedite the two-to-eight week delay minimize some of the side-effects (Machado-Vieira et al, 2008). Shortcomings as a pharmaceutical aside, fluoxetine at the very least replicates the effect of a placebo andit would

A common problem in the employment this therapeutic drugs in psychiatry is the management of side-effect profiles.Some of the many side-effects that have arisen from the practice of empirical drug-discovery have revealed themselves to be present in fluoxetine therapy: nausea, anxiety, decreased libido, dry mouth, dizziness, diarrhea, other flu-like symptoms, rhinorrhea, insomnia, and weakness are the FDA-required minor side-effects listed on fluoxetine scripts; anaphylaxis, bizarre behavior, gastrointestinal bleeding, decreased coordination, hallucinations, suicidality, and aggressiveness are only a handful of the 36 less common, “severe” side-effects (Drugs.com, 2011). One particular side-effect of interest caused a sizable controversy surrounding Prozac in the early 2000's: pediatric suicide. A series of well-reported stories of adolescent violence, suicide, and bizzarre behavior led to the investigation of this potential side-effect in patients under 25 years of age; an excellent resource documenting this hype exists at www.SSRIStories.com, which provides citations to over 4,300 such reports in the media. This press, coupled with a series of investigations which returned with conclusive evidence of doubled suicidal ideation and attempt behavior compared to an inert drug, prompted the FDA to append a Black Box Warning on all fluoxetine prescriptions in 2004 (Simon et al, 2004). This necessitated a reevaluation of the drug in pediatric practices: the consensus of several respected publications is that SSRIs carry increased risks for children, but remain a viable treatment (Cohen, 2007). Precautions are merited, as described by Daniel Pine's guidelines for

SSRI therapy in children prior to the FDA acknowledgment, because of the increased vulnerability of children to side-effects and their increased response to placebo: the advantage over placebo is weak, at best, and the risk-reward ratio is correspondingly bleak for pediatric treatment (2002).

The original development of SSRI's such as fluoxetine was motivated by the desire to reduce the negative side effects of existing treatments for depression such as MAOI inhibitors, and to reduce broad-spectrum effects of others. The development of understanding the fundamental mechamnisms of anxiolysis in SSRI's may aid in the goal of increasing effectiveness and reducing side-effects.

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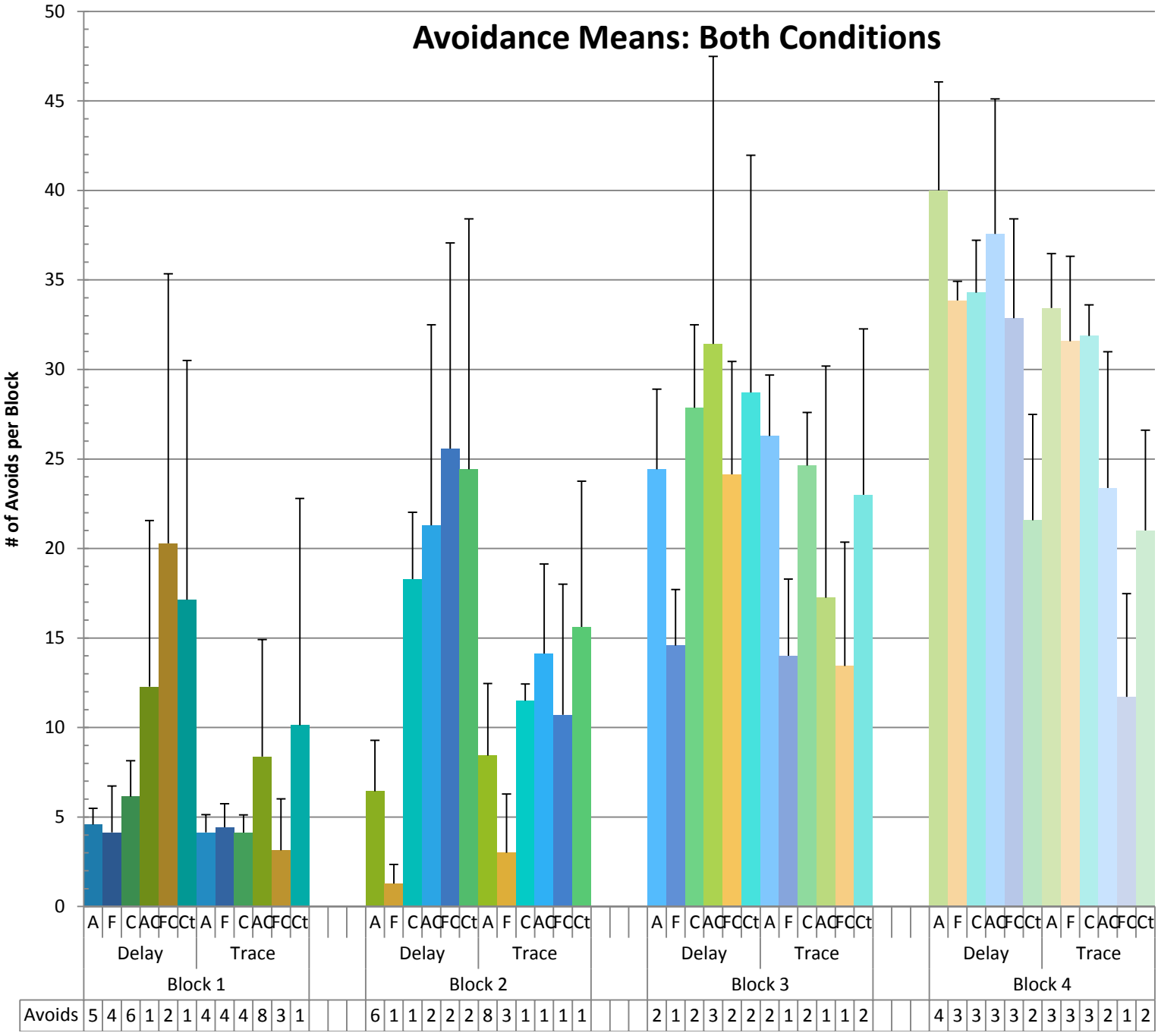
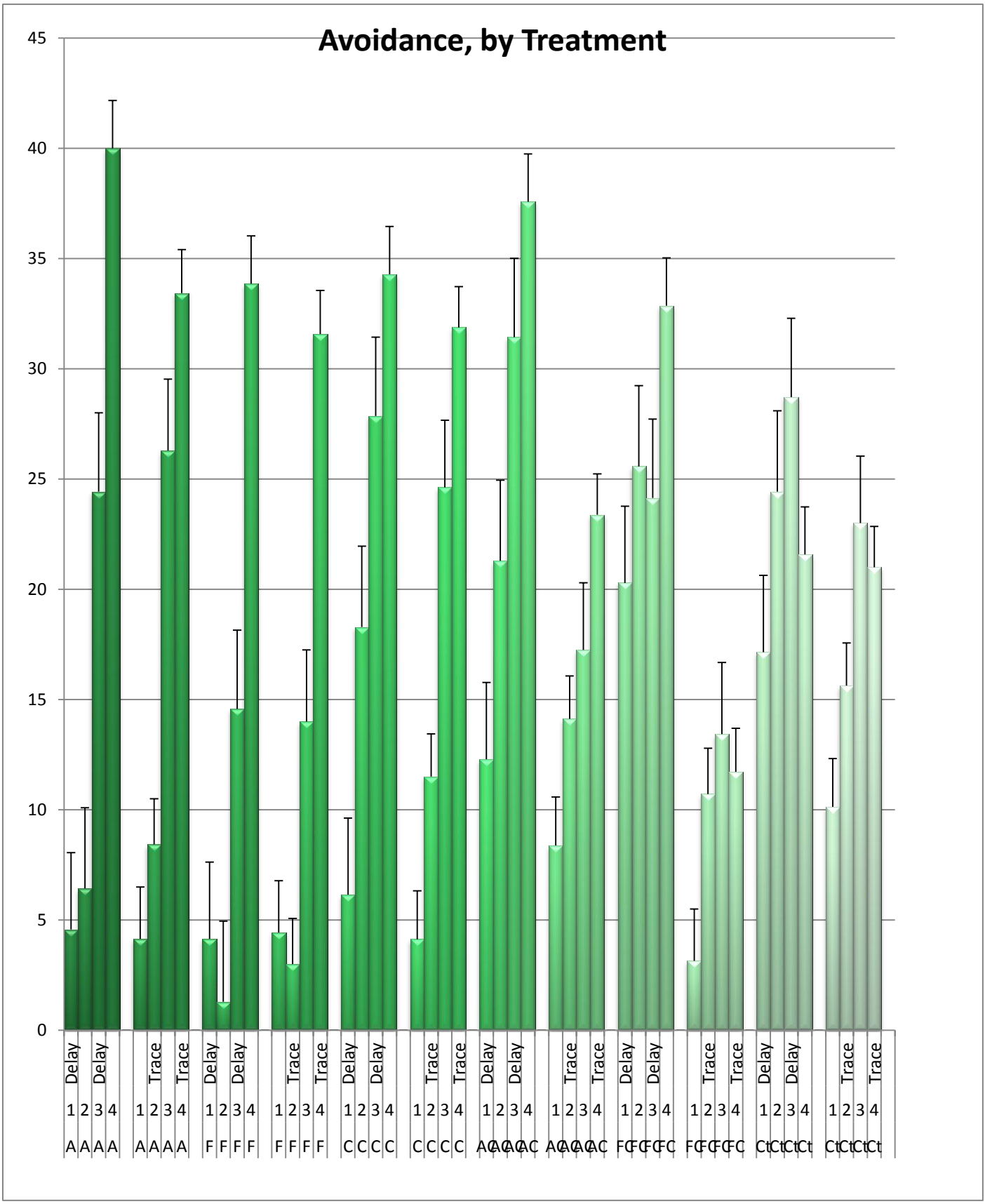
APPENDIX I:

Additional Graphs and Tables

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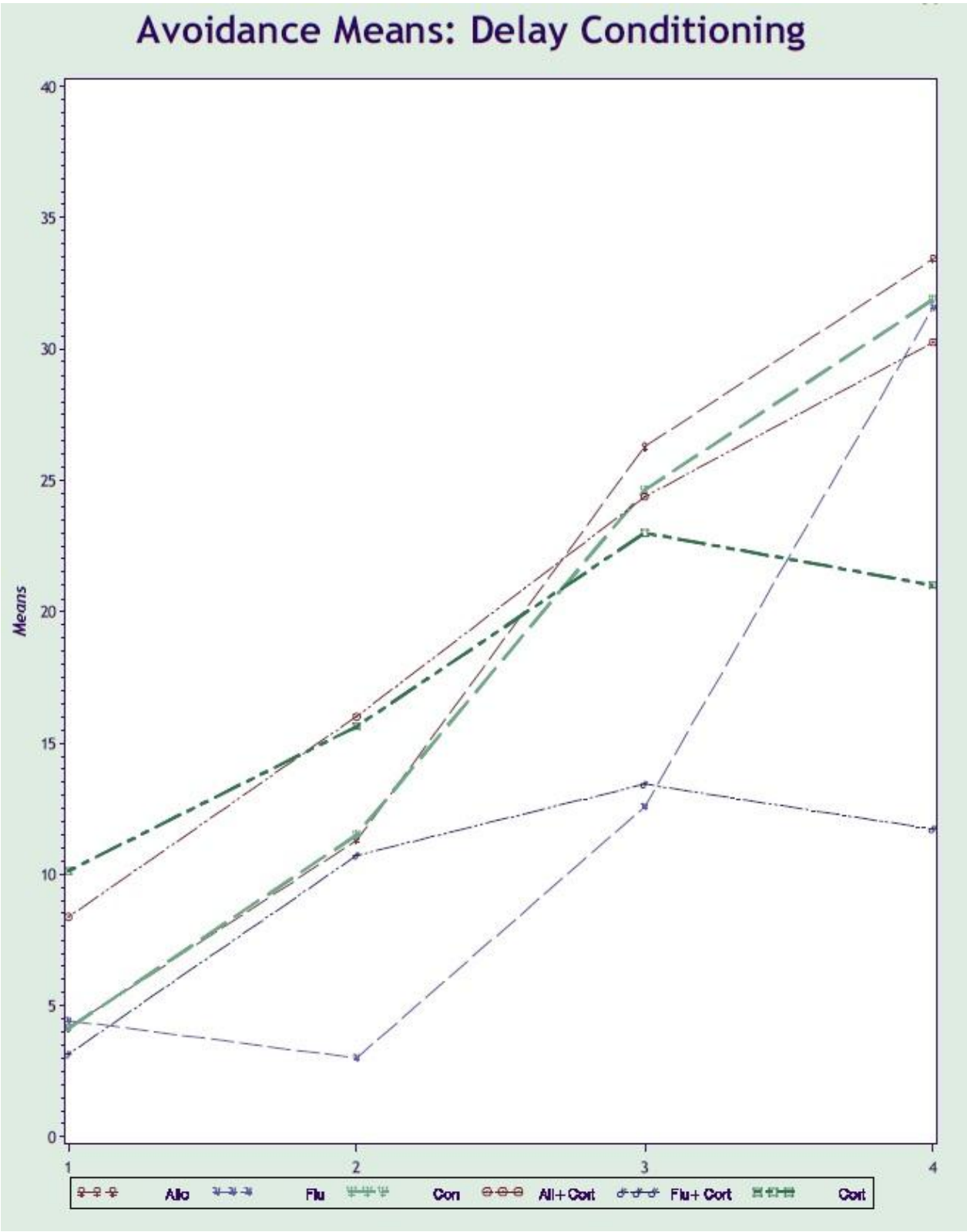
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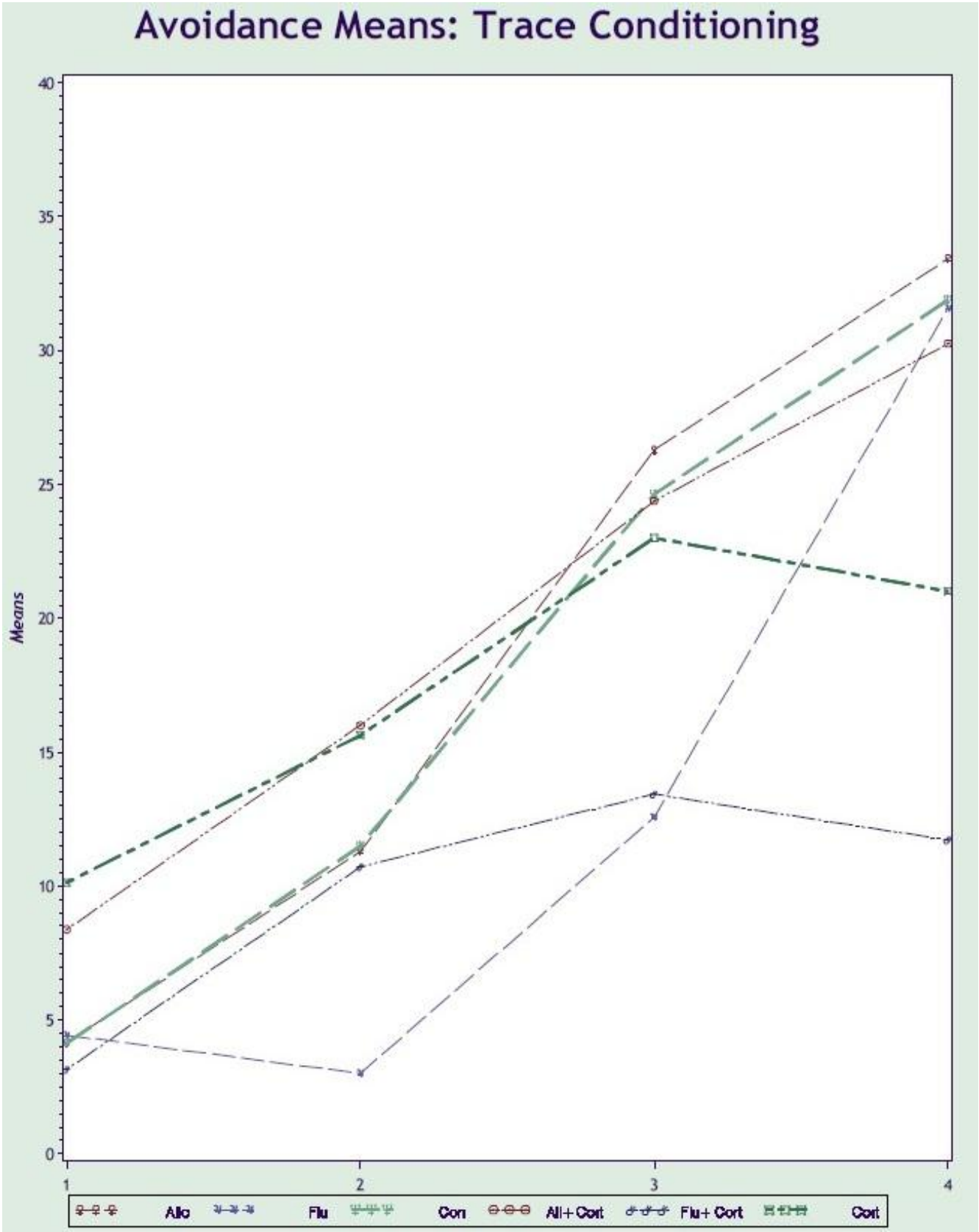
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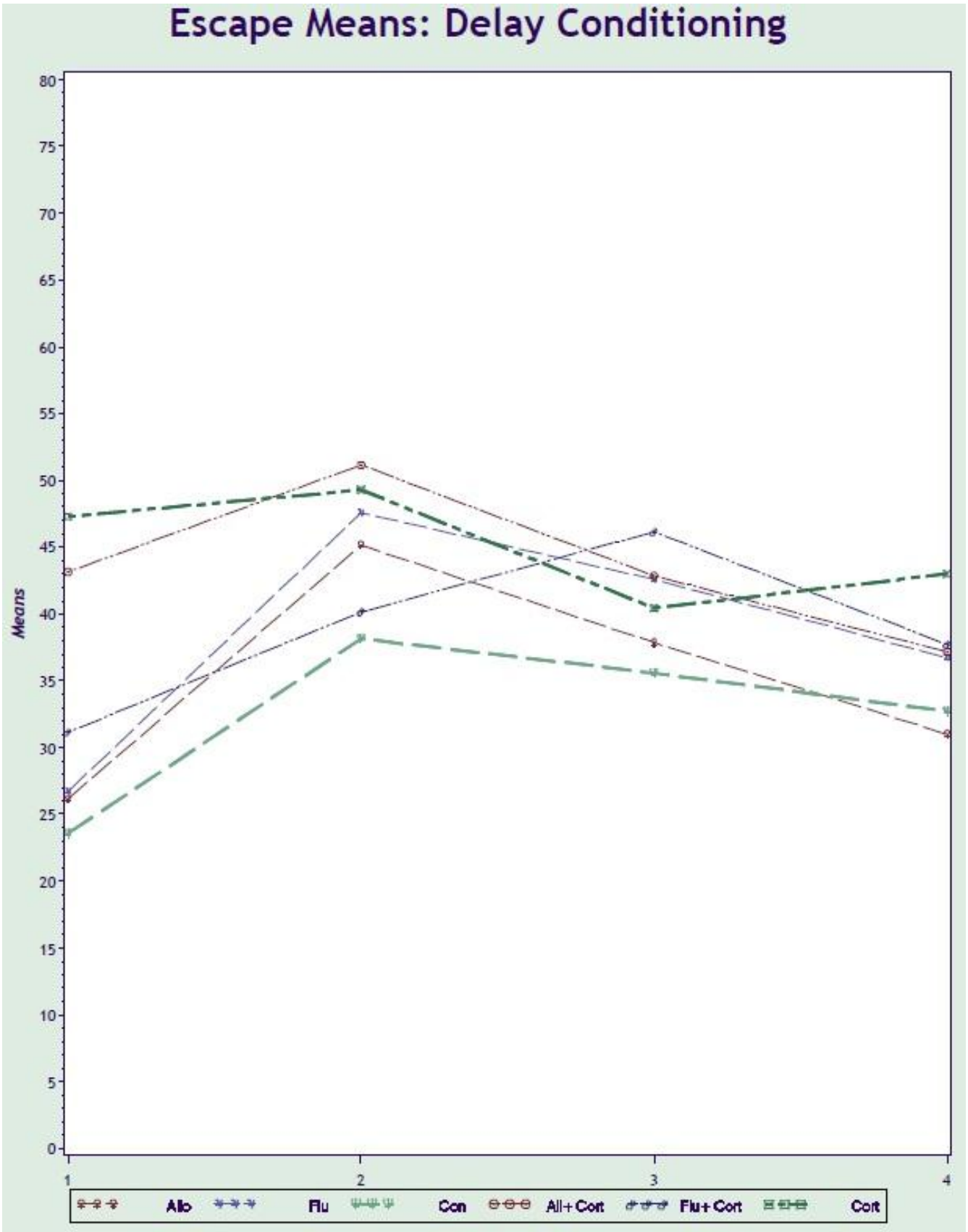
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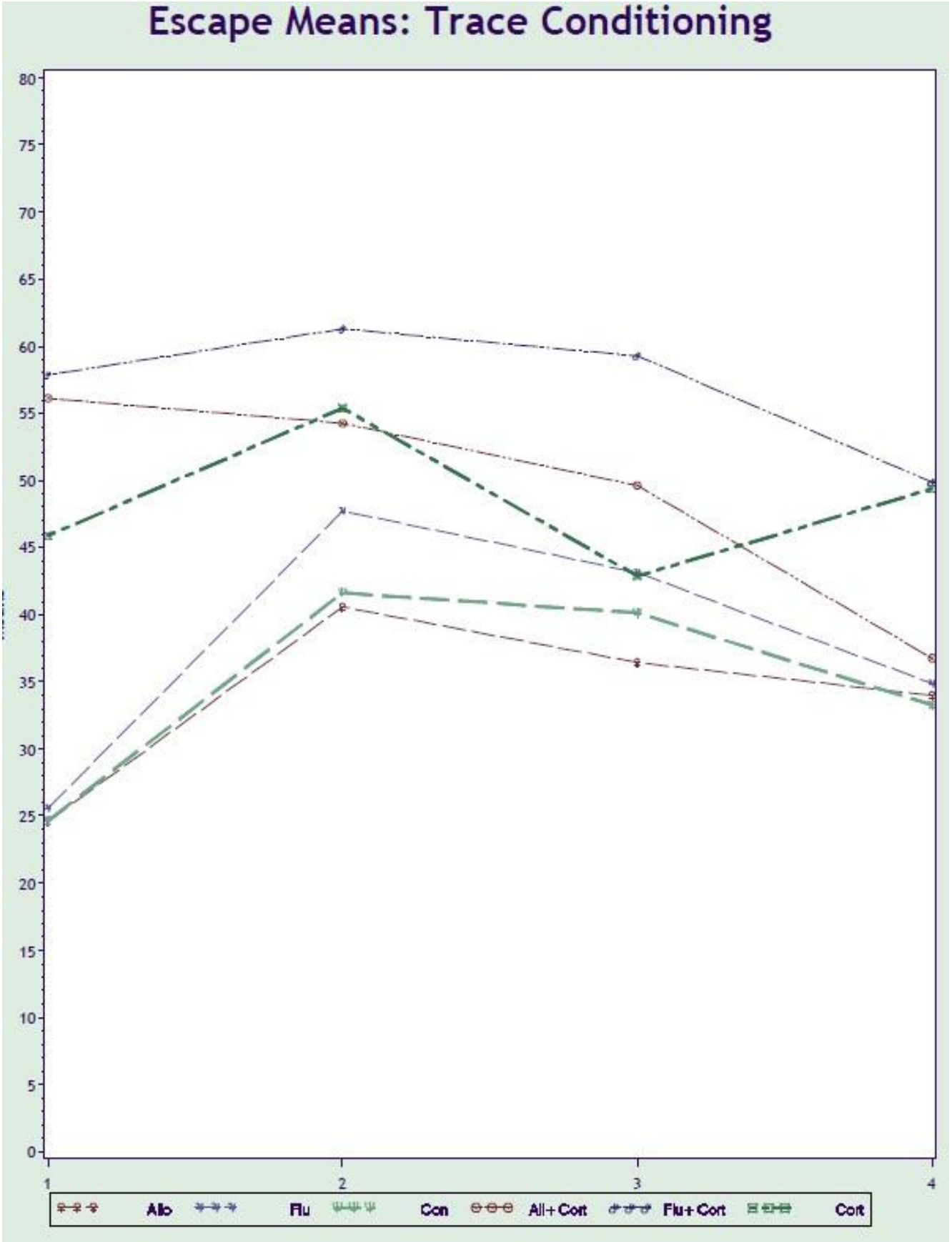
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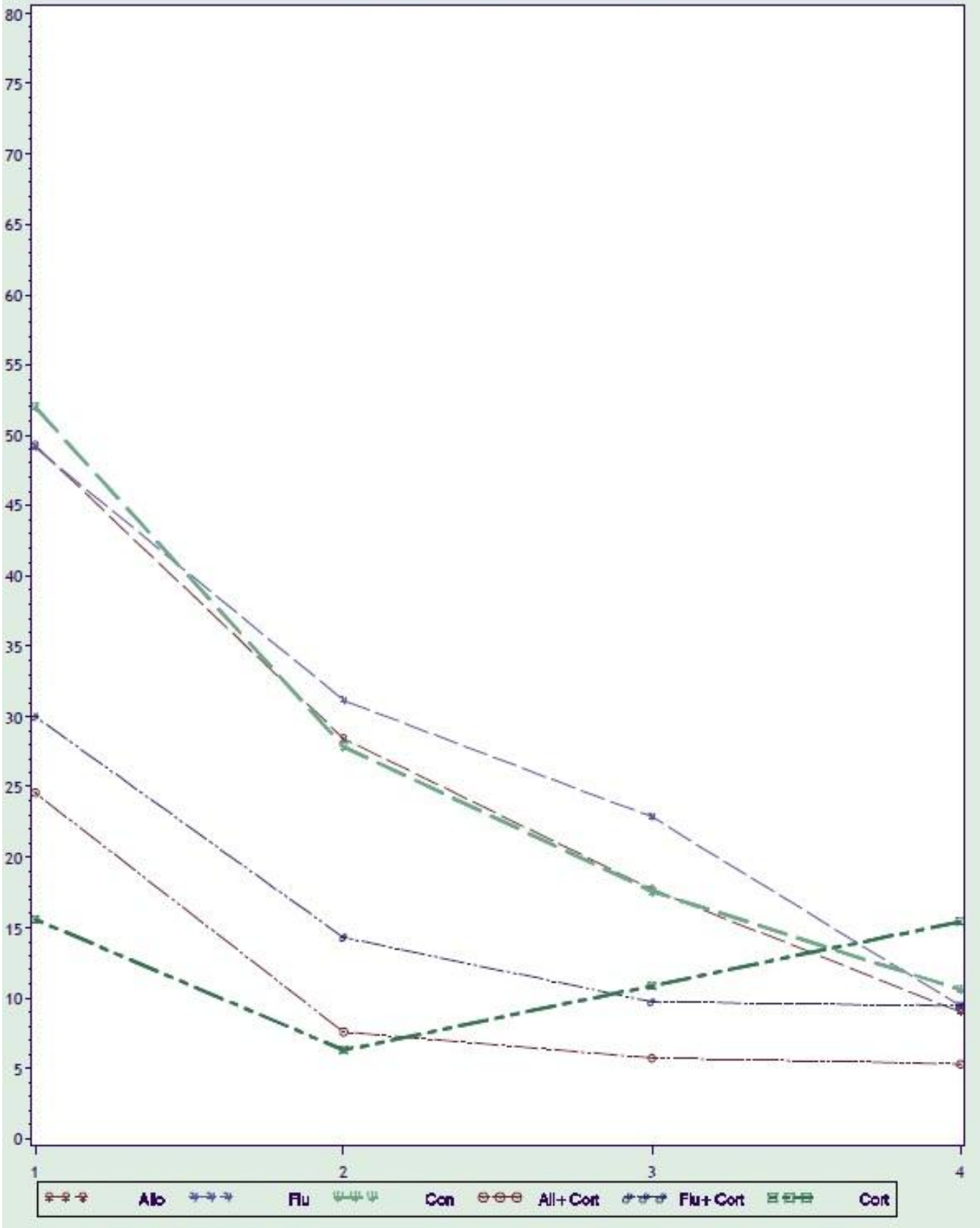

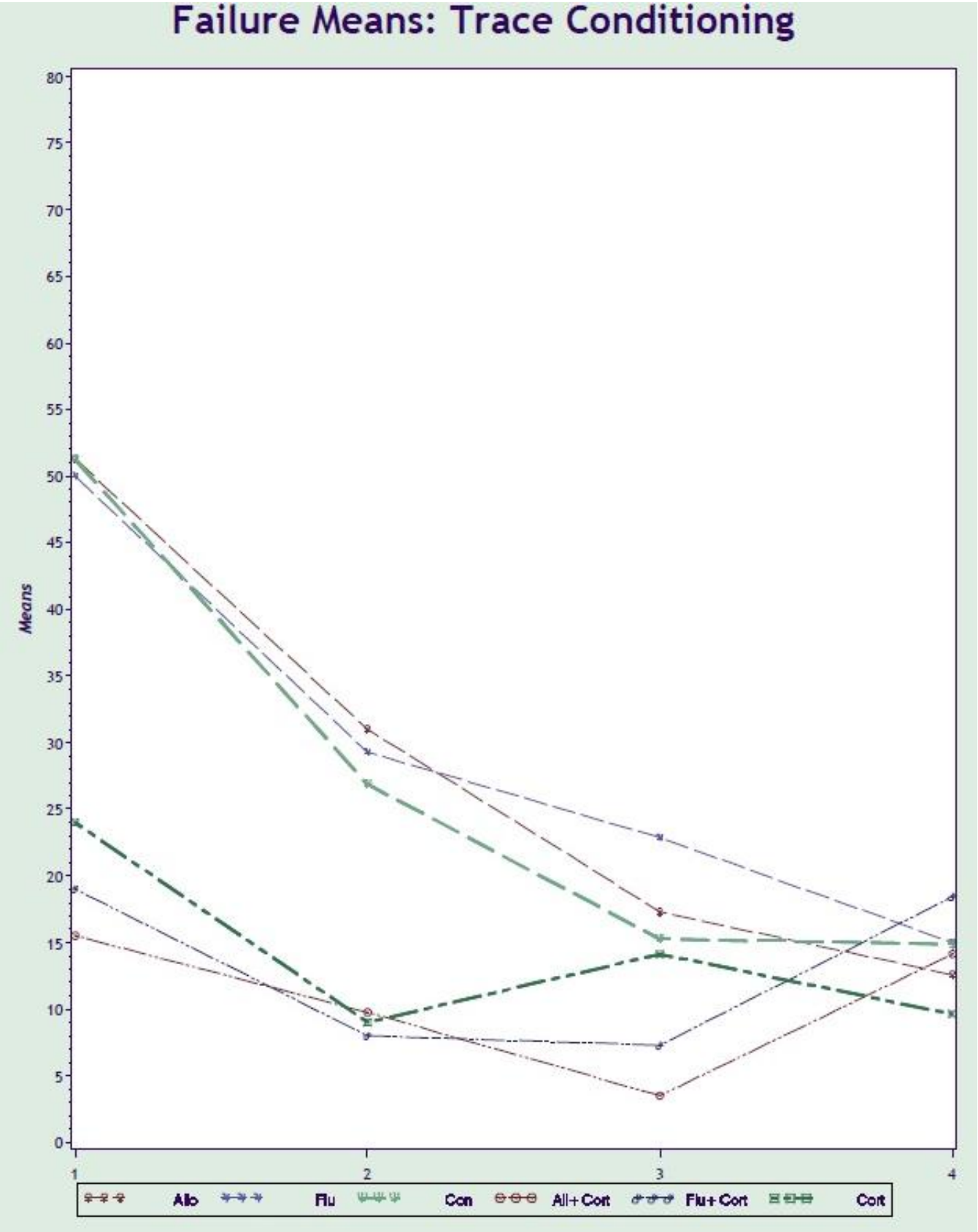
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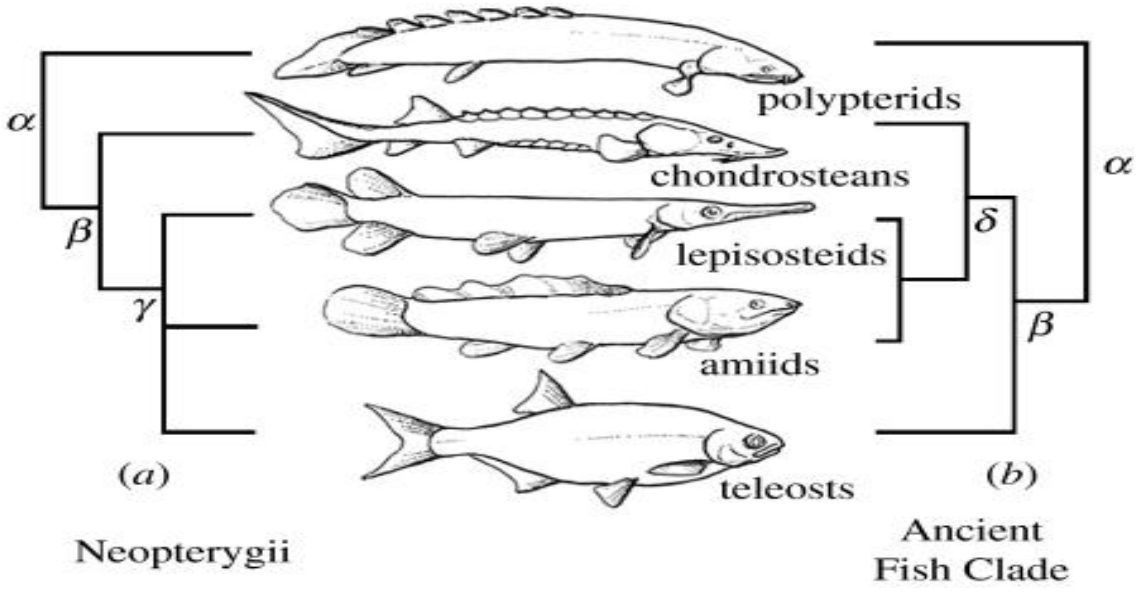
APPENDIX II:
The Teleostean Telencephalic Controversy

The vertebrate nervous system has many conserved features from hagfish through to the most complex manifestations in cetaceans and primates. In all vertebrates the neural crest develops to form three principal components of the brain: the prosencephalon, mesencephalon, and rhombencephalon. During embryogenesis these structures differentiate from the fledgling neural tube, generally through hypertrophy near areas of similar genetic expression and coordinated fate maps. The telencephalon particularly folds via evagination compared to the simple burgeoning of the diencephalon, leading not only to preliminary divisions but also the formation of the lateral ventricles. Interestingly, species of the class Actinopterygii do not exhibit evagination, but rather invaginate (or evert) the neural tube resulting in a unique configuration of the common neuroanatomical features typically associated with phylogenetically precedent and subsequent vertebrates. This distinctive arrangement leads to a number of important questions regarding the extent of this divergence from prior neuroanatomical morphologies, which may be extended to ask about the behavioral capabilities of this class compared to their near evolutionary cousins. These topics will be evaluated in depth over the course of this section to elucidate the nature of the long-controversial status of teleostean forebrain homologies.

Actinopterygii

The Actinopterygii are a diverse group of fish united by their radial fins, as is the literal translation of the class from ancient Greek, which are composed of true bone extending from their skeleton. The earliest fossil evidence of a true Actinopterygian was found approximately 425 million years ago in fresh water, where the fishes remained until around 340 million years ago when they began to venture into brackish and eventually sea water. There are two subclasses present among the Actinopterygii, the Chondrostei and Neopterygii, the former consist-



ing primarily of sturgeons and the latter representing two further infraclasses, the Holostei and Teleostei. The Holostei are further composed of two orders, Lepisosteiformes (gars) and Amiiformes (bowfins), and the Teleostei may be reduced indeterminately. (Cavin, 2008; Braford, 2009)

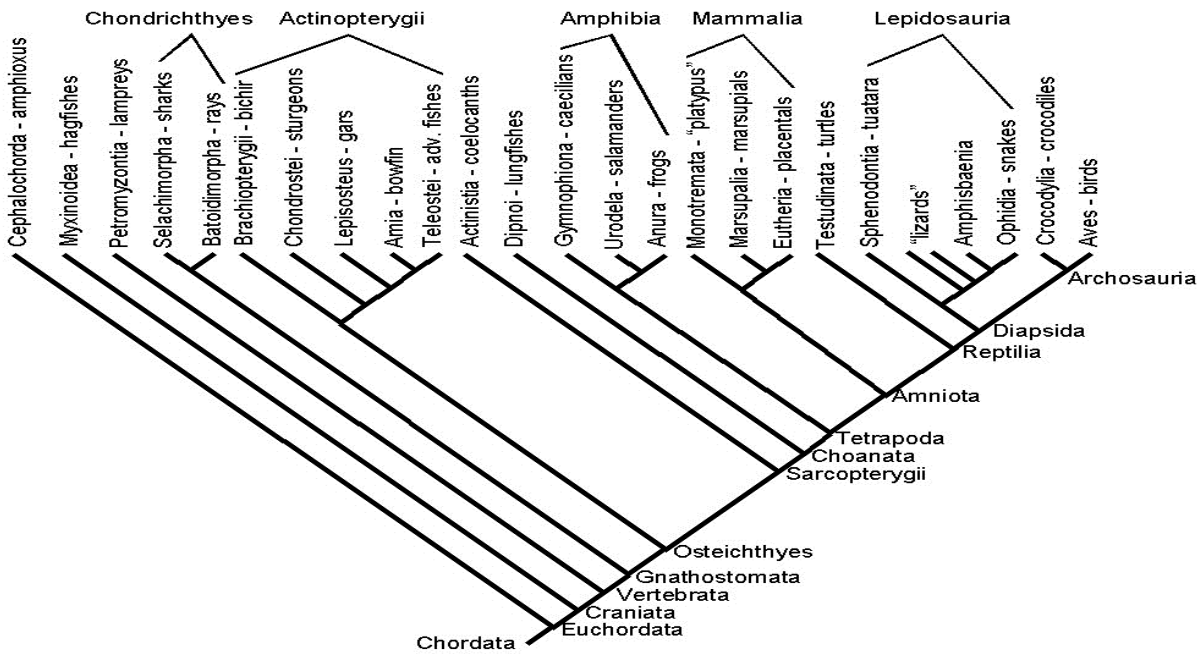
Teleostei is composed of at least 30,000 known species as diverse as eels and anglerfish to salmon and sea horses, completely dominating not only the class Actinopterygii but in fact the vast majority (>99% of total species) of extant bony fishes belong to this infraclass. The first fossil evidence for the teleosts dates them to 284 MYA, though mitochondiral DNA analysis predicts a divergence within the range of 268-326 MYA. The origin of Teleostei has long been considered to be derived from the crown group Neopterygii, which is comprised of the lepisosteids, amiids, and teleosts as sister groups. This has been debate, especially in light of recent mitochondrial DNA suggesting an opposing “Ancient Fish Clade” model as more parsimonious, though this model fails to statistically reject the opposing Neopterygian model; likewise, an analysis of various nuclear genes produces a model consistent with the Neopterygian hypothesis, but fails to reject the Ancient Fish Clade. While this issue remains to be resolved, recent Bayesian analyses favor the Neopterygian hypothesis as well as strong evidence from synapomorphic analysis

(Hurley et al, 2006). Regardless of which hypothesis one accepts, if one is to consider establishing homology across these groups it is necessary to identify the homologous organ in the ancestor to the teleosts, the polypterids, of which the only extant member is the bichir.

Considering the huge scope of the Actinopterygii, their behaviors vary widely according to their ecological niche and their cognitive capacities follow suit. Many different lifestyles have been adopted by different members of this class, such as the trigger fish which has a hugely developed optic tectum to measure the exact trajectory and force necessary to hit a flying arthropod, or the intense sexual competition that has led thousands of species of teleosts to wrestle for territory, build nests, negotiate sexual roles, and compete for hierarchal dominance (Barlow, 1961; Munakata et Kobayashi, 2010; Oliveira, 2009; Grosenick et al, 2007). The specific abilities of the Actinopterygii, typically Teleostei, will be discussed in terms of laboratory experimentation of cognition in order to elucidate the behavioral implications for many of the proposed homologies to Tetrapodia.

Chondichthyes

To fully evaluate the distinctive forebrain of the Actinopterygii, consideration of earlier gnathostome outgroups may shed light on the evolutionary basis of this oddity. There is substantial disagreement as to whether the Chondrichthyes, the cartilaginous fishes, belong as a sister group to tetrapods or Actinopterygii, which genetic evidence has not resolved (Mine, 2006). Regardless, this class is considered the nearest out-group to Actinopterygii (Nieuwenhuys, 1963; Wourms, 1997; Lisney et Collin, 2006). Elasmobranchs, an abundant subclass of Chondrichthyes, have a considerably larger brain-to-body ratio than most ray-finned fishes and exhibit a diverse set of skills: a set of acute sensory apparati for short- and long-range electroreception (i.e., bioelectric vs. migratory magnetic sense), vision adapted to predation, strong olfactory sense, ovoviviparous brooding, and social hierarchy in many species (Linsey et Collin, 2006;



Tricas, 2001; McComb, 2010; Pratt et al, 2001; Economakis, 1998; Guttridge et al, 2009). While sharks tend to have larger telencephalon-to-whole-brain mass ratios than Actinopterygii, this is predominantly due to drastically larger olfactory bulbs rather than non-sensory regions (Linsey et Collin, 2006).

As noted previously, the telencephalons of all Chondrichthyes undergo embryonic evagination of the telencephalon (Wourms, 1997). While this class parallels the Actinopterygii in many neuroanatomical features beyond the telencephalon, this ontogenetic paradigm shift makes it difficult to ascertain how much of the forebrain morphology is retained from this common ancestor of ray-finned fishes and tetrapods. Recent work on this front by Rodriguez-Moldes (2009) in the Lesser Spotted Dogfish, a common model elasmobranch, has revealed that this class may very well develop similarly to "higher species", incorporating many of the migratory and topological patterns previously believed to be of later descent:

- Sonic hedgehog (Shh) defines its alar-basal boundary with higher-concentrations in the basal region

- Paired box gene 6 (PAX6) is found to be expressed in heterogeneous densities in all prosomeric regions, but stands starkly in contrast to the mesencephalon where it is entirely absent
- Prosomere 2 (P2) has its caudal border defined by the fasciculus retroflexus and its rostral limens encircled by the zona limatans intrathalamica, the latter of which is defined by a gradient of Shh
- The first developmental period is defined by the absence of GABAergic cells in the alar component (pallium) and the absence of PAX6-expressing cells in the basal region (subpallium); by the second period, the former cells have migrated tangentially dorsal and the latter radially and tangentially ventral

The identification of the three prosomeres in the shark separated by the same semaphores found in Actinopterygii and even Tetrapodia indicates that this developmental process is almost certainly conserved across these diverse groups' development. Furthermore, this is the phylogenetically earliest example of tangential migration observed in any species, a complex process that requires many chemokines to be selectively expressed by radially migrated cells of specific fates and, foremost, a prerequisite for advanced axonal targeting (Handel, 2009; Kershaw, 2009; Li, 2009). These findings comprise reasonable evidence to conclude that many of the structures found in Chondrichthyan telencephalons may in fact be homologous to those of their descendants.

Coelacanths

The closest possible descendent to the Actinopterygii lies in its sister group of lobe-finned fishes, consisting of tetrapods, lungfishes, and coelacanths. In terms of telencephalic organization, one of two extant members of crossopterygii, *Latimeria chalumnae*, is the closest

relative. Long only considered a part of the fossil record, this coelacanth's discovery in 1938 ignited scientific curiosity into this armor-plated relic of the Mesozoic age and represented the potential for a long sought "missing-link" between teleosts and dipnoids (subclass of Sarcopterygii including lungfish). An analysis by Rudolf Nieuwenhuys concluded that this animal does indeed satisfy these criteria in some respects: its forebrain reveals a complex kind of evagination that is reminiscent of the eversion found in Actinopterygii and closely resembles the forebrain of the monopneumonian Ceratodus, a primitive lungfish (1965). The subpallium has undergone a true evagination whereas the dorsal pallium has everted in a manner akin to the Actinopterygii; altogether, this is considered to constitute "pseudoevagination". The subpallial structures are also noted to be exceptionally primitive, displaying a strong amalgamation of cells near the septal areas with almost no neural migration, which Nieuwenhuys asserts as being the most rudimentary subpallial organization of all extant Gnathostomes. A more recent evaluation by Northcutt and Gonzalez has supplemented these findings in light of recent advances in the Actinopterygian forebrain and the fortuitous procurement of two new specimens (2011): they conclude with a more complex model of the pallium which demonstrates at least four distinct cellular groups which are preliminarily considered homologous to the typical pallial divisions in tetrapods based on connectivity to the lateral olfactory tract and the eversion of the telencephalon (a discussion of these principles in teleosts follows below). The comparatively large medial pallium is thought to explain the advanced spatial capacities of most lungfish and amphibians, which

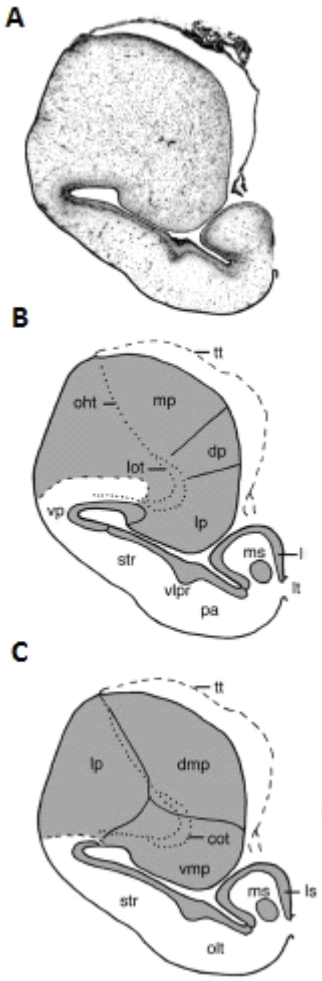


FIGURE 26: THE FOREBRAIN OF *LATIMERIA CHALUMNAE*. A) A NISSL-STAINED LEFT HEMISPHERE OF THE TELENCEPHALON, B) NORTHCUTT AND GONZALEZ (2011) INTERPRETATION OF THE FUNCTIONAL REGIONS OF THE PALLIUM, C) NIEWENHUYS' (1965) INTERPRETATION OF THE PALLIUM

are likely derived their enlarged hippocampal formations from an originating species common to all lobe-finned fishes or a preceding species. This new study also rebukes the supposed superior superoptic nucleus, instead believing it to be the medial amygdalar nucleus. This implies that the all of the major functional components of the amygdala were retained from a species predating the lobe-finned fishes, all extant members of which have been shown to possess these components.

While these features certainly make this fish interesting in its evolutionary context, it is not considered to be a direct ancestor of Tetrapodia but rather an offshoot that did not proceed to evolve any known species (Miles, 1965). While little is known of this species behavior due to difficulties in husbandry and incredible rarity, it is widely considered to be very primitive and unlikely to engage a more complex behavioral pattern than is afforded by its large size, armored scales, and residence in the mesopelagic volcanic caves, though it is has been observed to form peaceful social groups and possibly permanent homes (Fricke et al, 1990). The ancient coelacanth has thus presented a dead-end for researchers of brain evolution, though the apparent transitional qualities of its half-everted, half-evaginated telencephalon may represent a divergence from a common ancestor evolving from the everted condition of Actinopterygii; unfortunately, this is a difficult hypothesis to further given the uniqueness of this ancient species and the extreme paucity of scientific specimens.

Anatomical and Hodological Models of Pallial Arrangemen

While the simpler representatives of this class are analyzed less often and generally with the explicit intention of comparing to the most prevalent and advanced infraclass, Teleostei, there is much to be gained from observing the less-developed telencephalons present in the Polypteriformes (bichirs and Reedfish), Acipenseriformes (sturgeons and paddlefishes), Semionotiforms (gars), and Amiiformes (bowfins). There are many striking differences amongst the three species shown; namely, the single common ventricle decreases in size as the complexity of the brain increases from birchir to sturgeon to gar and as well as total pallial size and density increasing, as is particularly evident from *Polypterus* to more modern Actinopterygians. Importantly, it should be noted that the nissl-stain density becomes increasingly more visible at regions distal from the paraventricular zone (i.e., the ependyma), indicating increased mitochondrial metabolism in non-proliferative regions, thus neural greater migration; additionally it is particularly evident in the gar that there are distinct regions of various morphologies from the nissl stain alone. The teleost telencephalon is consistent with these trends and advances them significantly. Unfortunately, the pallial zones demarcated on all of these images remain controversial, especially in Teleostei.

From Polypteriforms to Acipenseriformes to Semionotiforms to Amiiformes to Teleosts there is an increasing trend toward the parcellation of the migrated rostrolateral preglomerular nuclei, or posterior tubercle (Demski, 1984; Braford, 2009). The posterior tubercle projects primarily to the pallium and the development of this diencephalic structure has been correlated to the development of more complex pallial morphology, standing in constrast to the dorsal preglomerular nuclei which are highly conserved from the basal species of Actinopterygii (Northcutt, 2007; Mueller, 2012). The basal fish also tend to have a large migrated nucleus medianus of the posterior tubercle, a nucleus with afferents from the optic tectum and efferents toward the areas of subpallium pars posteriori (Vp), Dl, and Dm; this nucleus shows a strong

trend toward increased volume as species advance, but is mysteriously absent or parcellated in the most advanced taxon, Teleostei (Northcutt, 2009). While the nucleus medianus dyes positively for calretinin, the only diencephalic nucleus with similar connections to the nucleus medianus, the lateral preglomerular nucleus, is monocellular and is calretinin negative. While the nucleus medianus may be split up into several nuclei or entirely replaced in teleosts, either outcome represents a paradigm shift in the hodoloy of the Actinopterygian prosencephalon, which suggests the presence of other critical forebrain changes in this most proliferant and diverse infraclass.

In the pallium of the basal species there are similar divisions to the better studied teleostean telencephalon and, employing Nieuwenhuy's labeling schema, several claims can be made: it is apparent that Dm and Dl are in approximately the same location in each taxon, that Dc is localized in vastly different regions, and that Dd is conspicuously absent in all but the teleots' and Lepisosteus' forebrain (Braford, 2009). This diversity complicates the assignment of the embryological pallial units, though the lesser degree of eversion allows for the simple localization of these units in *Polypterus*: ventral P1 is considered topological equivalent of VP, dorsal P1 is the LP, P2 is the DP, and P3 is the MP (Braford, 2009; Northcutt, 2007); this analysis is well-supported by hodological and genetic evidence (Nieuwenhuys, 2009). In the sturgeon it is thought by Northcutt and Braford that the situation is highly similar to teleosts, expressing all of the same regions except Dd and that these regions correspond to all four pallial divisions, possibly lacking the DP (Northcutt, 2007; Braford, 2009). Nieuwenhuys takes a different stance and asserts that the sturgeon possesses only two divisions, the LP and MP, corresponding to Dm and Dl combined with Dp, respectively (Nieuwenhuys, 2009).

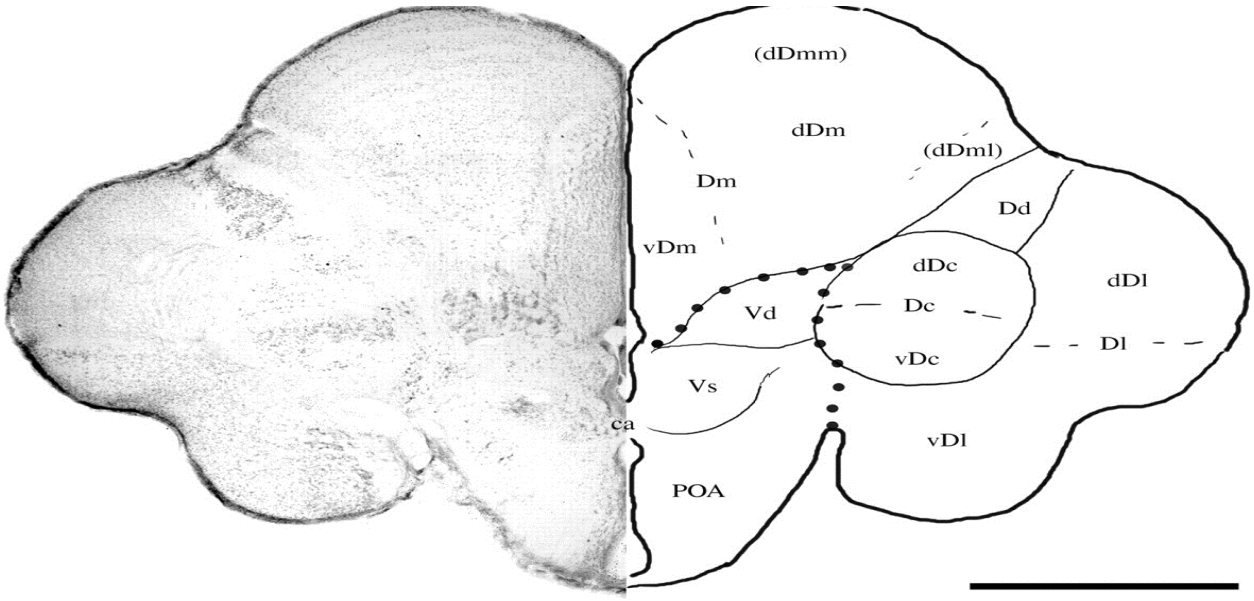


FIGURE 27: THE REGIONS DESCRIBED BY NIEUWENHUYS AT THE LEVEL OF THE PREOPTIC AREA. (ADAPTED FROM ITO ET YAMAMOTO, 2008)

Nieuwenhuys, Braford, and Northcutt have attempted to resolve the problem of teleostean telencephalic anatomy through attempting phylogenetic rationalizations and predicting the topological outcomes of a simple eversion of the basic telencephalic design of their nearest relatives, the so-called basal groups of the Actinopterygii, and the closest noneverted out-group, the Chondrichthyes. If a simple eversion had occurred in the teleosteon pallium, there are several possibilities for homologies to the typical four-fold divison. One could start with the MP as the most dorsal division the primordial state, thus making it the most lateral in adulthood and corresponding to either Dc or Dp, the former the potential result of a dorsomedial migration or the latter representing the most lateral proliferative region. The clearly migratory (due to its distance from the common ventricle) region Dc is thus problematic and indicates that simple eversion cannot solely explain the origin of this zone nor is it probable that the Actinopterygii have five histogenetic pallial divisions since the four divisions are inherited from more primitive taxa (Nieuwenhuys, 2009). From either Dc or Dp the regions would proceed medially in the order defined embryologically in a true eversion: MP, DP, LP, VP.

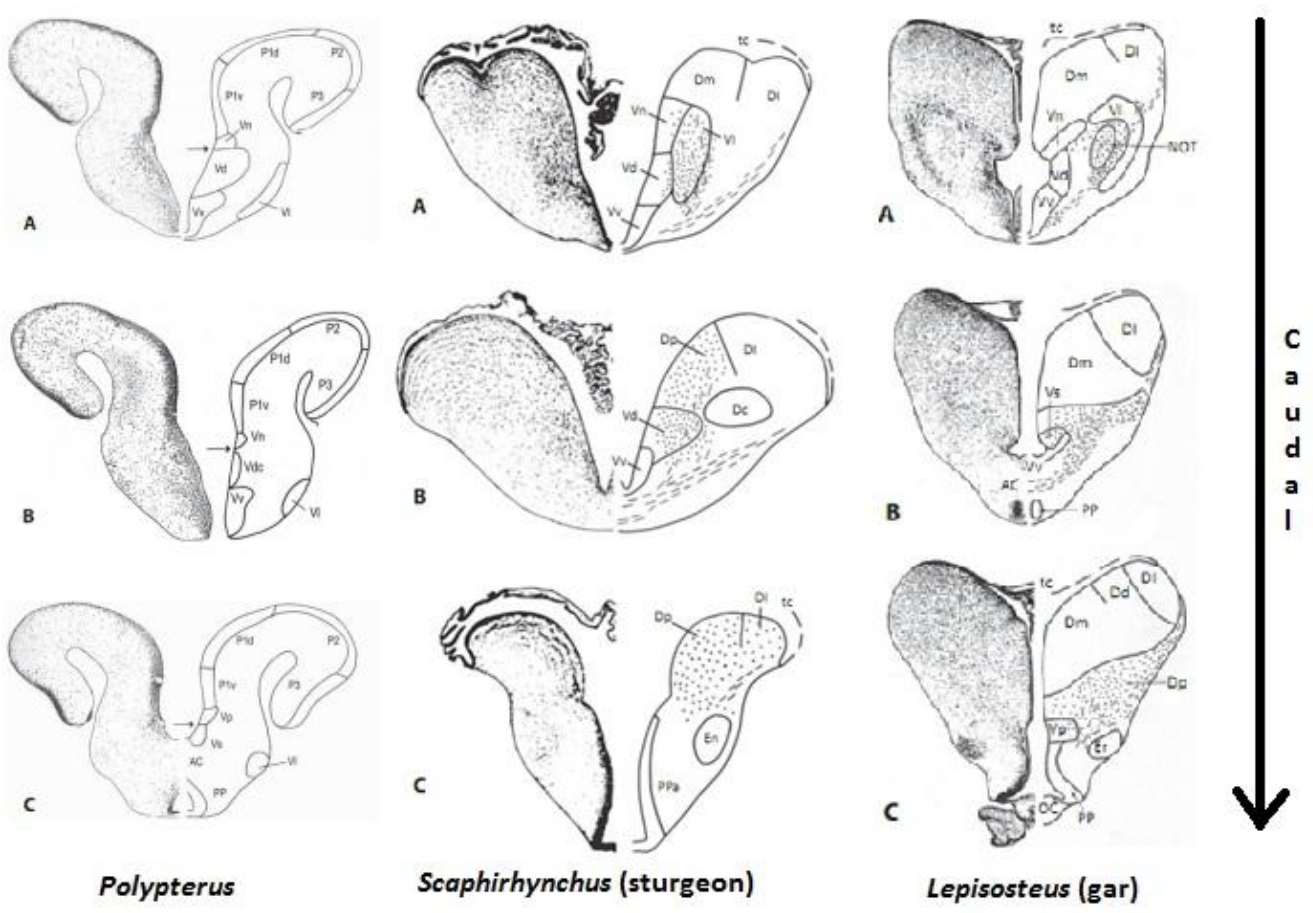


FIGURE 28. COMPARISON OF THREE BASAL ACTINOPTERYGIAN FOREBRAIN. LEFT SIDES ARE NISSL-STAINED, RIGHT SIDE ILLUSTRATES ANATOMICAL REGIONS PER BRAFORD AND NORTHCUTT. (ADAPTED FROM BRAFORD, 2009)

Various conclusions have been reached, with Northcutt and Braford adhering to the notion that Dm, Dp, Dl, and Dd correspond to the VP, LP, MP, and DP, respectively (Braford, 2009). This hypothesis excludes Dc as a pallial division and obviously defies simple eversion excluding Dc, which would be characterized by the order Dm, Dd, Dp, and Dl being equated to the VP, *LP, MP, and DP*. The reasoning for this is based on the connections present in these regions, especially the termination of the medial olfactory tract into Dp, and the topological awkwardness of this non-linear progression is justified by the comparatively larger Dm and Dl (or VP and MP) crowding the puny Dp into its caudolateral position, pressed against the ependymal wall.

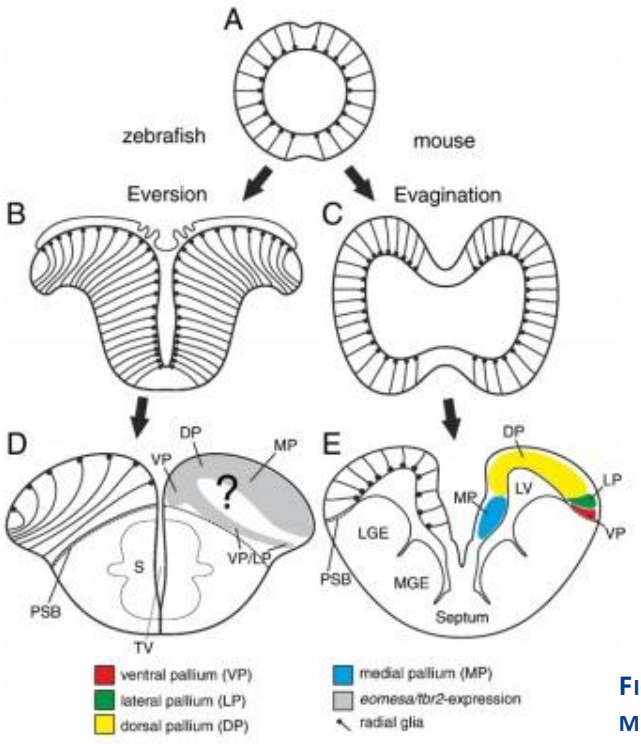
Nieuwenhuys provides the rationale for this argument, though he ultimately arrives at a different conclusion (Nieuwenhuys, 2009):

- In each of the taxa within Actinopterygii, there are distinct, homologous lateral olfactory tracts which present tightly against the ventricular space
- This lateral olfactory tract is not in a homologous region in evaginated brains, but rather another lateral olfactory tract (named based on the correct anatomical position in inverted brains) is present and homologous within all members of Gnathostoma with evaginated telencephalons
- *Polypterus* contains both the lateral olfactory tracts of everted and evaginated brains, referred to as the lateral and medial olfactory tracts respectively, in which the medial olfactory tract targets the dorsal part of P1 and the lateral olfactory tract terminates in the P3
- The evaginated lateral olfactory tract always sends efferents to the LP in all other Gnathostomes
- Dp receives strong secondary olfactory input from both the medial and lateral olfactory tracts
- Since Dm, Dd, and Dl are topologically equivalent to P1, P2, and P3, they must be homologous to the LP, DP, and MP, respectively; Dl and Dp both receive olfactory input and are thus probably extensions of the same division

Nieuwenhuys presumes a simple eversion beginning at Dm and terminating the at Dl, since he finds Dp to be an extension of Dl and omits the VP. This is certainly supported by his analysis of the trajectory of these two unique olfactory tracts, but hodology cannot define homology, instead only suggesting it. In his own words earlier in the same publication: “[Connectivity] is an

auxiliary criterion, [sic] because a cell mass may change one or more of its connections, [sic] without losing its morphological identity." He admits the weaknesses of his argument in his summary analysis of the whole state of affairs:

> ***"…details concerning which telencephalic cell masses arise from which zone are lacking for teleosts and for actinopterygians in general. Because of uncertainties regarding the embryonic origin of several pallial cell masses and therefore their primary topological position (see table 1), it will be of great interest for comparative anatomists to establish whether these four basic pallial zones are also present in representatives of each of the five actinopterygian clades (fig. 1), and if so, to trace the migratory paths of their derivatives. Given the considerable variation in forebrain structure in these clades, it will not be sufficient to confine these studies to a single species (and to proclaim this species subsequently 'the actinopterygian model species')."***



Nieuwenhuys also admits to being unable to trace the apparent histological evidence of Dp's migration as suspected by Northcutt and Braford (1980). Altogether, he stands alone in his assertion that Dm is homologous to the LP and he is the first to claim Dp as an extension of Dl. (Nieuwenhuys, 2009)

FIGURE 29: CONTRASTING THE STATE OF AFFIARS OF THE MOST POPULAR MODEL OF THE TELEOSTEAN DORSAL TELENCEPHALON WITH THE MOUSE. (ADAPTED FROM MUELLER ET AL, 2010)

Embryonic Origins of Pallial Zones

Recent genetic evidence by a scientist thoroughly engrossed in this field of research, Dr. Thomas Mueller, and his German collaborators have recently identified a new proliferative zone that represents a significant advancement to this heavily conjectured question. By dually staining the pallium of the most common model teleost, the zebrafish, with nicotine adenine dinucleotide phosphate diphorase (NADPHd) and parvalbumin, this new publication has exposed the embryonic origins of three essential regions in question, Dp, Dd, and Dc. NADPHd binds nitric oxide synthase(an essential enzyme for the formation of nitric oxide gas and a basic component of many kinds of GABAergic, acetylcholinergic, and glutamatergic cells in the CNS), thus providing a trace in the adult teleost's brain near the area of Dd that descends ventrocaudally through progressively more caudal tranverse sections to form the dorsal border of Dc. This provides Dc with an embryonic origin and identifying it as a histogenetic zone. By employing a marker for DNA-uncoupling to test for neurogenesis, Bromodeoxyuridine (Brd-u), the scientists also observed the migration of cells originating from the proliferative zone at Dl to the paraventricular area at Dp, thus eliminating Dp as a candidate for an independent pallial zone. Combined with previous evidence, Mueller and colleagues present their findings, defining the pallial zones thus: the VP is Dm (the homologue of the amygdala of tetrapods), the LP is the combination of Dp and Dd (the homologue of the piriform cortex), the DP is Dc (homologous to the isoform cortex), and the MP is Dl (the homologue to the hippocampus). This coincides with the behavioral evidence presented as well as with the models of eversion put forth by Northcutt and Braford, who previously recognized Dp as a migratory region. (2011)

Northcutt, the long-standing authority on the teleostean doral pallium, has since published a response to Mueller and his colleagues' recent findings (2011). Northcutt is quick to note that that teleostean dorsal pallium is unlikely to be resolved through research in a single

species of cyprinid; he continues to correctly state the the question of tetrapodian homology relies on the demonstration of these four distinct pallia in the preceding clades, the chondrosteans (polypteriforms and acipenseroids) being the most essential as the closest extant taxa to the original ancestor of both actinopterygii and tetrapods. Northcutt expounds a series of doubts regarding the conclusion that Dd is simply the narrow zone from which Dc derives its neural progenitors: the Dd has not been conclusively identified in non-teleosts; likewise, Dc/DP has not been found in polypteriforms despite previous attempts to find a pallial connection to the optic tectum; finally, the sulcus ypsiloniformi, which Mueller and others suggest is the entry point from DP's proliferative zone overlapped with the hypertrophied MP and VP, is only present in teleosts. While Northcutt acknowledges Mueller et al's work as valuable, he finds the claims of the discovery of the dorsal pallia in teleosts to be extravagant. Mueller rebuts that is is less disputed if the distal outgroup chondrichthyes possess a dorsal pallium or even agnathans, but concedes that the identification of which remains to be proven in studies of chondrosteans (2011).Despite uncertainties regarding the exact layout of the pallia, he states that it the existence of regions homologous to the amygdala, hippocampi, and piriform cortex of tetrapods has never been in doubt, merely anatomically implacable.

In conclusion, it is apparent that while much debate surrounds the exact locations of the hippocampal and amygdalar formations of the many diverse species in Actinopterygii remains ambiguous, it is often taken for granted that the behavioral and genetic evidence, while incomplete, suggests that the commonly used model teleost species have regions in the brain that are at least analogous and possible homologous to regions in the mammalian or "higher vertebrate" brain. While the questions of the unique teleostean telencephalon excite further inquiry, the answers are unlikely to emerge from theoretical derivations of embryological processes, which

have already been thoroughly evaluated, but rather from further genetic and hodological evidence to confirm or deny preexisting theories.