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Review Article

Nonpeptide corticotropin releasing hormone type 1 receptor antagonists as medications and imaging agents

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Abstract

The stress response is commonly thought of as a fight or flight reaction to external adverse events. Recent discoveries show that prolonged activation of the stress system is detrimental to health and a common feature of medical and psychiatric disorders. Corticotropin-releasing hormone (CRH) is a principal regulator of the stress system as both a hormone and neuropeptide neurotransmitter. Overproduction of CRH causes chronic activation and overproduction of hormones from the hypothalamus, pituitary and adrenal glands. The discovery of nonpeptide antagonists that block the actions of the CRH type 1 receptor (CRHR1) has led to a greater understanding of the physiology of the stress system and has also become a target for drug development. The utility of *in vivo* markers for drug action has led to an increased interest in functional neuroreceptor imaging. Radiotracers for specific receptors for single photon and positron tomography imaging can localize and quantitate specific molecular targets. Imaging specific molecular sites where drugs act will aid in the diagnosis of disease states that are not well characterized by other clinical assessments. Functional neuroimaging will provide an objective measurement of therapeutic response to new medications such as those targeted to block CRHR1. CRHR1 tracers will be invaluable probes to better understand the stress system in health and disease.

Introduction

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide produced in the brain and in the periphery that coordinates the body's response to stress. When CRH is synthesized and released from the hypothalamus, it activates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which stimulates adrenal release of cortisol, the principal adrenal steroid hormone in primates. CRH and CRH-like molecules are produced in the central nervous system (CNS) and act as peptide neurotransmitters. CRH and its specific receptors are widely disseminated throughout the CNS. The regional actions of CRH in the CNS are varied and only partially understood. In addition to regulation of the hypothalamic-pituitary-adrenal (HPA) axis, CRH coordinates other hormones as well as autonomic, immune and behavioral responses to stress. Through its actions on the sympathetic nervous system, CRH affects a wide array of cardiovascular, metabolic and behavioral effects. Aberrant CRH action is implicated in many diseases including addiction, depression, anxiety and eating disorders and is likely to be important in many others as reviewed in Chrousos (1).

Our goal has been to understand the physiological role of CRH and the pharmacological actions of the CRHR1 antagonists. We have studied the action of antalarmin, a potent CRHR1 antagonist, and developed a radiolabeled CRHR1 ligand as an *in vivo* imaging agent. The ability to localize and quantify this CNS receptor will lead to an improved understanding of CRH and CRHR1 specific drug actions.

Table I: Binding properties of members of the CRH neuropeptide family.

Peptide binding (K_1, nM)	$hCRHR_1$	$rCRHR_{2\alpha}$	$mCRHR_{2\beta}$
CRH (rat/human)	3.3	42	47
CRH (sheep)	1.1	230	320
Ucn (rat)	0.32	2.2	0.62
Ucn (human)	0.4	0.3	0.50
Ucn II # (human)	>100	1.7	0.50
Ucn II # (mouse)	>100	2.1	0.66
Ucn III $ (human)	>100	21.7	13.5
Ucn III $ (mouse)	>100	5.0	1.8
Urotensin I (fish)	0.4	1.8	5.7
Sauvagine (frog)	0.70	0.52	0.1

#Stresscopin related peptide; $Stresscopin. Adapted from Reul, J.M., Holsboer, F. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2002, 2(1): 23-33.

Endogenous CRH compounds and their receptors

The investigation of CRH physiology and pharmacology has been limited by the availability of specific endogenous and nonpeptide ligands for CRH receptors. The peptide CRHs, urocortin (Ucn), stresscopin (Ucn III), stresscopin-related peptide (Ucn II), urotensin I and sauvagine all share similar chemical characteristics, although they display different actions at and affinities for CRH receptors (Table I). Though not specific for the CRH receptors, the CRH binding protein (CRH-BP), a 37 kD protein, binds CRH with high affinity, limiting the biological action and exposure of CRH to its receptors (2).

In 1993, CRHR1 was cloned (3, 4) and subsequently a second CRH receptor, CRHR type 2 (CRHR2) was discovered and sequenced with at least 3 specific receptor subtypes identified (5, 6). CRH receptors belong to the G-protein receptor super-family and contain 7 transmembrane regions. They have been identified in both the CNS and in the periphery. CRHR1 has 415 amino acids and has a structure similar to that of calcitonin/vasoactive intestinal peptide/growth hormone-releasing hormone receptors. CRHR1 signaling may involve both G-protein and cAMP activation. CRHR2 has 411 amino acids with about 70% homology to CRHR1 and operates via a similar second messenger cascade. The 3 CRHR2 subtypes have been identified in brain, pituitary, spleen and circulating lymphocytes. Higelin *et al.* report that antisauvagine-30, an N-terminally truncated version of sauvagine, is a specific CRHR2 antagonist (7). A recently discovered peptide, stresscopin or Ucn III and its related peptide(s) have been discovered and proposed as the endogenous ligands for ligands for CRHR2 along with urocortin (8, 9). It is interesting to note that CRH does not appear to be the principal endogenous ligand for the CRHR2. Specific endogenous peptide ligands for CRHR2 have been discovered although no nonpeptide antagonist for CRHR2 has been identified yet (10).

These peptides do not cross the blood brain barrier and CRHR1 and CRHR2 receptors are widely distributed in the primate brain in a distribution which is distinct from that observed in rodents. Both CRHR1 and CRHR2 are found in the hypothalamus and are important for hypophyseal ACTH release. CRH is produced in the hypothalamus and is secreted into the portal plexus of veins to activate the pituitary. In addition to the pituitary, a high CRHR1 density is found in neocortex, hypothalamus, amygdala, insula and hippocampus (11, 12). It appears that as primates evolved, CRHR1 became more dense in the neocortex, with receptors heavily expressed in areas of brain ascribed to higher cognition (*e.g.*, frontal and prefrontal lobes) as well as in more “primitive” brain regions sites associated with emotion, memory and visceral functions (*e.g.*, amygdala, hippocampus, insula and brain stem). It is important to note that there is significant interspecies variability in the neuroantomical distribution of CRH receptors. As an example, the rat anterior pituitary expresses mainly CRHR1 while humans express both CRHR1 and CRHR2.

Physiology and pathophysiology

The investigation of the integration of the central and peripheral regulation of the HPA began in earnest with the discovery of CRH in 1981. Still the subtle actions of stress hormones (glucocorticoids, ACTH and CRH) and neurotransmitters and their roles in activating the CNS remain unclear. As mentioned above, CRHR1 and CRHR2 receptors are widely distributed and densely concentrated in the primate brain in manner distinct from the rodent. A high density of CRHR found in neocortex, hypothalamus, pituitary, amygdala and hippocampus (11, 12). The circuitry of CRH neurons in the CNS is complex and involves many pathways in the neocortex as well as CRH cell bodies in the central nucleus of the amygdala which send projections to the brain stem and limbic structures. CRH neurons from the central nucleus of the amygdala course caudally to brainstem areas that regulate the autonomic nervous system. The locus ceruleus, raphe nuclei and parabrachial nucleus constitute the major sites of origin for NE and serotonin (5-HT) neurons and are important in regulating sleep, arousal, cardiovascular and visceral autonomic functions. Recent neuroanatomical studies showed colocalization of these centers with CRH cell projections. Rostral projections to the hypothalamus, especially the paraventricular nucleus, direct neuroendocrine regulation. NE, dopamine and 5-HT neurons project through the hypothalamus to the frontal and prefrontal cortices, and contribute to cognition and executive functioning (13).

CRHR1 activation enhances stress-induced learning and the CRHR1 knockout (KO) mouse fails to display anxiety-like behavior even when challenged by environmentally stressful stimuli. When an animal is repeatedly exposed to inescapable stressors such as foot shock, endogenous CRH is released into the CNS and activates

the CRHR1. When peptide CRH is administered to the rodent brain it precipitates a similar behavioral profile; blockade of the CRHR1 with antalarmin, a nonpeptide antagonist, suppresses both the formation of conditioned fear and the behaviors seen with CRH administration (14, 15). Activation of hippocampal CRHR1 enhances stress-induced learning with evidence that CRHR1 in the amygdala and hippocampus mediates anxiety responses in stress (16).

In contrast, CRHR2 activation in the lateral intermediate septum (LMS) in the mouse tonically impairs stress-induced learning. However, the local injection of high doses of CRH into the LMS, an area concentrated with CRHR2, precipitates anxiety-like responses. The different actions of CRH in numerous brain regions indicate that CRH and its receptors have complex effects on behavior and physiology. The CRHR2 can be blocked specifically by antisauvagine-30, however peptide antagonists for CRH are rapidly degraded, have poor bioavailability and thus have limited therapeutic utility (17).

Cortisol production and its actions in CNS also mediate behavioral response. The actions of glucocorticoids (GC) are complex but appear to be influenced by early life experiences and even prenatal exposure to stress. The density of the GC and other steroid receptors such as the mineralocorticoid receptor in the hippocampus, amygdala and neocortex, have a role in the function of the HPA and are influenced by stress in early life. Thus GC action at the GC receptor modulates CRH, either magnifying or diminishing the acute stress signal to the brain based on the individual's prior exposure to stressful events (18). The precise actions and interactions of these feedback events are unknown but appear to be critical in genesis and perpetuation and neuropsychiatric disease.

Neuropsychiatric disorders

Overproduction of CRH in the CNS often occurs in patients with anxiety, depression, eating disorders, substance abuse and addiction. The normal integration of the stress response often becomes deranged in these conditions. It is believed that overproduction of CRH in the CNS and the aberrations in stress hormones are causal and merely a consequence of the disease state(s). Both hyper- and hyporeactive stress responses have been observed in depressive disorders.

Substance abuse

Alcohol abuse increases CSF CRH (19) causing activation of the HPA and resulting in chronic cortisol overproduction; in severe alcoholic disease, this can induce a psuedo-Cushings syndrome. Preclinical studies show that CRH is overproduced when the administration of an addictive substance is abruptly stopped. This activation of CRH neurons mediates the stress-like behavioral and endocrine profiles associated with drug withdrawal syndromes (20-25). Additional evidence suggests that activation of CRH neurons contributes to the motivational and reinforcing properties fundamental to substance abuse. Comorbidity with other psychiatric disorders characterizes a majority of substance abusers. Although CRH overproduction most probably contributes to the symptoms in these patients, more research is need to further understand stress and addiction.

Anxiety

Investigations undertaken in our laboratory revealed that administration of antalarmin to macaques exposed to intense social stressors significantly attenuated behavior associated with anxiety and fear (*i.e.*, body tremors, grimacing, teeth gnashing, urination and defecation). Conversely, antalarmin increased exploratory and sexual behaviors that are normally suppressed during stress. Antalarmin reduced the expected increase in CSF CRH as well as the pituitary-adrenal, sympathetic and adrenal medullary activation. Because CRH plays a major role in the physiological responses to physical and psychological stress, CRHR1 antagonists like antalarmin may prove to be a valuable therapy for anxiety (15).

Depression

The clinical development of nonpeptide CRHR1 antagonists has thus far been in industry-sponsored trials. The results from these trials are proprietary and few findings have been made available. Zobel *et al.* presented data from a brief clinical trial using a pyrrolopyrimidine with potent CRHR1 antagonist action. In a dose escalation paradigm, the authors found that at tolerable doses the medication showed efficacy in reducing both anxiety and depression in patients without evidence for central suppression of the HPA axis (26). This study, although limited in design and statistical power, demonstrates the efficacy of CRHR1 antagonists in the treatment of depression. Given that HPA hyperactivity predicts relapse to depression, CRHR1 medications may be important in the management of refractory depression (27). Activation of the HPA and an intact hormonal response to stress is essential for survival in life-threatening circumstances such as acute injury, overwhelming infection, psychological and/or physical danger. Further investigation of new antagonists will be necessary to assure that the HPA response remains intact in the setting of severe life-threatening stress.

Depression is heterogeneous, expressing a constellation of symptoms. Modulation of the HPA should prove to be a powerful tool in the understanding of depression. Future studies using CRHR1 medications and imaging will characterize both the depressive symptomology and the neuroendocrine features to improve treatment outcomes and to elucidate the biological basis of the disorder(s).

Eating disorders

Eating disorders are life-threatening diseases. They include deranged behavioral responses to food intake, altered appetite and satiety with severe metabolic aberrations in energy expenditure and nutritional requirements. Eating disorders are extraordinarily difficult to treat. Investigators believe that in conditions such as major depression and anorexia nervosa, the overactivation of the HPA system can result in metabolic derangements that include changes in body weight (28-30).

Leptin, a peripheral hormone secreted from adipose tissue, suppresses appetite. The metabolic effects of leptin are thought to be mediated through interactions with several orixigenic and anorexigenic neuropeptides including CRH. Recent observations confirm the existence of a bidirectional loop between CRH and the leptin system, indicating an interaction between the stress system of the CNS and the adipocyte (31-34). Animal studies show that CRH suppresses feeding. Also, CRH receptors are expressed in adipocytes human cultured adipocytes and peripheral administration of CRH was shown to modulate energy expenditure in human subjects. Nonetheless, the interactions between leptin, CRH and the HPA axis remain poorly understood and are likely to be complex. Glucocorticoids secreted from the adrenal under specific conditions may act to suppress leptin production possibly through decreasing hypothalamic CRH secretion which diminishes the satiety signal (34).

Imaging

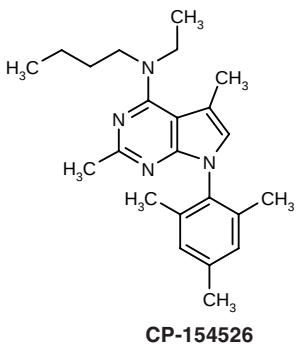
The physiological role of CRH in many clinical conditions led our team to investigate the feasibility of developing a radiolabeled CRH analogue for *in vivo* imaging. Positron emission computed tomography (PET) and single photon emission tomography (SPECT) are imaging technologies first adapted to measure blood flow and glucose metabolism. However with the development of specific receptor ligands, these techniques have been adapted for localization of brain and somatic receptors. PET involves labeling molecules with a positron-emitting radionuclide such as [^{11}C] ($t_{1/2}$, 20.4 min), [^{18}F] ($t_{1/2}$, 109.7 min) or [^{76}Br] ($t_{1/2}$, 960 min, 16 h), with detection of the high-energy dual photons by coincidence counting. Two 511 KeV photons are produced by positron annihilation. SPECT imaging uses radionuclides which emit single photons. There are advantages to each form of imaging technology. PET tracers with short physical half-lives permit multiple imaging studies within a short period of time. In addition, they emit dual high-energy photons that can be detected in a manner that allows a very high-resolution image. SPECT uses equipment that is widely available clinically and is less technically complicated. The logistic limitations of *in vivo* imaging can be formidable. Labeling with short-lived radiotracers constrain the pharmacodynamic properties that a specific receptor ligand can possess. Tracers with slow penetration into the brain and slow uptake at the receptor are of limited usefulness as *in vivo* markers. Also the short physical half-life of most PET radionuclides limits the time necessary for radiotracer synthesis and the distances that PET tracers can be transported. SPECT imaging uses radiotracers with longer physical half-lives, which generally allows longer synthesis time and the possibility to transport radiotracers to sites distant from the radiopharmacy.

However, one uncommon radiotracer bromine ([^{76}Br]) has the advantage of being a positron emitter with a long physical half life ($t_{1/2}$, 16 h). Bromine's long physical half-life affords more time for both synthesis and transport while still retaining the imaging characteristics of a positron emitter. [^{76}Br] is not however without technical limitations, it releases positrons and gamma photons with a variety of energies. These higher energy emissions may limit the amount of the radiotracer administered to patients following usual radiation guidelines.

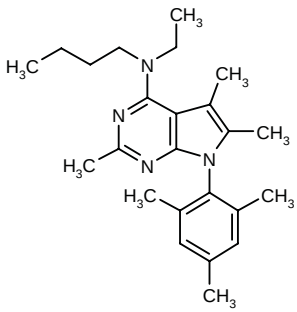
Radiochemical development of CRHR1 imaging agent

To date there are no specific *in vivo* imaging agents for the CRHR1 receptor. These studies were undertaken to develop a CRHR1 specific compound agent that would be amenable to rapid labeling with iodine or positron emitters. Initial studies to develop a labeled CRHR1 ligand focused on the chemical structures initially developed by Pfizer Pharmaceuticals (Fig. 1).

As previous mentioned pharmacological, behavioral and neuroendocrine studies in our laboratory have used



CP-154526



Antalarmin

Fig. 1. Chemical structures of CP-154526 and antalarmin.

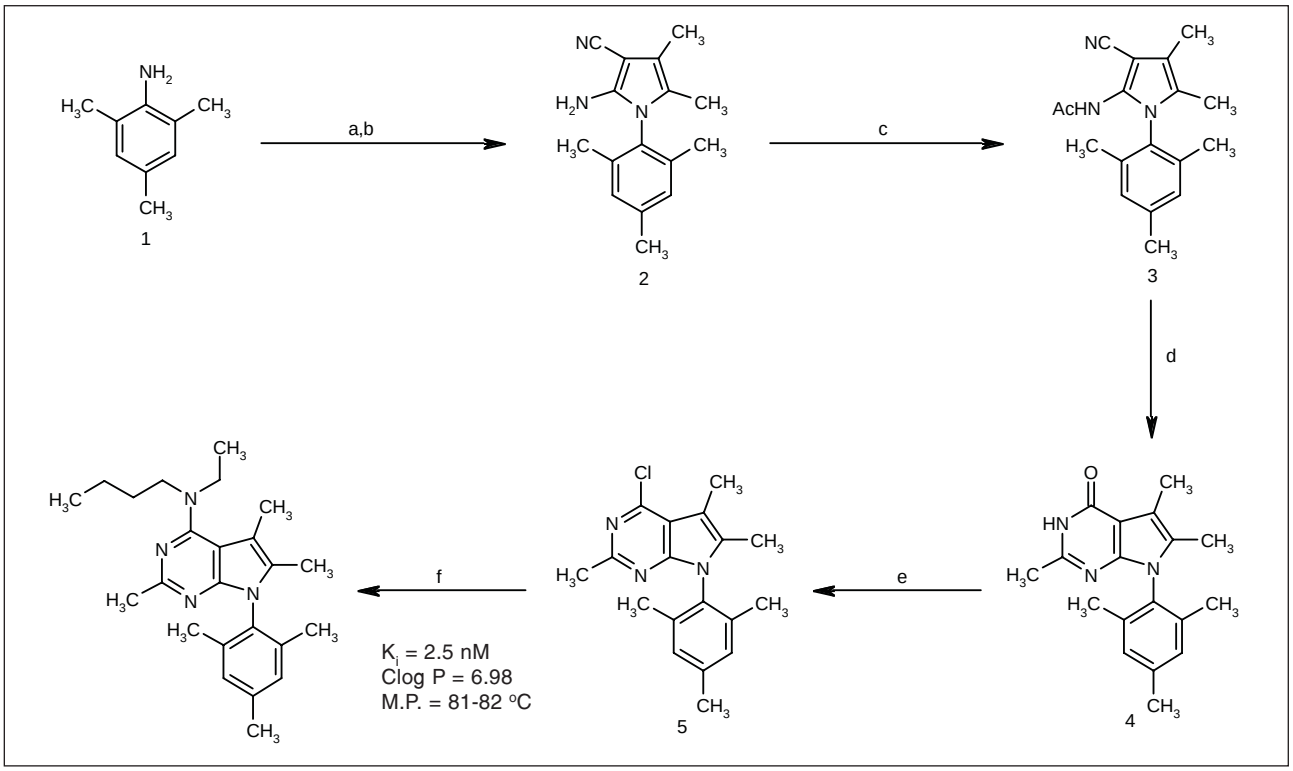


Fig. 2. Synthesis of crystalline antalarmin. (a) 3-Hydroxy-2-butanone; (b) malononitrile; (c) acetic anhydride, acetic acid; (d) phosphoric acid; (e) phosphorus oxychloride; (f) N-ethylbutylamine. According to the method of Chen, Y.L. International Patent Application WO 9413676, 1994 and crystallization as described in Bornstein, *et al.* Endocrinology 1998, 139: 1546-1555.

antalarmin, a CRH antagonist originally published by Pfizer. Antalarmin (*N*-butyl-*N*-ethyl-*N*-[2,5,6-trimethyl-7-(2,4,6)-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]amine) is a highly lipophilic antagonist with high CRHR1 binding affinity in the nanomolar range. The synthesis of antalarmin and its analogs with the functional side chains are shown in Figures 2 and 3.

Competitive binding studies show that antalarmin and related analogues have high affinity and specificity for CRHR1 and are able to block CRH biological actions *in vivo* and *in vitro* (35-37).

The initial steps in imaging ligand development involved investigation of the suitability of synthesizing and labeling compounds similar to antalarmin. One candidate compound incorporated fluorine at a site that would allow a rapid synthesis of [^{18}F] in the side chain, suitable for *in vivo* imaging purposes (38) (Fig. 3). This compound has been designated LHW-154 and was demonstrated to have high affinity for the CRHR1 at 0.91 nM. (38). To perform the necessary *in vitro, in vivo* and *ex vivo* tracer experiments, tritiation of LWH-154 was required (39). This synthesis enabled production of [^{3}H]-LWH-154 with a specific activity of 69 Ci/mmol.

Rodent distribution studies with [^{3}H]-LWH-154 showed that the fractional CNS penetration of the compound was low (< 0.5%) within the limited window of the [^{18}F] physical half-life. A significant portion of the total activity trapped in liver, suggesting extensive metabolic conversion. Subsequent calculations found that the molecule was highly lipophilic with a calculated log of partition coefficient (Clog P = 6.8 ± 1.33), above the optimal range for CNS penetration. Thus, in spite of its subnanomolar affinity, LWH-154 would not make a suitable radiotracer for imaging purposes. A subsequent investigation examined the requirements for lowering the lipophilic properties of compounds related to antalarmin while maintaining high affinity at the CRHR1 site (38). The next set of investigations sought to overcome the slow penetration of the molecule in to the brain. The use of radiotracers with longer physical half-lives would be one strategy to overcome slow penetration of a lipophilic molecule. The use of [^{123}I] with a physical half-life of 13 h would enable a longer time for the tracer to enter the CNS and reach receptor equilibrium. [^{123}I] is a commonly used isotope for SPECT imaging and many commercially and clinically available radiotracers incorporate it. To take advantage of these factors, other CRHR1 antagonist molecules were synthesized to incorporate iodine and the Neurocrine platform molecule was modified (Fig. 2) (39, 40). Precursor synthesis was accomplished and initial iodinations were performed with [^{125}I] ($t_{1/2}$, 60 days). Preclinical *in vitro, in vivo* and *ex vivo* experiments were repeated with similar results. In addition to limited CNS penetration, the uptake of the tracer in the CNS failed to mirror the known distribution of CRHR1.

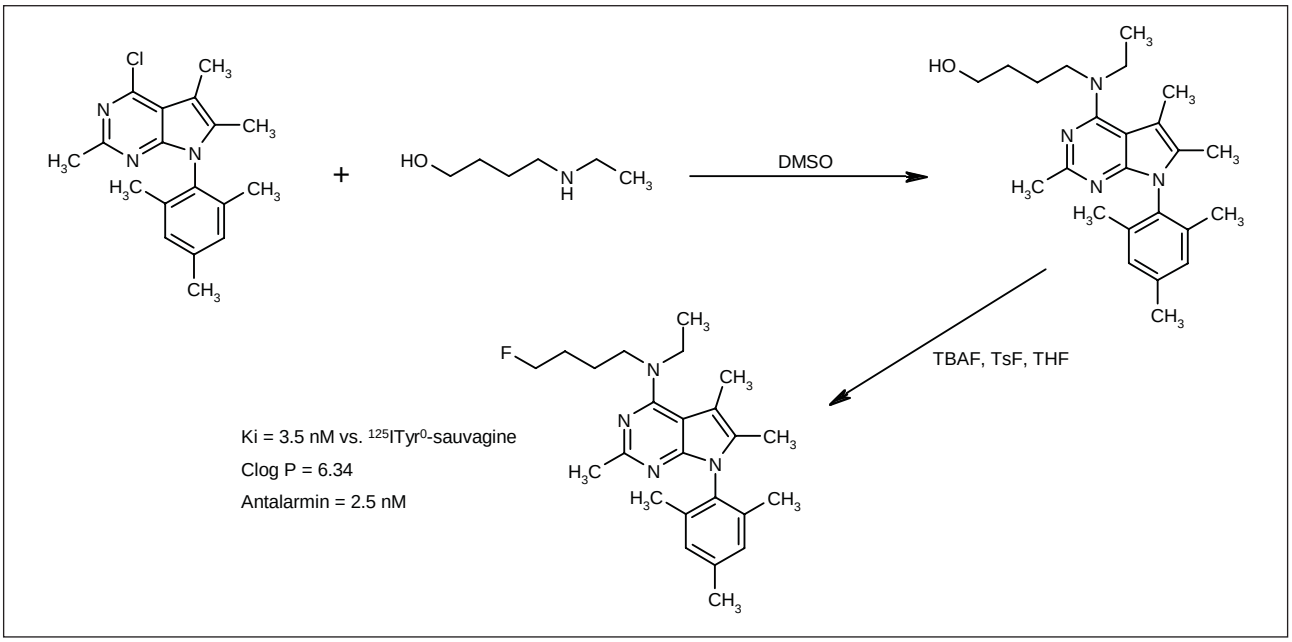


Fig. 3. Synthesis of LHW-154.

Current experiments are under way to exploit the advantages of the longer physical half-life of [^{76}Br] to label CRHR1 molecules with optimal Clog P and binding characteristics to enable *in vivo* imaging.

Future directions

The promise of specific neuroreceptor diagnostic imaging for neurological and psychiatric disorders has yet to be realized. Functional CNS imaging is primarily a research tool, though now metabolic and blood flow studies of the brain are routinely used diagnostically for a limited numbers of medical and psychiatric indications (*i.e.*, dementia, cerebrovascular accidents, oncology). Functional neuroreceptor imaging remains a research tool but eventually the ability to target molecular sites will find clinical applications. As CRHR1-specific medications prove valuable in disease treatment the ability to quantify molecular targets and initiate specific therapy will be the diagnostic tools of the future. The availability a neuroreceptor PET ligand as a diagnostic test that predicts therapeutic response for a medication class would be a major advance in therapeutics. Specific ligand imaging studies will aid clinicians in monitoring therapy and understanding the behavioral characteristics and neuroendocrine responses of different stress related disease. Increased knowledge of the functional nature of the CRH neuron in real time will elucidate the physiology of human stress response. Imaging data of CRH may show commonalities in HPA and autonomic nervous system activation in somatic and psychic disease states and predict individual and group vulnerability to stress. This work has implications for the understanding sociocultural response to stress as seen in different human populations.

Depression as many neuropsychiatric diseases comprises a constellation of nonspecific and often ambiguous clinical symptoms and signs that sometimes point to a diagnostic category. Biologic and clinical markers of depression are few and often nonspecific and do little to predict therapeutic and behavioral responses. Functional diagnostic imaging for known sites of drug action will prove helpful in delineating the mechanisms of disease, better categorization of disorders and prediction of therapeutic response.

Current theories of the receptor dynamics suggest that receptor density (up or downregulation) and/or affinity magnifies or reduces the second messenger signals sent to the neuron. The development of specific radiolabeled agonists and antagonists will be valuable probes to better understand neuropharmacodynamics.

Classic feedback physiology of endocrine systems has been known for over 50 years. Negative feedback of adrenal cortisol to the pituitary corticotroph (ACTH secreting cells) and to hypothalamic CRH neurons has been understood. However, the action of cortisol and its associated receptors on central CRH neuron remains largely unknown though GC is critical in the modulation of internal and external stressor. The outflow of cortisol and their interactions with the CRH neuron are future areas that require study. The ability to trace one aspect of the control the CRHR1 receptor of the stress axis in the CNS will be a tremendous advance in the understanding of the integration of the HPA with the development, vulnerability and manifestation of diseases as varied as neuropsychiatric, immune, cardiovascular, reproductive and others yet unrecognized. This ability to trace CNS neuroendocrine physiology with the knowledge that these and

perhaps other medications can modify disease manifestation and perhaps vulnerability will represents a considerable advance in medicine.

These ligands will characterize specific membrane receptor proteins which interact with CRH to produce biological actions. The use of CRHR1 antagonists as medications and specific imaging agents will provide tools to study the biological underpinnings crucial to understanding the biology of stress. Ultimately, these studies will lay the groundwork for understanding, diagnosing and treating patients with a variety of stress related psychic and somatic diseases.

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