Physiology & Behavior 146 (2015) 86–97

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Nuance and behavioral cogency: How the Visible Burrow System inspired the Stress-Alternatives Model and conceptualization of the continuum of anxiety

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HIGHLIGHTS

- Bob Blanchard used the Visible Burrow System (VBS) to model highly nuanced behavior.
- The Stress Alternatives Model (SAM) was inspired by results from VBS experiments.
- The SAM explores dynamic stress responses and anxiety through dichotomous choices.
- Nuanced behaviors are helpful in parsing complex behavioral conditions like anxiety.
- An anxiety gradient exists spanning contextual settings in SAM experimentation.

ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form 24 March 2015
Accepted 25 March 2015

Keywords:
Social stress
Anxiety
Aggression
Learned escape
BDNF
NPS
CRF
CRF_1
Antalarmin
Yohimbine
Exercise
Bob Blanchard

ABSTRACT

By creating the Visible Burrow System (VBS) Bob Blanchard found a way to study the interaction of genetics, physiology, environment, and adaptive significance in a model with broad validity. The VBS changed the way we think about anxiety and affective disorders by allowing the mechanisms which control them to be observed in a dynamic setting. Critically, Blanchard used the VBS and other models to show how behavioral systems like defense are dependent upon context and behavioral elements unique to the individual. Inspired by the VBS, we developed a Stress Alternatives Model (SAM) to further explore the multifaceted dynamics of the stress response with a dichotomous choice condition. Like the VBS, the SAM is a naturalistic model built upon risk-assessment and defensive behavior, but with a choice of response: escape or submission to a large conspecific aggressor. The anxiety of novelty during the first escape must be weighed against fear of the aggressor, and a decision must be made. Both outcomes are adaptively significant, evidenced by a 50/50 split in outcome across several study systems. By manipulating the variables of the SAM, we show that a gradient of anxiety exists that spans the contextual settings of escaping an open field, escaping from aggression, and submitting to aggression. These findings correspond with increasing levels of corticosterone and increasing levels of NPS and BDNF in the central amygdala as the context changes. Whereas some anxiolytics were able to reduce the latency to escape for some animals, only with the potent anxiolytic drug antalarmin (CRF_1R-blocker) and the anxiogenic drug yohimbine (α_2 antagonist) were we able to reverse the outcome for a substantial proportion of individuals. Our findings promote a novel method for modeling anxiety, offering a distinction between low-and-high levels, and accounting for individual variability. The translational value of the VBS is immeasurable, and it guided us and many other researchers to seek potential clinical solutions through a deeper understanding of regional neurochemistry and gene expression in concert with an ecological behavioral model.

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1. Introduction

Animal behavior is always far more complex than we initially imagine. That complexity is derived from the environmental stimuli and internal physiology that motivate it, gene-by-environment interactions,

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http://dx.doi.org/10.1016/j.physbeh.2015.03.036
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evolutionary history, and adaptive significance for the individual behaving [1]. Despite these levels of complexity, numerous experimental protocols depend on extremely simplified stimuli ("painful, novel, or sudden") and outcomes, to produce high throughput designs for assessing translational relationships with human disorders [2]. Studies such as these have recently been determined to have limited clinical validity [3–5]. Context and small discrepancies in behavior (nuance) often make distinctive differences in specific neural activity and the adaptive value of the response for the individual [6–20]. Lorenz suggested that the full range of behavior in contextually appropriate naturalistic settings was necessary to understand the underlying mechanisms [21]. Behavioral paradigms and experimental designs created to study the neurochemical correlates of these nuanced and distinguishing behaviors came from the critical analyses of innovators like Bob Blanchard.

The Blanchards recognized the critical nature of nuanced behavior in their work on animal models of anxiety [16,18,22–37]. Anxiety is ostensibly a continuum of apprehensive behavior extending from subtle reactions related to stressors in the environment. Anxious states are therefore constructed along a stress/fear gradient that spans from foreboding to terror [2,39–40]. The complex nature of anxiety and anxious behavior comes from the potentially unlimited possibilities for stimuli to elicit a response. The high variability of stimuli produces highly variable responses, with a dramatic range of response magnitudes, especially related to neural and physiological activity. Anxiety covers a spectrum of related conditions that include general anxiety, social anxiety, panic, posttraumatic stress disorder (PTSD), and other related syndromes which carry a substantial societal and individual burden, affecting as much as 25% of the population [41]. They are highly comorbid with each other and with other psychiatric conditions, particularly mood disorders. Social anxiety alone has an estimated incidence of approximately 18%, and comorbidity as great as 80% with major depression [41].

Parsing this continuum between normal situational anxiety and that which leads to serious psychiatric disorders in an ethologically valid manner is no small task, as the experimenter must tie the complex and poorly understood symptomatology in humans to observable behaviors in animals. McKinney and Bunny proposed such validity criteria, which were later revised by Willner [42–45]. In addition to relating human symptoms to animal behavior (face validity), effective models should also utilize treatments which produce parallel results in humans and their animal homologues (predictive validity), and relate the symptoms and treatments to the systems involved (construct validity). In anxiety and depression, construct validity proves to be one of the hardest standards to meet, as the bio-behavioral inputs are multifaceted and poorly understood. This is revealed by often contradictory findings in studies [3–5,26] modeled upon experiments which seek to explain a single gear in a large and duplicitous machine. Recent clinical trials suggest that the predictive power of most single niche tests for psychological disorders in animal models is low [3–5]. A new focus, then, must be aimed where behavioral parallels between disorders and models converge, including nuance within a larger picture of behavioral complexity, as it becomes the crucial element in assessing model validity [12].

To model such complexity and accomplish ethological validity, Bob Blanchard recognized a need for systems which model behavior in a semi-natural context, accounting for many of the factors which Tinbergen and Lorenz had laid out many years before. He set out to create such a system, where behavior could be observed in a malleable social context, with variables offering the chance to study neural and endocrine correlates of behavior for individuals in differing social status. The Visible Burrow System (VBS) provided a unique experimental paradigm and apparatus that allowed for highly nuanced behavior within a laboratory setting and detailed evidence for a rodent's psychosocial state, as well as the opportunity to examine the neural and endocrine cascade effects that connect the behavior with potential predictive and construct validity [2,19,46–50]. For defensive behaviors and those related to social anxiety, the escape response in the VBS showed the most broadly homologous potential for mammalian taxa which could be modeled with laboratory animals. Escape behaviors provide a direct and active response compared to those stimulated by novel conditions and exploratory capacity [26]. Conceptions of the importance of social stress and stress coping strategies helped pave the way for understanding how dichotomies in behavior may be predictive of psychosocial disorders [51–54]. The inspiration of the ethologically and ecologically valid Visible Burrow System model combined with the prospect that dichotomous stress coping strategies could be predictive of psychological disorders led us to develop the Stress-Alternatives Model (SAM), a conceptual model and apparatus for assessing anxious and depressive behaviors and their effects on decision-making, which allows for parsing anxious behavior into contextual niches along an anxiety gradient [55].

2. Visible Burrow System

The Visible Burrow System (VBS) was created about 25 years ago, with the express purpose of providing a semi-natural situation affording many of the crucial features of a typical natural environment, in which groups of rodents could live for substantial periods of time. It was based in part on much earlier studies in which unfamiliar conspecific intruders had been introduced into large groups of laboratory rats for the purpose of polarizing the aggressive tendencies of the colony male(s) vs. the defensiveness of the intruder [11]: However, these initial studies had not used the VBS' combination of 'open space' (maintained on a 12:12 h light–dark cycle) with chambers and tunnels (maintained under red light; not visible to the rodent subjects used). This addition of chambers and tunnels, in total areas of about the same dimensions (about 1 m on a side) as the earlier tests, greatly enhanced the range of possible behaviors, and enabled a finer analysis of the behavioral effects of dominance-subordination relationships among male rats in these groups. Blanchard & Blanchard [2] detailed the reduced eating, drinking, and offensive aggression of subordinate males, with particular attention to differences of these animals with reference to space. Subordinates tended to remain largely in the tunnels/chambers, especially while the dominant male of each group utilized the open or 'surface' area. This, it did freely, as did females, with much of the copulatory behavior within the group in this location. A second focus of this study involved analysis of changes in behavior for group members as a function of status, following a brief (15-min) presentation of a cat in the open area.

Cat presentation was followed almost immediately by flight to the burrows by any rat located in the open area. Circa 22-kHz ultrasonic cries were made by animals in the burrows, remaining strong for 30 min to about an hour after cat presentation. As these cries declined, individual rats gradually began to approach the open area through the tunnels, peeping out very briefly to scan, and then retreating to the depths of the tunnels. However, no rat re-emerged onto the surface for at least 5–6 h, and the first to emerge was invariably the colony dominant male. Many of the remaining animals had not emerged by the end of the 20 h video recording period, a factor that obviously contributed to decreases in levels of the sorts of normal activities that tended to occur on the "surface".

Approaches to the surface through tunnel entry points involved a head poke and visible scanning in which the animal's head moved from side to side, clearly affording a look at all of the surface area. During its initial full-body re-emergence the dominant colony male continued to limit its exposure to the open area by "corner runs". These utilized a feature of the original burrow systems, that the tunnels and chambers were arranged along two adjacent sides of the open area, such that a tunnel entry point nearest the inside corner was only a very short distance from the entry nearest the same corner on the adjacent wall. Moreover, these "corner runs" were very rapid, as had been the prior instances in which subjects in the burrow system peeped out onto the surface area. In both cases, some visual scanning of the surface could be accomplished in a time that afforded little opportunity for the cat,

had one still been present, to detect the rat and attempt to seize it. The corner runs may, in fact, have served an additional purpose, of soliciting a potentially present but not visible cat to show itself by movement; thus affirming its presence even though repeated scans by the rats had suggested otherwise. These actions, and also a specific "stretched attend" posture involving a lowering of the body and locomotion interspersed with periods of immobility during later forays onto the surface, were given the label "risk assessment activities" as they were hypothesized to represent attempts to determine if a threat was present, and to localize and possibly identify it, while remaining as safe as possible. Other activities, such as burying [56] or tossing substrate at [57] novel or ambiguously threatening stimuli have been included under this functional grouping, in that these appear to represent attempts to determine if this stimulus is alive, thus responsive to being buried or hit. An additional defensive behavior noted in the initial VBS study was behavioral inhibition, involving suppression or reduction of normal activities such as eating, drinking, sexual activity or aggression. Notably, this is not the same as freezing, since it does not involve the same degree of immobility and also tends to occur over much longer periods; in fact a similar time period as risk assessment, both lasting much longer than the freezing response in this particular context. It likely reflects interference with normal ongoing activities from risk assessment as well as because of proxemic avoidance, as many of these activities tend to occur in the "surface" area.

The VBS has subsequently been used in a number of studies aimed at measuring some aspect of defensive behavior in more detail, or looking at differences in defense between lab rodents of different species, strain, or social status (e.g., dominant vs. subordinate) [26,50,49]. There are, in fact, some notable species and strain differences in the pattern of defenses seen in the VBS, in addition to the status differences outlined above: First, mice, as befits their status as less social animals, show a lack of tolerance among males when females are present. Thus mixed sex mouse groups usually consist of a single male with multiple females, or, multiple males without females. Second, mice fail to emit the 22 KHz "alarm cries" given by the latter; presumably on the basis that such cries, drawing attention to the emitter in a predatory context, may be less adaptive for less social species, reflecting the reduced adaptive value of warning other group members. A third difference is that the VBS actually had to be slightly redesigned to make mice less likely to be caught and mauled by the cat during its 15-minute sojourn in the "surface"; they tended to peep out of the tunnel entries for so long, and so soon after retreating to the burrows, that the cat actually caught some of them [personal observation: Caroline Blanchard]. With reference to strain, the reduced affiliative behaviors of BTBR T + tf/J mice, long noted in specialized social preference tests [58] were well demonstrated in the VBS, in which BTBRs showed striking reductions in virtually every category of approach/proxemic behaviors, along with spending more time alone [49].

The differentiation of defensive behaviors made originally in the VBS and subsequently documented in a more focused system, the Mouse Defense Test Battery [59] has since been applied to human responses to threat, in the form of a scenario study [28] indicating that people may show much the same pattern as rodents of defenses to similar contexts. This has been replicated in Wales [60] and in Brazil [61–63] suggesting that culture is a relatively minor factor in the relationship between context and specific defenses, and further emphasizing the possibility that these relationships between context and behavior are features of the brain systems controlling defense patterns.

The major direct use of the VBS has been to evaluate changes in the brains or neurochemical systems of males as a function of their status as dominant or subordinate animals [48,64–66]. It continues to represent a strong model for evaluation of social stress effects. However, the more general analysis that the VBS provides for defensive behaviors has also been viewed as potentially applicable to number of psychiatric conditions [12,61,67]. Against this background of broad-based usefulness, one must consider a critically important feature of the VBS: It is definitely not "high throughput". Might it be possible to combine the analytic value of the VBS with a dichotomous test situation? This was the focus for the development of the SAM.

3. Stress-Alternatives Model (SAM)

The SAM was inspired by the VBS and its underlying concepts, as a model built upon species-specific understanding of ecology and social behavior. In a seminal 1989 paper on the VBS, Bob said "the first step in such an analysis is the systematic measurement of a wide range of defensive and non-defensive behaviors, over time, in a situation designed both to incorporate important features of the natural environment of the subject species and to permit systematic manipulation of important variables" [2]. Our reading of this and other papers on the VBS, as well as discussions with Bob, suggested that the subjects of study were clearly making important decisions based on each unique individual, social context, and every nuanced behavior over time [6–20,68]. The fundamental concept in the design of the SAM (Fig. 1) is to provide an arena that allows for the fullest expression of social interaction, including socially anxious and depressive behaviors, with a specific but simple dichotomous choice, to escape social subordination or remain [55]. These specific behavioral outcomes, escape and submission, are evolutionarily conserved and expressed in vertebrate organisms from fish to humans.

3.1. SAM apparatus (rodents)

The SAM combines elements of several behavioral tests, with variable stressors forcing the animal to make decisions. If they are alone in the Open Field (OF) of the SAM arena, anxiousness associated with the open space generates movement mostly along the edge as in any other Open Field test, but the SAM arena affords escape from this anxiety through 2 escape routes at each end of the oval space, which lead to small safety chambers (Fig. 1A). However, the initial escape produces another anxiety which directly contrasts with the motivation to escape OF anxiety: novelty of the escape route and its unknown destination significantly delays its first use (initial novel escape anxiety) [55,69]. Trials in the SAM apparatus may be conducted in the presence (for social interactions) or absence (for controls) of a larger aggressive male conspecific, but access to escape holes is consistent. The escape routes are too small for the large aggressor, and the oval arena promotes dynamic interaction as the smaller animal cannot hide or be cornered, or the escape routes blocked. For social interactions an aggressive male is placed into the SAM inside the oval arena but outside an opaque cylindrical divider. A test animal is placed inside the cylindrical divider and allowed 30 s to acclimate. All training days (1–4) as well as the test day (5), are conducted in the SAM apparatus (one trial/animal/day). Behavioral observations are manually and digitally recorded. On training days, after the test or control rodent is in place, a tone (2500 Hz at 75 dB) serves as the conditioned stimulus (CS), and is sounded for 15 s, followed by 15 s of silence. After the silence, the opaque cylinder separating the two animals is removed (presentation of the unconditioned stimulus [US]) allowing the animals to interact for a maximum of 300 s; submissive animals remained for the entire 300 s with the large aggressor. The time allowed for social interaction is calculated to minimize injury to the test mouse (the average latency to attack is ~30 s). Attacks are defined as a successful bite by the large aggressor on the test animal. A novel aggressor is used for each interaction (used once per day), to limit habituation; animals often display more interest in novel conspecifics [70,71]. Interactions are scored for intensity of attacks made and latency to escape. On test day, test animals are placed in the SAM apparatus as on training days, with the exception that no aggressor is present. The CS is given, and latency to escape recorded with brain and blood samples collected after 300 s.

4. SAM experiments

4.1. SAM experiments — rainbow trout

In early iterations of SAM experiments on rainbow trout, a standard large fish tank with opaque dividers was used that included only one escape hole (Fig. 1B) [72]. Trout are social species in which larger individuals are quick to establish territorial dominance over smaller, subordinate individuals. In these experiments, the large aggressor was three times the size of the smaller test fish. In a natural environment, larger fish patrol the center of the water column, where they are less likely to be subject to predation and have a greater range of feeding opportunities. Smaller fish are forced into less-optimal zones near the water's surface, edge, or the bottom. This was observed consistently in the SAM, wherein the smaller test fish had to choose between remaining with the larger aggressor and escaping through a hole to an empty chamber.

This SAM model is distinguishable from those involving other species as it offers a three-dimensional behavioral space (including corners), wherein the animals were fed, giving them territorial affinity according to the unique ecology of the rainbow trout. As such, the escape hole needed to be located near the top in an area the subordinate fish would frequent, while the other SAM models would feature two escape holes. Experiments with the fish model revealed a behavioral split (50:50) between escaping and submitting test fish [72], a result that was found in later experiments on rats and mice as well (Fig. 2). Those trout choosing to remain submissively with the larger aggressive conspecific exhibited a classically conditioned stress hormone (cortisol) increase stimulated on test day by the conditioned stimulus (cessation of water flow) alone. Similarly, submissive animals also exhibited elevated serotonergic and dopaminergic activity in the striato-amygdalar complex, hypothalamus, and raphé [72], and increased gene expression for brain derived neurotrophic factor (BDNF) and AMPA receptor subunit $GluA_1$ in the hippocampus [73,74]. Submissive behavior can be reversed in two-thirds of the individuals treated with the anxiolytic corticotropin releasing factor type I receptor (CRF_1) antagonist antalarmin, such that they began escaping, and at a rate much faster than initial escape latencies in control or vehicle-treated animals [75,76].

For those trout that did escape, the latency to escape decreased over seven days of SAM trials; from an average over 600 down to 35 s, with most of the improvement in learning time occurring within the first three days. The propensity for escape is not determined by the immediate intensity or frequency of aggression, or its overall severity (Fig. 3). Instead, the likelihood of escape is most closely correlated with the activity of the patrolling larger fish, such that in early trials escaping fish would leave the arena without regard to the orientation of the larger conspecific, but in later trials would only escape when the aggressor could not observe the escape (Fig. 4) [76].

4.2. SAM experiments — rats

Similar to the fish tank used for trout experiments the earliest iterations of rodent-experiments in the SAM took place in square or rectangular arenas with definite corners and only one hole for egress (Fig. 1C).

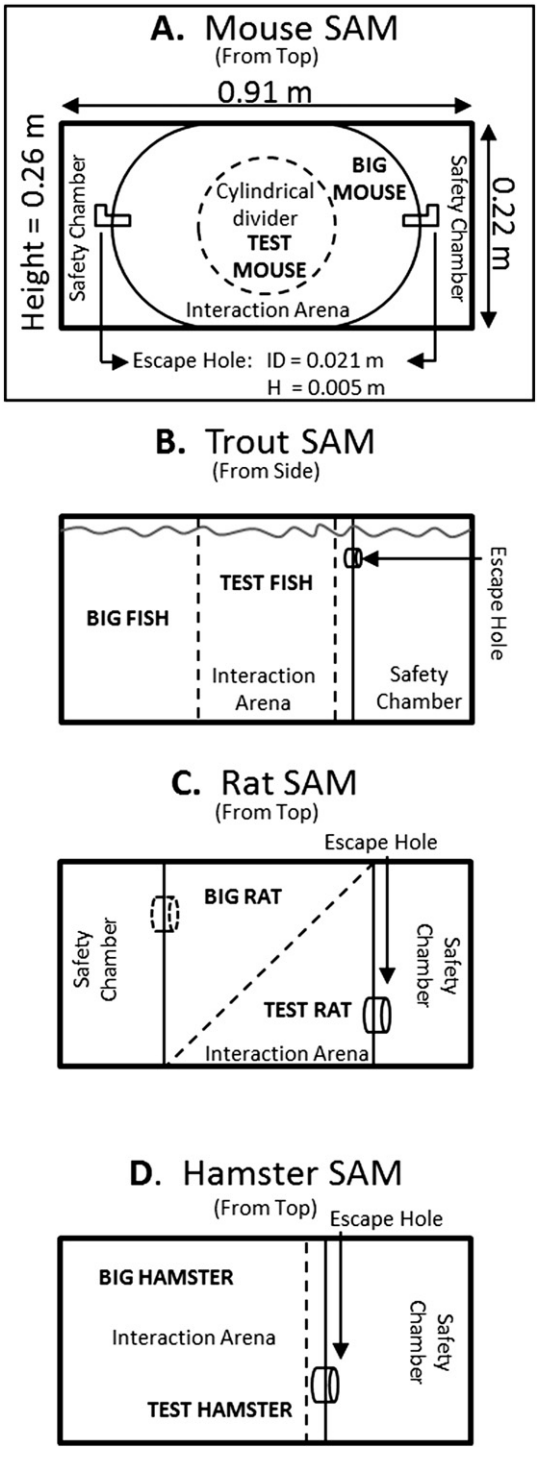

Fig. 1. Schematic depictions of developmental iterations of the Stress-Alternatives Model (SAM) designed for mice, trout, rats, and hamsters. A) The current SAM apparatus designed for rodents (dimensions are for the mouse version), including mice, rats, and hamsters. This version has no corners, an oval open field with flexible dimensions and two escape routes only large enough for the smaller test animal. A test rodent is placed inside the center circular opaque divider (dashed line); the divider is removed after presentation of a tone (conditioned stimulus, CS) to initiate trials. Trials may be social interactions with the addition of a larger conspecific placed outside the circular divider prior to the start. Arena and escape routes are novel for the initial trial, and L-shaped tunnels obscure the destination, creating some anxious behavior (delayed latency to escape) on their initial use. B) The SAM was first tested with rainbow trout in a 284 L aquarium where a test animal is fed. A removable opaque divider (dashed line) was used to separate the larger aggressor and intruder prior to interaction; its removal followed cessation of water flow (CS). A second removable opaque divider (dashed line) allows access to an escape hole large enough for only the smaller test fish, located near the top of the water column, which leads to the safety chamber. C) Initial experiments with rats utilized a square arena, and a single escape hole and safety chamber. A second escape hole and safety chamber were added to prevent the larger aggressive animal from blocking access to the safety chamber. A diagonal opaque divider (dashed line) separated the large rat from test rats, and was removed following a short tone (CS), prior to social interaction. D) Golden hamsters reliably escape social aggression, and this version of the SAM was designed to study the effect of forced interaction and submission versus escape. A removable divider (dashed line) could be used to block access to the escape hole and safety chamber for smaller individuals, forcing some hamsters to submit to the larger aggressor.

The preliminary trials required adjustments for the height of the escape hole relative to the floor of the chamber, the size of the arena, as well as the strain and condition of rat that would produce an effective amount of aggression. Two escape routes on opposite ends of the arena were determined to be necessary: this is because the larger aggressive male would block access to the hole or physically pull the smaller animals from it as they attempted escape. Once this adjustment was made, tested Sprague–Dawley rats segregated into groups of escaping and submissive animals, showing a similar ratio (50:50) to that seen in the trout experiments (Fig. 2). Intensity-graded behaviors were used to calculate the magnitude of actions associated with escaping, submission, and aggression to compare differences in magnitude across time for escaping and non-escaping groups. As in the VBS, our social interaction paradigm reveals marked differences in individual responses to the stress of aggressive interaction with a conspecific (Fig. 5).

In animals that did not escape from the larger aggressor, the greatest frequency and magnitude of behaviors were those associated with submission (Fig. 5 top). In both escapers and non-escapers aggressive behavior remained low throughout the social trial. During the first quarter of the social interaction animals that eventually escaped also rapidly displayed submissive behavior, though the magnitude and frequency decreased dramatically over the session. Submission in non-escaping animals remained high throughout the duration of the social trial. Conversely, escaping animals were initially submissive during the interaction, but quickly began exploring the escape option (Fig. 5 bottom). The intensity and frequency of escape behaviors increased until the animal actually left the behavioral arena.

4.3. SAM experiments — hamsters

The next sequence of experiments explored this dynamic in another model organism commonly used in aggression studies, the golden hamster [77–85]. Hamsters are territorial and solitary, except when mating, extending our comparisons to a less-social species which always utilized the escape hole if given the opportunity. We therefore designed an experiment in which escape was either possible or totally blocked to examine the contrasting effects of submission compared with the possibility of escape (Fig. 1D). The latency to use the escape route was similar to all other experiments using this model: slow during the first trial, and significantly faster each day thereafter [86]. The total amount of aggression received by the test animal did not vary depending on the possibility to escape, however the latency to contact in these interactions was significantly longer when escape was available. Additionally, and counter-intuitively, there was more social interaction between the large aggressor and the test animal when escape was possible than when it was not.

In the hamsters, hippocampal BDNF expression was significantly higher in fast compared with slow-escaping individuals [86]. This relationship was also influenced by stress hormones, such that hippocampal BDNF and TrK_B expression were also significantly related to the level of cortisol. Not surprisingly, the amygdala also played an important role in which TrK_B and AMPA receptor subunit $GluA_1$ exhibit an inverse relationship with the speed of escape. Elevated expression of $GluA_1$ occurred only in individuals that were not afforded an escape opportunity, suggesting that this change in AMPA structure may have been associated with enhanced fear learning associated with social defeat [72,87,88].

4.4. SAM experiments — mice

Results from the hamster experiments indicated a strong role for the contributing actions of BDNF, TrK_B, and glucocorticoids in determining

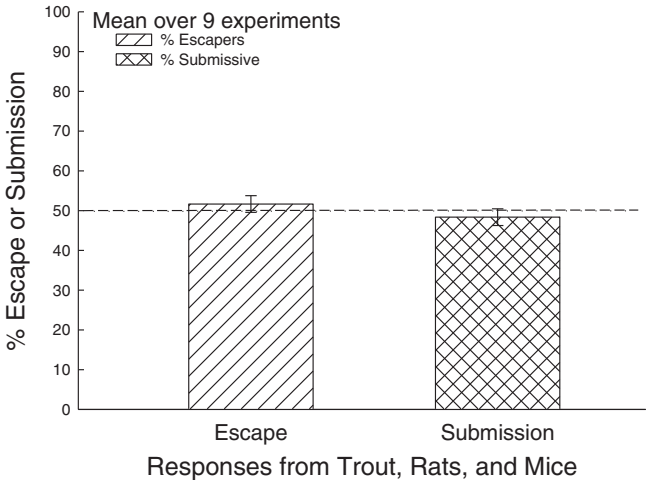

Fig. 2. Across species tested in the SAM, approximately 50% escape, and 50% remain submissively. Animals escape or remain submissively when presented with a larger aggressive conspecific in SAM social interactions, where an escape route is available that is too small for the larger animal to pass. Means are averaged over nine experiments (3 for trout, one for rats, and 5 for mice). The ranges were greatest for mice, and extend from 43.3 to 60% for escape and for submission extend from 40 to 56.7%.

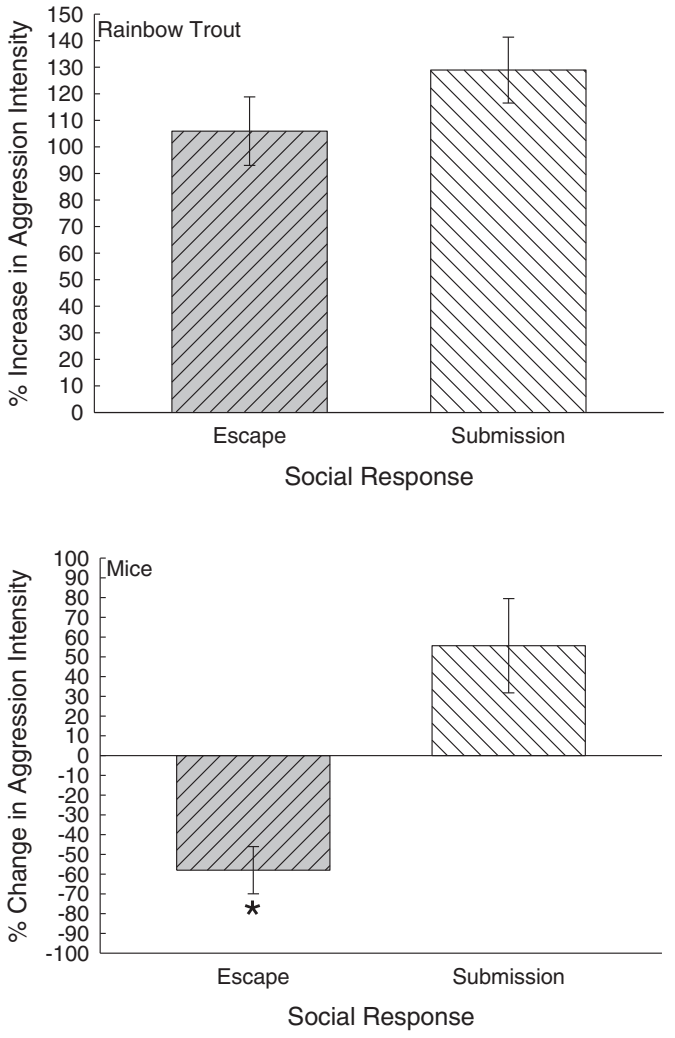

Fig. 3. Intensity of aggression doesn't drive escape behavior. The original raw data have been normalized to the initial/baseline levels of aggression, such that the means represent change in intensity of aggression received (derived by: maximum intensity/average total intensity of aggression/time) as related to escape or submission in SAM social interactions. The data have also been normalized for time spent in the arena. Data from all trials have been collapsed, as there were no significant differences between trials. Top) No significant differences in the increase in intensity of aggression between escaping (gray bar, upward hatching) and submissive (white bar, downward hatching) rainbow trout. Bottom) Mice that escaped aggression received significantly ($P < 0.05$) reduced aggression intensity over time, while those that submitted received more.

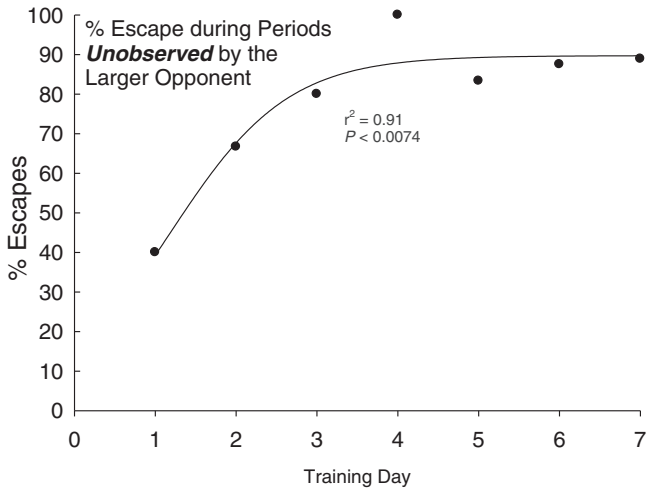

Fig. 4. Percent of trout escaping from aggression while unobserved (line-of-sight) by the larger opponent in SAM interactions. As training days progressed, trout increasingly escaped only when unobserved by the dominant patrolling fish (curvilinear sigmoidal regression $r^2 = 0.91$; $P < 0.0074$).

behavioral outcomes in the SAM. Similar to trout and rats, C57BL/6 mice exhibit a 50/50 split between expressing preference for social submission and escaping, and these animals learn how to use the escape holes between the first and second day with a similar decrease in escape latency (Fig. 2) [55].

For experiments with mice, the current formal design of the SAM apparatus was developed (Fig. 1A); which includes an open field intended as an anxiogenic factor, as with other open arenas [89–91]. Mice alone in the arena escape to the safety of the smaller outside chambers [55]. The initial escape is motivated by the OF, but inhibited by the novelty of the escape route and its unknown destination, which significantly delays its first use (initial novel escape anxiety; Fig. 6A) [55,69]. Conversely, when test mice experienced social aggression from a larger conspecific, half of them reliably remain in the OF arena with the aggressor (Fig. 2). The other half escape with decreasing daily latency as the initial novel escape anxiety wears off (Fig. 6B), which was not driven by the amount of aggression received (Fig. 3). The submissive behavior of half the mice was surprising, as mice not experiencing social interaction clearly preferred the safety of the side-chambers to the open field. We hypothesized that the behavioral split resulting from this choice, in this and other species, was due to the level of anxiety individually experienced. In the SAM, mice in the novel open field are apprehensive of passing through the escape hole, as it is initially unfamiliar and they are unsure of what's on the other side. The rapid decrease in latency to escape is indicative of the decreased anxiety that comes from the familiarity of the model, and can be facilitated by anxiolytics such as neuropeptide S (NPS), which when administered intracerebroventricularly (icv) reduces latency to escape in the initial and subsequent trials (Fig. 6A) [69,92]. Escape, although initially anxiogenic, also affords relief from fear of the novel OF and escape routes in the initial trial and attenuates apprehension of the familiar OF and route in subsequent trials (Fig. 6A, B). The decision-making process is altered by adding social aggression to the model, which increases the stress experienced by the individual, as indicated by increased glucocorticoid levels (Fig. 6D, E). Escape also mitigates the stress and anxiety generated by social aggressive interaction (compare left bars, Fig. 6E, F).

When mice were confronted with social aggression, escape followed a trend of decreasing latency similar to other species, as they became familiar with the model (Fig. 6B). However, the 50% which did not escape (Fig. 2) also followed a similar trend to those seen in other species (Fig. 5), with early submissive behavior presaging social defeat. We hypothesized that this was because the anxiety of the novel initial escape in the presence of the aggressor was greater than the fear that came

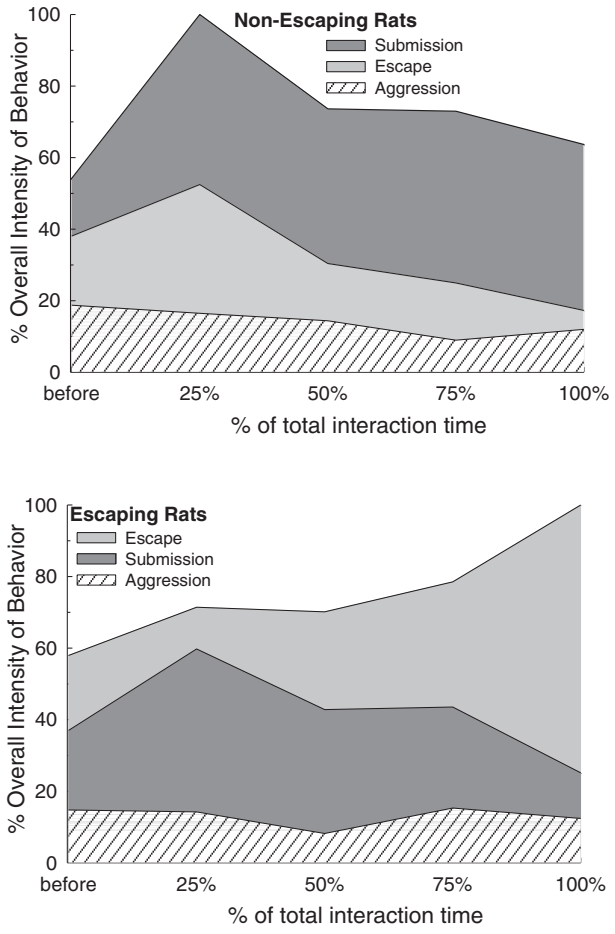

Fig. 5. Expression of phenotypic behavioral attributes over time (% total interaction) in Sprague Dawley rats in SAM is distinctive depending on whether the interactions result in submission and no escape (top) or escape (bottom). The original raw data are normalized to represent a percentage of the total behavior in any given quartile of the total duration of the interaction (% total interaction time). Only one quartile exhibits 100% intensity of behavior (at 25% of the interaction duration for submissive rats, and 100% for escaping rats), other quartiles had lower total behavioral responses. The overall intensity of actions associated with submission (dark gray), escape (light gray), and aggressive (hatched) behaviors is represented by the thickness of the layer for each type of behavior. Top) Non-escaping rats quickly establish submissive behavior as most intense, and actions related to submission dominate the interaction throughout. Bottom) Escaping rats initially show submission-related behaviors along with escape behavior, then quickly switch to more escape-related behaviors, ultimately resulting in escape.

from social interaction. Exercise is known to reduce anxiety, and the short-term (6 days) addition of a running wheel, reduced initial and subsequent latencies to escape during social stress (Fig. 6B). However, the anxiolytic effect of wheel running (Fig. 6B, E, F) did not change submissive behavior into escape by those initially choosing to remain with the larger aggressive conspecific. These transitions between low and high intensity anxious behavior (changing submissive behavior into escape; escape behavior into submission) are possible with anxiolytic and anxiogenic drugs (Fig. 7 top). The anxiolytic CRF_1 receptor antagonist antalarmin allowed 40% of formerly submissive animals to escape (and escape quickly; Fig. 6C), while the anxiogenic norepinephrine (NE) α_2 receptor antagonist yohimbine inhibited 67% of formerly escaping animals (slow escape time; Fig. 6C) and made them behave submissively [69,92]. Our results have confirmed that novel escapes are accompanied by more anxiety than familiar ones. Likewise, the inability of some animals to escape from an aggressor indicates that this experience (social anxiety) is more intense than the anxiety produced by the open field alone. These data combined with those of our other experiments indicate that the submissive animals are experiencing more anxiety than those which choose to escape. Taken together, with submission to social defeat

as the most intensive anxious behavior, and escape from a familiar empty arena as the least anxious, these experiments describe a gradient of anxiety, which is ameliorated differentially by endogenous neuropeptides, physical apparatus, and pharmaceutical interventions.

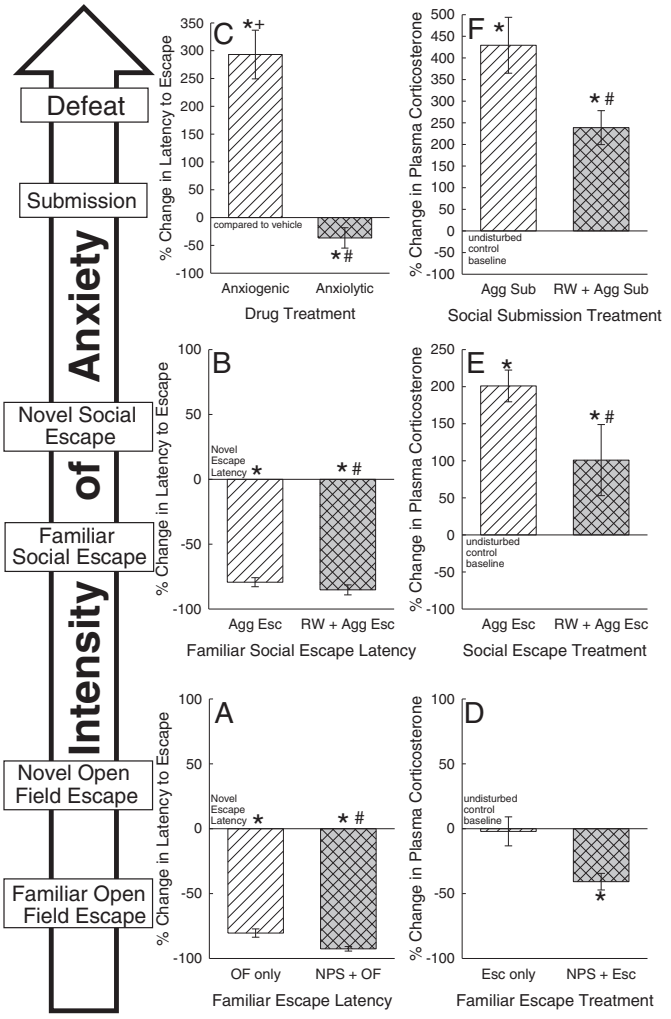

Fig. 6. Familiarity reduces anxiety. A gradient of anxious behavior (open field → social interaction → social defeat) is verified by latency to escape and plasma corticosterone concentrations in mice (*read from the bottom up*). The data are normalized to novel escape latency (A, B), latency to escape after control vehicle drug treatment on day 3 (C), undisturbed control baseline corticosterone concentrations (D, E, F). Left column, latency to escape: A) Open Field (OF) test using SAM reveals significantly faster escape from the OF (left hatched clear bars) on repeated trials (familiarity), and even faster escapes after anxiolytic NPS treatment (double hatched gray bars; *significantly [$P < 0.05$] different from novel escape latency normalized to = 0%; #significantly reduced from OF only). B) Faster escape occurs with repeated trials (familiarity) during social aggression (Agg Esc; clear hatch), and even faster escapes after anxiolytic running wheel treatment (gray double hatch; *significantly different from novel escape latency during social interaction normalized to = 0%; #significantly reduced from Agg Esc). C) Anxiogenic treatment (yohimbine = α_2 antag; clear hatch) increases latency to escape in previously escaping animals, while anxiolytic treatment (antalarmin = CRF_1 antag; gray double hatch) reduces latency in previously submissive animals (*compared to vehicle baseline, +compared to anxiolytic, #compared to anxiogenic). Right column, plasma corticosterone concentrations: D) Familiarity with escape (clear hatch) results in low corticosterone secretion (not different from undisturbed controls, and the addition of the anxiolytic NPS (gray double hatch) significantly reduces corticosterone concentrations (*compared to undisturbed controls). E) Potent glucocorticoid response after repeated exposures to aggression and escape (Agg Esc; clear hatch), is reduced by exposure to a running wheel (gray double hatch; *compared to controls; #compared to Agg Esc alone). F) The largest increase in corticosterone is found in submissive animals following social interaction (Agg Sub; clear hatch), which is reduced by escape (compared to 6E clear hatch), or access to running wheel (gray double hatch; *compared to control concentrations, #significantly reduced compared to Agg Sub).

Anxiogenic interventions increase plasma levels of corticosterone (Fig. 6), central amygdalar gene expression of neuropeptide S (NPS; Fig. 8) and BDNF (Fig. 9), all of which are ameliorated by treatments that functionally reduce anxious behaviors (Figs. 6–9) [69,92]. The correspondence of anxiogenic and anxiolytic treatments with changing plasma corticosterone levels verify the model, and corroborate the existence of a gradient of anxiety which spans low-to-high intensities. Where test animals fell on this scale was dependent on both the experience of the individual and their nuanced behavior in the context of social aggression.

The experience of repeated social defeat had lasting effects on the anxiety of the aggressor mice as well [93,94]. Larger CD1 mice, paired with a novel smaller mouse for ten consecutive days of trials quickly altered their response to the social context, by reducing latency to attack and increasing the intensity of attacks, targeting the head of the smaller mouse more often than the rump. Interestingly, two months after all aggressive interactions ceased, these aggressive CD1 mice still showed heightened anxiety as measured by the elevated plus maze (EPM) as well as enhanced gene expression of NPS in the central nucleus of the amygdala (CeA) and vasopressin (AVP) in the hypothalamic region associated with aggression in mice (ventrolateral ventromedial hypothalamus, vlVMH) [93,94]. This protracted gene expression for AVP in the vlVMH was positively correlated with anxious behavior on the EPM after two months. Neuropeptide S in the CeA was negatively correlated with the intensity of aggressiveness, which corroborates results showing the inhibitory effect of NPS on aggression [93–95].

5. Discussion

Bob Blanchard was extremely helpful when we discussed the early iterations of the SAM model with him. It is not surprising; Bob and the VBS led the way for a myriad of researchers, including us. The nuanced behavior that Bob described in his earliest work led him to develop the VBS. The subtle and overt interactions between any aggressor and victim suggest that there is much more to social interaction than just victory and defeat. With the VBS, Bob began to describe to all of us the subtle differences between how each cohort of rats or mice behaved, and the decision-making that they were engaged in. In our own work, the nuanced behavior that we observed in rats suggested that social decision-making was an important part of behavioral outcomes, especially when escape is available. This was clear in the description of VBS behavior with respect to which social arenas and tunnels each cohort of animals would use. That led us to the idea that a singular decision-making process, to escape aggression or not, would be particularly illuminating for both aggression and decision-making.

Bob Blanchard's VBS created a context-rich arena that allowed for the nuances of behavior to be observed and manipulated. Through the establishment of social systems, individuals sorted themselves into groups according to rank and status. The semi-natural setting allowed for this self-segregation, and therefore the study of context-dependent decisions. During social interaction in the VBS development of social ranks and status constituted a dramatic change in social context from the initial, novel condition, which could be augmented by the presence of a cat, forcing re-segregation of territory and interaction among groups. We designed a system which follows this example, wherein individuals self-segregate during the process of decision-making into those that escape social aggression and those that remain and submit to it. As Blanchard used the VBS to demonstrate the importance of the dominant–subordinate interaction in the development of stress, use of the SAM revealed that anxious decision-making varies individually and contextually.

Bob Blanchard used the VBS and the Mouse Defense Test Battery to show how behavioral systems like defense are dependent upon context and behavioral elements unique to the individual [28,47,59]. Similarly, individual behavior and context make dramatic differences with respect to escape and submission in the SAM. In both fish and mammalian SAM

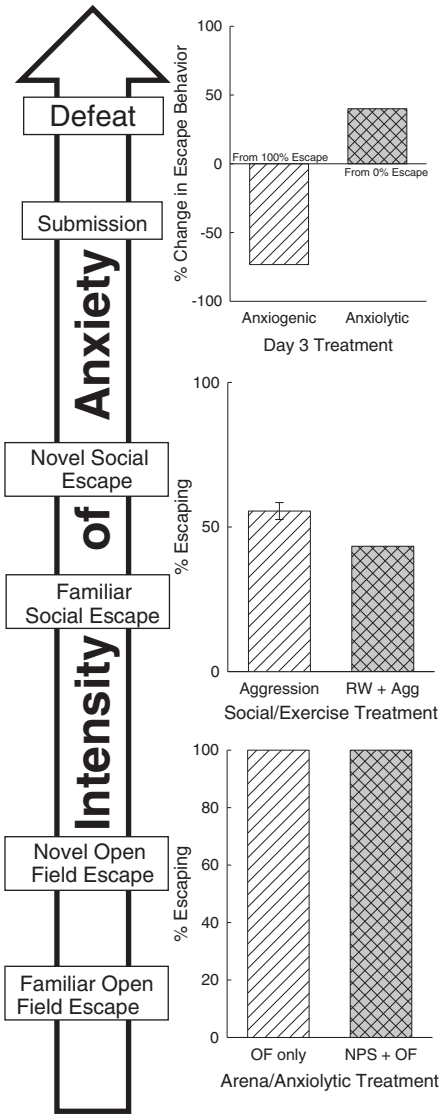

Fig. 7. Escape behavior (in the SAM) reflects anxiety depending on social context. A gradient of anxious behavior in mice is verified by % of mice escaping (*read from the bottom up*). Bottom) Neuropeptide S (NPS) injection (icv) does not affect the % of mice escaping from open field (OF) in the absence of social interaction (100%). Middle) Social interaction (Agg) reduced the % of escaping mice to approximately 50%. The addition of a running wheel did not affect the % of mice escaping from social aggression. Top) Injection (ip) of anxiogenic and anxiolytic drugs influenced the % of animals escaping; such that the anxiogenic yohimbine ($=\alpha_2$ antag; clear hatch) dramatically inhibited escape (67% reduction, compared to 100% escape prior to treatment), and anxiolytic antalarmin ($=CRF_1$ antag; gray double hatch) promoted escape (40%) in previously non-escaping animals (0%).

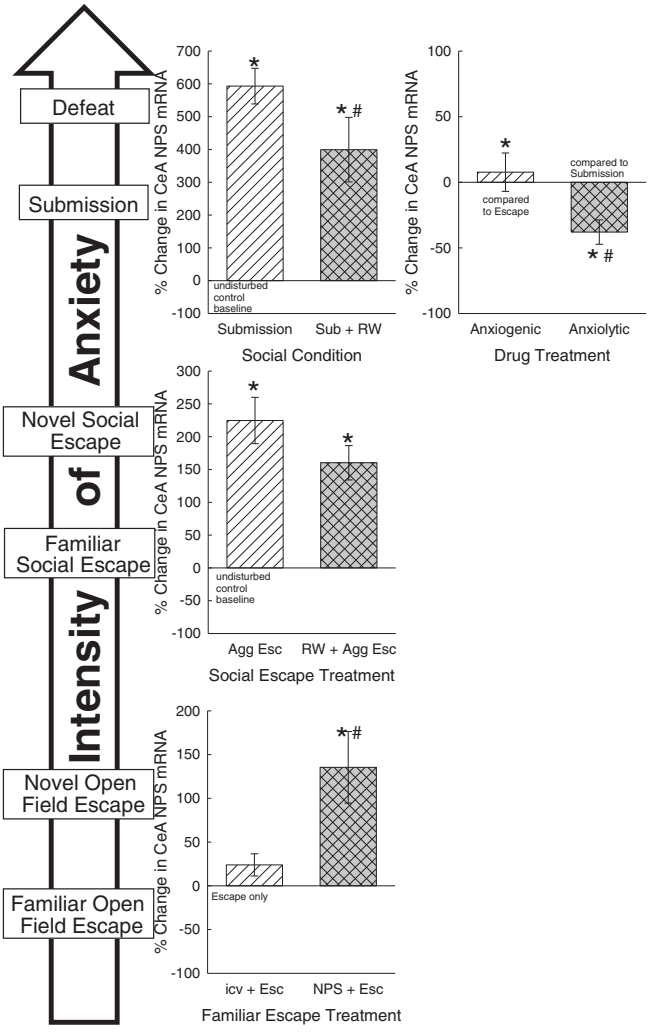

Fig. 8. A gradient of anxious behavior in mice is verified by neuropeptide S (NPS) gene expression (mRNA) in the central amygdala (CeA) (*read from the bottom up*). Bottom) NPS injection (icv; gray double hatch) significantly increases NPS mRNA expression compared to vehicle (clear hatch) when escaping from the Open Field (*compared to undisturbed controls; #compared to vehicle icv escape). Middle) Escaping aggression (clear hatch) increases NPS mRNA expression. Access to running wheel (gray double hatch) shows a trend toward attenuating NPS mRNA response in CeA to escaping aggression (*compared to undisturbed controls). Top left) Submitting to aggression (clear hatch) significantly increases NPS mRNA expression. Access to running wheel (gray double hatch) significantly reduces NPS mRNA response in submissive individuals (*compared to undisturbed controls, #compared to submission alone). Top right) Injection (ip) of anxiogenic and anxiolytic drugs on day 3 influenced NPS mRNA expression. Escaping animals treated with anxiogenic yohimbine ($=\alpha_2$ antag; clear hatch) had increased NPS mRNA expression (*compared to undisturbed controls but not baseline of escaping animals). Submissive animals treated with anxiolytic antalarmin ($=CRF_1$ antag; gray double hatch) had reduced NPS mRNA expression (*compared to submissive animals, #compared to anxiogenic treatment).

experiments, the results surprisingly suggest that no facet of intensity, frequency, or duration of aggressive behavior promotes escape (Fig. 3). In the VBS, like the SAM, interactions very quickly determine outcomes; even though in the SAM submissive animals receive the same amount of aggression as escapers. Pre-tests on standardized anxiety platforms like the elevated plus maze (EPM) suggest that both SAM outcomes are derived from animals with equal levels of generalized anxious behavior. While submissive animals demonstrate early and consistent preference for submissive behaviors (Fig. 5 top), escaping animals also demonstrate a subtle understanding of the social environment in order to affect escape. Escaping fish learn to anticipate the patrolling actions of larger aggressors, and with experience, only choose to escape when the larger fish isn't watching (Fig. 4). This observer effect [96] is likely an important part of all social interactions, especially those that include aggression.

The observer effect has implications for both victim and aggressor, with protracted aggressive behavior producing long-term consequences for defeated animals [97], and anxious phenotypes for aggressors [93]. These long-term anxious phenotypes may derive from gene-by-environment interactions that result in protracted enhancement of gene expression in specific portions of the brain. The reason that social interactions have such high impact on the participants is that the pain and potential mortality involved are amplified by the unpredictable and uncontrollable nature of the interaction [98]; and such stressors are always the most potent [51,99]. The validity of research on nuanced social interaction stems from its ability to evoke such a strong response in an individual. The interactions are potently stressful, and nuances in behavior is what makes them uncontrollable and unpredictable [98].

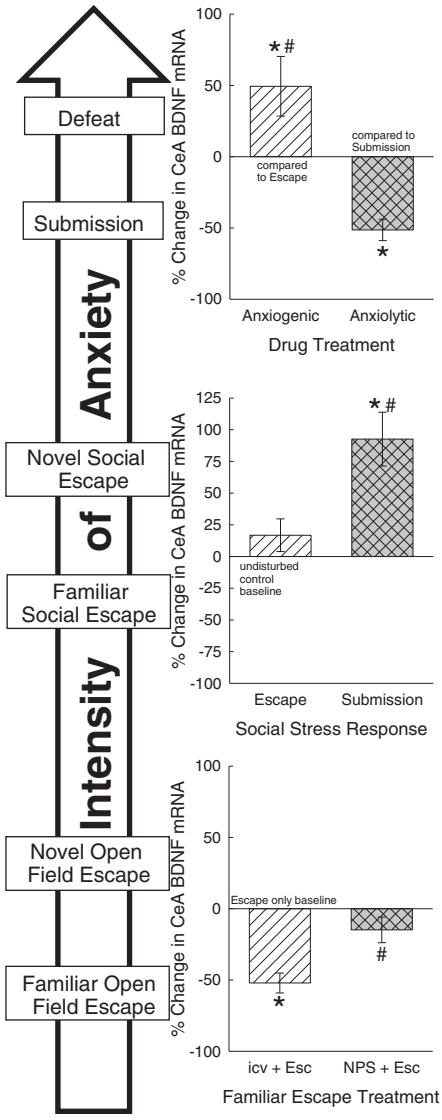

Fig. 9. A gradient of anxious behavior in mice is verified by brain-derived neurotrophic factor (BDNF) gene expression (mRNA) in the central amygdala (CeA) (*read from the bottom up*). Bottom) Injection (icv) of NPS (gray double hatch) significantly attenuated the reduction of BDNF mRNA expression caused by icv injection of vehicle (clear hatch) when escaping from the Open Field (*compared to escape only controls; #compared to vehicle icv escape). Middle) Submitting to aggression (gray double hatch) significantly increases BDNF mRNA expression (*compared to undisturbed controls, #compared to escaping animals; clear hatch). Top) Injection (ip) of anxiogenic and anxiolytic drugs on day 3 influences BDNF mRNA expression. Escaping animals treated with anxiogenic yohimbine (=α_2 antag; clear hatch) had increased BDNF mRNA expression (*compared to escaping animals, #compared to anxiogenic treatment only). Submissive animals treated with anxiolytic antalarmin (=CRF_1 antag; gray double hatch) had reduced BDNF mRNA expression (*compared to submissive animals).

Bob Blanchard created an opportunity for researchers to study how aggression and social interaction lead to the production of anxious behavior in a naturalistic model [16,18,22–37]. The colony dynamics of the VBS revealed the neural and physiological underpinnings of the stress response, and cemented many concepts which are now considered foundational in our understanding of the behavior of social species [17,19,47,48,64–66,100–110]. Changes in animal behavior following the introduction of a cat showed how anxiety correlated with changes in behavior via risk assessment [2]. Blanchard recognized the value of naturalistic models for elucidating psychological disorders, and called for the creation of other new models which incorporated similar dynamics [2]. The SAM was originally designed for the purpose of examining social decision-making, a critical factor in maladaptive outcomes associated with anxious and depressive behaviors. Results from the SAM show something remarkable yet intuitive: anxious behavior in model organisms, like in humans, is not static and uniform, but rather reflects the intensity of the context in which it occurs. The intensity of context is amplified as the potential for harm or mortality increases and is heightened even more when the events are unpredictable and uncontrollable [51,52,69,98,99]. More intense situations result in a greater magnitude of apprehension, and yield a gradient of anxiety, built around the outcome of individual decision-making via risk-assessment. In addition to the increased concentration of plasma corticosterone that occurs at each stage of the anxiety gradient (Fig. 6D–F), gene expression in the CeA is also markedly influenced by the intensity of anxious behavior being expressed. Gene expression for NPS in the CeA, a known endogenous anxiolytic [95,111–118] in a brain region tightly linked with expression of fear and anxiety [119–125], is progressively increased with incrementally increasing anxious behavior (Fig. 8). Accompanying increased expression of NPS mRNA were increases in BDNF expression in the CeA for anxiogenic conditions such as social defeat and α_2 antagonist treatment, which may be ameliorated by escape or CRF_1 antagonism (Fig. 9). Together, the results suggest a system of progressively modified anxious behaviors that are plastic to environmental conditions and stress-related neuromodulation.

Bob Blanchard, from his very earliest work, led the way to translational models that have the greatest clinical relevance available today, because they model social interaction [3,126]. Bob noted that "Subordinate males are characterized by particular wound patterns, severe weight loss, and a variety of behavioral changes, many of them isomorphic to target symptoms of clinical depression" [19]. The VBS clearly led the way to the social defeat model of depression and anxiety commonly used [127–130]. Translational studies aimed at elucidating anxiety and depression may be facilitated by the combination of open field, social escape, and social defeat elements that are combined in the SAM apparatus.

6. Conclusions

The integrative and translational value of this work is immeasurable. The Visible Burrow System fundamentally changed the way we think about social stress and affective disorders, making Bob Blanchard a pioneering behaviorist, in that he found a way to study the interaction of genetics, physiology, environment, and adaptive significance in a model with broad validity. The Stress Alternatives Model isolates some of the unique behavioral components of the Visible Burrow System into a situation with a clear dichotomous outcome. The split between escaping and submissive animals highlights an adaptive behavioral split that is evolutionarily conserved, and may predict psychosocial disorders [51–54]. In the SAM, individuals must weigh the anxiety of their initial escape against fear of their aggressor. We assert that the stress being experienced by submissive individuals is greater than that experienced by escapers, and this is corroborated by our findings. The gradient of anxiety that spans the contextual settings of escaping the open field, escaping from aggression, and submitting to aggression corresponds with increasing levels of plasma corticosterone and increasing levels of NPS and BDNF in the central amygdala. Whereas some anxiolytics were able to reduce the latency to escape for some animals (putatively illustrating a decrease in anxiety regarding the act), only with the potent anxiolytic drug antalarmin (CRF_1 antagonist) and the anxiogenic drug yohimbine (α_2 antagonist) were we able to reverse the outcome for individuals.

In an era when scientific legitimacy is increasingly based on results, Bob Blanchard reminded us that the means by which we study behavior are equally important. He did research that was better at capturing the dynamism of a natural environment, and drew important parallels between the mechanisms of defensive-behavior in social species. As we continue to investigate the neural mechanisms involved in producing anxiety and potential therapies for anxiety-related disorders, Bob

Blanchard's influence on how we design our model systems and behavioral paradigms, specifically, using naturalistic settings to explore translational interactions of physiology, genetics, and environment on social adaptations will continue to inform us.

Acknowledgments

This work was substantially inspired and influenced by Robert J. Blanchard. The section on the Visible Burrow System (Section 2) was written by D. Caroline Blanchard, to whom we express many thanks for this contribution. Dr. Blanchard also provided substantial help by making suggestions and corrections to the manuscript. We also thank Samuel Young for contributions to analysis of the effect of aggression on escape. Information in this article is covered by certain pending patents owned by the South Dakota Board of Regents and managed by the University of South Dakota. This research was funded by NIH grants R15 MH104485 and P20 RR15567, a CBBRe Research Enhancement Pilot grant, and by an anonymous donor to the Summers' lab via the USD Foundation.

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