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Neural circuits of anxiolytic and antidepressant *pherine* molecules

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Review

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Abstract

In this review, we describe proposed circuits mediating the mechanism of action of *pherines*, a new class of synthetic neuroactive steroids with demonstrated antianxiety and antidepressant properties, that engage nasal chemosensory receptors. We hypothesize that afferent signals triggered by activation of these peripheral receptors could reach subgroups of olfactory bulb neurons broadcasting information to gamma-aminobutyric acid ($GABA_{ergic}$) and corticotropin-releasing hormone (CRH) neurons in the limbic amygdala. We propose that chemosensory inputs triggered by pherines project to centrolateral (CeL) and centromedial (CeM) amygdala neurons, with downstream effects mediating behavioral actions. Anxiolytic pherines could activate the forward inhibitory $GABA_{ergic}$ neurons that facilitate the release of neuropeptide S (NPS) in the locus coeruleus (LC) and GABA in the bed nucleus of the stria terminalis (BNST) and inhibit catecholamine release in the LC and ventral tegmental area (VTA) leading to rapid anxiolytic effect. Alternatively, antidepressant pherines could facilitate the CRH and $GABA_{ergic}$ neurons that inhibit the release of NPS from the LC, increase glutamate release from the BNST, and increase norepinephrine (NE), dopamine (DA), and serotonin release from the LC, VTA, and raphe nucleus, respectively. Activation of these neural circuits leads to rapid antidepressant effect. The information provided is consistent with this model, but it should be noted that some steps on these pathways have not been demonstrated conclusively in the human brain.

Introduction

Natural steroidal chemosignals active in human nasal receptors

In the early 1990s, we reported that naturally occurring steroidal molecules in humans androsta-4,16-dien-3-one (ER670, PH56 or androstadienone (ADO)) and estra-1,3,5,(10,16-tetraen-3-ol (ER830, PH78 or estratetraenol (ETE)), administered in concentrations below olfactory threshold can induce depolarization of the local electrogram recorded from the nasal chemosensory mucosa in human subjects.[1] We called these naturally occurring molecules "putative pheromones." In pharmacology *in vitro* studies using isolated living human nasal chemosensory cells, ADO and ETE induced robust transient calcium (Ca^{++}) membrane currents supporting a membrane (nongenomic) effect of these steroidal compounds.[2]

In subsequent studies, using an experimental miniprobe that is the extension of a computerized olfactometer for local and topical administration of volatile substances while simultaneously recording the local electrogram from receptors (EGNR) in the nasal chemosensory mucosa,[1,3] we reported a rapid depolarizing effect of odorless steroids ADO and ETE on the nasal electrogram of human volunteers. This rapid nongenomic effect was followed by rapid activation of autonomic nervous system (ANS) reflexes and subtle behavioral changes that were distinct for ADO and ETE.[1,4,5]

ADO and ETE are inactive when administered systemically. In a pharmacokinetic study in human volunteers, ADO administered intranasally at 1-hour intervals during 12 consecutive hours was not detected in plasma samples collected at hourly intervals during dosing (HPLC [high-performance liquid chromatography]-mass-mass, assay sensitivity = 2.857 ng/mL).[6] Furthermore, intranasal and systemic administration of ADO and ETE to laboratory animals (rodent, lagomorph, canid, swine) in doses 100-fold higher than the dose to use in clinical studies did not induce any behavioral or ANS effects. It was concluded that odorless ADO and ETE induced species-specific pharmacological effects through activation of nasal chemosensory cells.[5,7]

Later independent contributions to this field confirmed similar ANS changes, subtle psychological effects, and distinct activation of the hypothalamus (HYP) measured with PET, after intranasal administration of ADO and ETE to human volunteers.[8–19]

Other reports showed non–sex-specific effects of putative pheromones ADO and ETE influencing the perception of emotional stimuli in human volunteers.[20–22] The non–sex-dimorphic effects of ADO and ETE were recently questioned in work using these steroidal molecules at high concentrations,[19] but this is not supported by previous publications using ADO and ETE in concentrations below the olfactory threshold.[3,5] In a recent article,[23] men with

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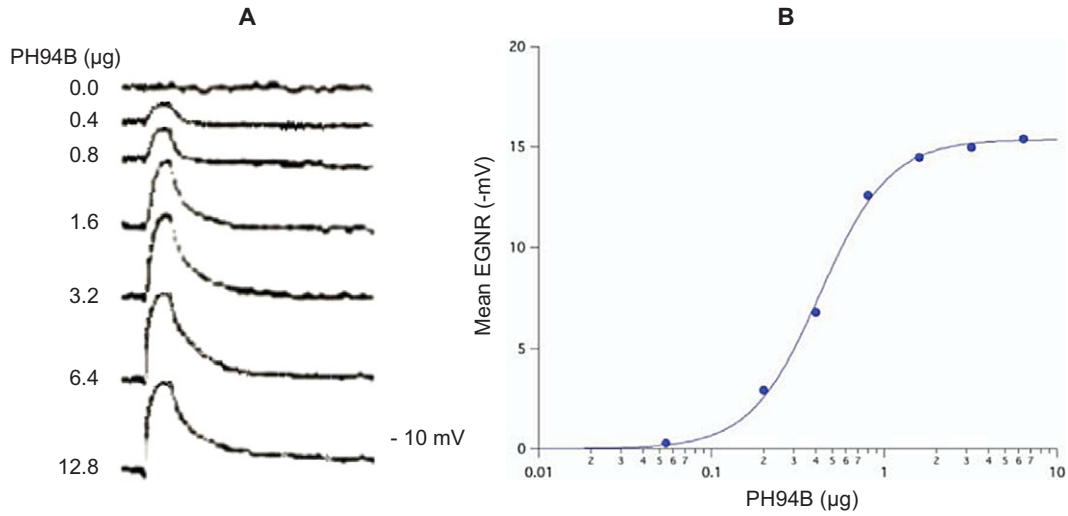

Figure 1. (A) Representative electrogram (EGNR) traces recorded from the surface of the dorsomedial chemosensory mucosa of the nasal septum in a young adult male volunteer during local administration of control (0.0) and different doses of PH94B. (B) Dose-dependent relationships of PB94B nasal spray on the amplitude of the EGNR in clinically healthy male and female subjects (n = 20). ED_{50} = .4 µM; Hill coefficient = 1. The EGNR was recorded using the Multifunctional Miniprobe (MM®), which is an extension of a computer-driven olfactometer. The therapeutic dose range to use in clinical studies was obtained from the dose–response relationships of PH94B.

"high social anxiety" (mean LSAS [Liebowitz Social Anxiety Scale] score of 53.2) reported increased sensitivity to threat and avoidance after nasal delivery of ADO. The adverse effects of ADO could be explained by the high concentration used (above olfactory threshold), although the authors masked ADO odor with eugenol. These results are not consistent with the positive effects of low doses of odorless ADO (below olfactory threshold and without using odor masking) reported in women volunteers.[3,5] Also, the "high social anxiety" subjects had lower baseline LSAS scores than those required (LSAS ≥ 60) in studies of the therapeutic effect of intranasally administered *pherines* in subjects with social anxiety disorder (see next section: mean LSAS on entrance was 97.9).[24,25]

Pherine molecules

Our preliminary findings led to the development of a new class of more potent neuroactive steroids, focusing on the following advantages: (a) 100% availability of ligand at the peripheral receptor sites immediately after intranasal administration, (b) ultralow dose needed to induce pharmacological effects due to direct administration to the receptor sites, and (c) fast oligosynaptic neural paths from nasal receptors to basal forebrain areas contributing to rapid onset of effect.

The new synthetic neuroactive steroids[a] were odorless and specifically designed and formulated to engage human nasal chemosensory receptors (nongenomic effect), looking for rapid and more potent behavioral and ANS effects than their naturally occurring predecessors. The substances were screened *in vitro* and *in vivo,* and those without toxicity in laboratory animal studies and lacking binding affinity to steroidal hormone receptors were studied in human volunteers for profiling their pharmacological effects on nasal receptors, ANS reflexes, and psychological effects and to gain information about their possible therapeutic indication. Neuroactive steroids meeting the above profile were included in a new family of therapeutic pharmaceuticals that we called *pherines.*[4]

Pherines are formulated in a water-based excipient for intranasal administration in spray form using a metered spray pump. Low microgram quantities of *pherine* administered locally and topically to the surface of the nasal chemosensory mucosal lining produce robust, dose-dependent depolarization of the electrogram (mass receptor potential) ENRG (Figure 1) followed by selective and dose-dependent brain activation of a behavioral and ANS response.[4,7,24,25,27–29]

Pharmacokinetic studies in human volunteers, administering to the nasal receptor area different doses of androsta-4,16-dien-3β-ol (PH94B or Aloradine), a neuroactive steroid from the androstane family of *pherines,* produced dose-dependent and reversible activation of the EGNR (ED_{50} = .4 µM; Hill Coefficient = 1). The dose–response relationships were used to find the effective dose range to administer in clinical studies (Figure 1).

An *in vitro* pharmacology study[30] in primary cultures of isolated human nasal chemosensory cells using the radiometric Ca^{++} indicator Fura-2 showed significantly increased intracellular Ca^{++} in response to PH94B, and this effect was dose dependent (ED_{50} = 1.0 µM) and similar to that reported for other neuroactive steroidal compounds acting on nasal receptors.[31]

Activation of nasal receptors by PH94B was followed by decreased sympathetic tone (assessed using physiologic sinus arrhythmia), decreased cardiac and respiratory rate, decreased frequency of electrodermal activity events and electromyogram, and increased body core temperature.[4] In a separate study, intranasal PH94B also induced specific and dose-dependent activation of brain areas that was different from control and from the effect of primary odors (Figure 2).

In a pharmacokinetic study in healthy volunteers, with PH94B administered intranasally in spray form at 1-hour intervals during 12 consecutive hours, the concentration of the *pherine* in plasma samples collected at hourly intervals during dosing was below the detection level of the analytical method (HPLC-M-M; assay sensitivity = 2.857 ng/mL).[32]

The behavioral effects of PH94B were assessed in a double-blind placebo-controlled study involving 90 subjects meeting DSM-IV criteria for social anxiety disorder. Subjects underwent two sets of laboratory-based challenges involving public speaking and social interaction.[24] During the first set (visit 2), all subjects were pretreated with placebo in a single-blind fashion 15 minutes before each challenge. Those demonstrating significant symptoms were brought back a week later (visit 3) and randomized to PH94B or placebo

[a]For the definition of neuroactive steroids, see Reference 26.

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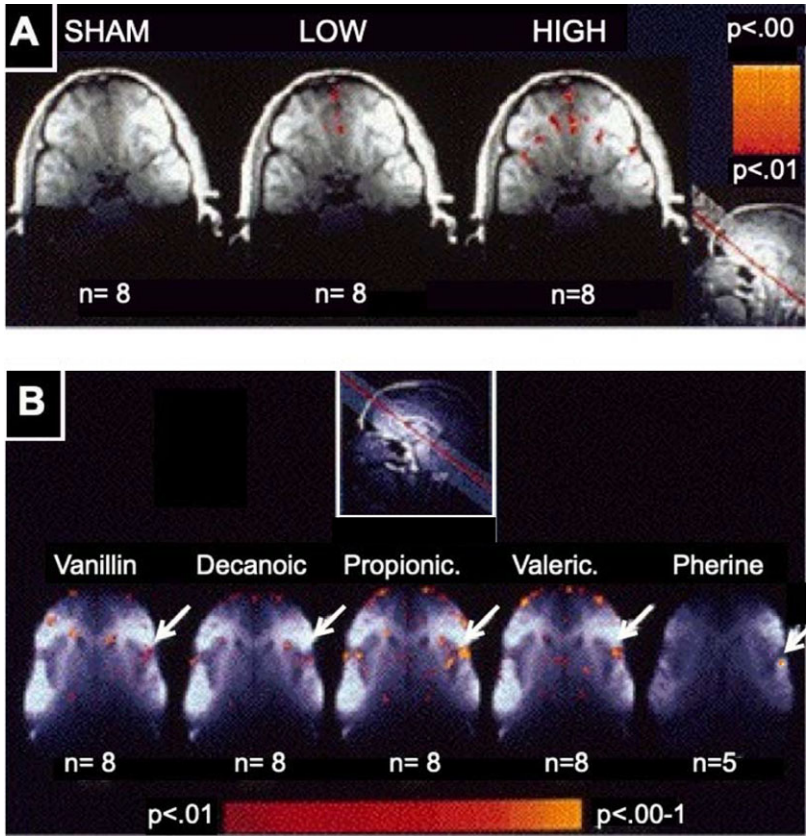

Figure 2. Selective and dose-dependent brain activation induced by odorless pherine PH94B (A) is different from control (SHAM) and brain activation induced by primary odors shown in (B). The results are averaged functional MRI images from human healthy volunteers (n = 8). Warmer colors on the color bars correspond to increased brain activation.

pretreatment, each followed 15 minutes later by second rounds of performance and social challenges. PH94B was significantly more effective than placebo in reducing both performance and social anxiety as rated by subject self-reports and investigator ratings.[24]

A subsequent 4-week double-blind crossover study[25] was conducted to obtain a preliminary estimate of the efficacy of PH94B when used in real-world situations for a longer period of time. Subjects meeting DSM-IV criteria for social anxiety disorder (n = 22) were randomized to use 1.6 to 3.2 microgram (μg) intranasal PH94B or placebo on an as-needed basis 15 minutes before confronting stressful performance or social situations in their daily life for 2 weeks, after which they were crossed over to the opposite treatment for an additional 2 weeks. Despite the small sample, PH94B demonstrated significant treatment efficacy in several ways. On the primary outcome measure, subjects with marked social anxiety disorder experienced significantly less peak anxiety during social and performance events in their daily lives when using PH94B than when pretreated with placebo (Effect Size = .658, r = .832).[25] Also, between-groups comparisons for the first 2 weeks of treatment showed effect sizes in favor of PH94B on the LSAS total score (effect size = .812) and LSAS avoidance subtotal score (effect size = 1.078), and significantly more subjects rated themselves as treatment responders on the Patient Global Improvement evaluation. What was particular noteworthy is that the degree of improvement seen with PH94B compared to placebo on the LSAS seen in this trial after 2 weeks was comparable in magnitude to that seen after 12 weeks with Food and Drug Administration (FDA) approved medications for Social Anxiety Disorder such as paroxetine[33] and sertraline.[34] These findings extend those of the study that was based on laboratory challenges and shows intranasal PH94B efficacy in real-life situations.

In vitro pharmacology studies using isolated living human nasal chemosensory cells show that PH10 (pregn-4-en-20-yn-3-one), a neuroactive steroid *pherine* molecule from the pregnane family, induces significant dose-dependent inward membrane currents (ED_{50} = .2 μM, Hill Coefficient = 1),[35] and significant dose-dependent depolarization of the electrogram (ENRG) recorded from the nasal chemosensory mucosa in men and women volunteers. The ERG response is followed 15 minutes after intranasal administration of 3.2 μg PH10 by increased plasma NE, 5-HT, and DA, increased sympathetic nervous system tone and frequency of electrodermal activity events.[27]

In a more recent placebo-controlled, parallel group dose ranging trial in 30 adults with major depressive disorder intranasal PH10 (low dose: 3.2 μg/day and high dose: 6.4 μg/day) reduced Hamilton Depression scores substantially more than did placebo.[28] The effect size at the end of the 8-weeks trial was: Effect $Size_{High\ Dose\ vs\ Placebo}$ = .95 and Effect Size Low Dose vs Placebo = .74), suggesting rapid antidepressant activity. Drug–placebo separation appeared during the first week of treatment (Effect $Size_{Low\ Dose\ vs\ Placebo}$ = .72, and Effect $Size_{High\ Dose\ vs\ Placebo}$ = 1.01).

Body

Olfactory neural circuits

Traditionally, it is accepted that in most mammals, olfaction is accomplished by two subsystems: main olfactory system (MOS)

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broadcasting sensory inputs from odor chemosignals to the main olfactory bulbs (OB) projecting to the olfactory tubercle, piriform cortex, medial amygdala (MeA), and cortical amygdala (CA)[36,37]; and the accessory olfactory system (AOS) conveying pheromone chemosignals to the accessory olfactory bulbs (AOB), which in turn reach basolateral amygdala (BLA) neurons that project to the anterior and ventromedial HYP, bed nucleus of the stria terminalis (BNST), medial preoptic area (MPA), striatum (ST), locus coeruleus (LC), parabrachial nucleus (PN), and prefrontal cortex (PFC).[38,39] Activation of these neural circuits is involved in the modulation of different social behaviors.

More recently, it was reported that, in mammals, there are subsets of OB neurons that share synaptic connections in the same limbic amygdala nuclei as the AOS, and olfactory activity behaviors originally assigned to the AOS are mediated through the main olfactory epithelium and the MOS.[40–48] Therefore, since humans do not have an identifiable AOB, we hypothesize that this could be the neural path by which chemosensory inputs triggered by pherines could reach the amygdala nuclei.

Numerous studies show that the MeA, a key structure in the control of social behavior, projects to $GABA_{ergic}$ neurons in the centrolateral amygdala (CeL) that trigger the forward inhibitory $GABA_{ergic}$ circuits directly mediating fear and anxiety and also to the BNST and the HYP involved in the regulation of innate defense responses[49–51] (Figure 3).

It has been shown that olfactory projections to the cortical amygdala can also trigger BLA neurons[52] which synapse with the important contingent of $GABA_{ergic}$ forward inhibitory neurons in the lateral (CeL) and medial (CeM) division of the central amygdala involved in the modulation of fear and anxiety.[42,44,48,49,53–56] More recent evidence shows that $GABA_{ergic}$-PKCδ-positive OFF-neurons in the CeL facilitate the release of neuropeptide S (NPS) in the LC and GABA from anterolateral BNST through forward inhibition of $GABA_{ergic}$ neurons in the CeM, and there is concurrent inhibition of NE, DA, and 5-HT release from the midbrain and decreased sympathetic system tone through inhibition of neurons in the posterior HYP.[42,51,53–61]

Furthermore, neural inputs from the BLA reach $GABA_{ergic}$–PKCδ-negative ON-neurons in the CeL. It has been reported that CeL outputs via an intercalated feed-forward series of $GABA_{ergic}$ interneurons and also through CRH neurons can stimulate glutamatergic neurons in the BNST oval area and in the prefrontal cortex, with concurrent stimulation of NE, DA, and serotonin release from the midbrain (LC, VTA, and RN), (Figure 3). Activation of these circuits leads to simultaneous inhibition of $GABA_{ergic}$ neurons in the BNST and NPS-releasing neurons in the LC, increased sympathetic system tone, and a neuroendocrine response,[53,60–62] all in agreement with the neurocircuits of mood disorders.[63]

Proposed mechanisms of pherine activity

We hypothesize that the PH94B-induced rapid decrease (latency ≤ 400 ms) of sympathetic tone[64] and rapid improvement (latency = 10-15 minutes) in performance anxiety and social interaction anxiety[24,25] are triggered by sensory inputs originating in nasal chemosensory neurons that stimulate subset of OB neurons projecting to the MeA and BLA. There is evidence that MeA and BLA neurons trigger the forward inhibitory $GABA_{ergic}$–PKCδ-positive OFF-neurons in the CeL and CeM amygdala, which downstream effects mediating behavioral actions that directly mediate social behavior, fear, and anxiety[51,52,56,63,64] (Figure 3). The modulation of neural circuits involved in the pathogenesis of social anxiety disorder[55–57,59,65–68] appears to be consistent with the PH94B-induced acute anxiolytic effects and autonomic nervous system changes reported in our clinical studies in patients diagnosed with social anxiety disorder.[24,25]

We also hypothesize that the rapid change in sympathetic tone and dose-dependent improvement in Hamilton Depression scores (HAM-D) scores induced after intranasal administration of PH10[28] are the result of activation of glutamatergic neurons in the BLA that in turn trigger the $GABA_{ergic}$–PKCδ-negative ON-neurons in the CeL. The antidepressant effect of PH10 through activation of nasal chemosensory receptors is supported by other independent studies showing an important association of the olfactory system and mood disorders.[63,68–71]

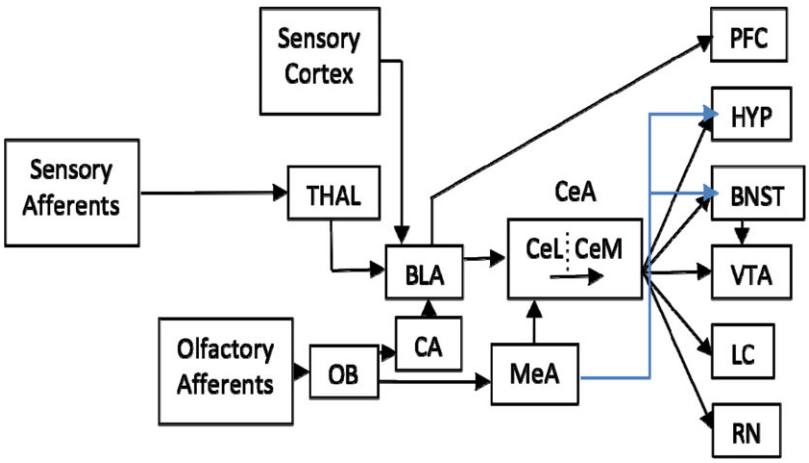

Figure 3. Schematic diagram showing the olfactory connections to the limbic amygdala and related areas. The olfactory bulb (OB) connections to the limbic amygdala are shorter and bypass the thalamus thus being a fast (shorter latency) neural input to the basal forebrain compared to other sensory afferent systems.

Abbreviations: BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA, cortical amygdala; CeA, central amygdala; CeL, centrolateral amygdala; CeM, centromedial amygdala; HYP, hypothalamus; LC, locus ceruleus; MeA, medial amygdala; OB, olfactory bulb; PFC, prefrontal cortex; RN, raphe nucleus; THAL, thalamus; VTA, ventral tegmental area.

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Conclusions

There is extensive evidence showing the human olfactory system's role in social behavior, food ingestion, appetite regulation, awareness of the surrounding environment, and detection of hazards.[72,73] Unlike other sensory systems, olfactory inputs do not have a synaptic relay in the thalamus to be routed to the cortex. Rather, they are wired to the limbic amygdala, HYP, and hippocampus, which provides olfaction with a unique and potent power to influence mood, acquisition of new information, and its use in many different contexts including social interaction, fear, emotions, and the memory components of behavior.[74–80]

Thus, it is reasonable to assume that in humans, there are functional neural circuits reporting afferent information from olfactory chemosensory receptors, via the OB, to the basal forebrain areas (Figure 3) that influence behavior, mood, and emotions, and that the neuroanatomical areas involved are the same as the dysfunctional areas described in laboratory animals with bilateral olfactory bulbectomy[81–83] and in subjects with congenital absence of OB, who develop anxiety and depression in early life.[84–88] The documented olfactory bulb-amygdala connections[29,40,41,43–52,54,56,66,67,69,89] seem to be compatible with the neural circuits involved in the pathophysiology of social anxiety disorder, specific phobias, generalized anxiety disorder, and depression.[57–59,61,63,65–67,73,84]

Therefore, neural circuits from nasal chemosensory neurons to OB cells that project axons to the MeA and BLA and to $GABA_{ergic}$ PKCδ-OFF-neurons in the CeL and the CeM could explain the anxiolytic effect of *pherine* PH94B[24,25] through the modulation of GABA and NPS and the decreased sympathetic tone. Also, the rapid antidepressant effect of PH10[28] could be explained by the activation of $GABA_{ergic}$–PKCδ-negative ON-neurons and CRH-releasing neurons in the CeL that induce release of glutamate from the BLA and the oval neurons in the BNST and catecholamines from the midbrain and concurrent increase of sympathetic tone. Since pherine molecules are species specific, animal testing would not be helpful in elucidating the relevant brain pathways. Therefore, further studies using functional magnetic resonance imaging (fMRI) and spectroscopy magnetic resonance imaging (sMRI) during nasal administration neuroactive pherine molecules to human subjects are needed to confirm the functional connectivity and changes in central nervous system (CNS) neurotransmitters proposed here.

Specific chemosensory inputs triggered by pherines could be an important portal for the modulation of neurotransmitter release in the telencephalon and ANS function and behavior. The effects of *pherines* in the CNS, bypassing the hurdles of systemic administration and the brain–blood barrier, and their safety and tolerability demonstrated in clinical studies provide a novel approach to address the interrelationships between the dysfunctional neuronal circuits in patients with anxiety disorders and mood disorders. *Pherines* also open a new approach for administration of therapeutic pharmaceuticals, with the advantage of rapid onset of efficacy and more accurate and safe therapeutic effect for the management of neuropsychiatric conditions that require acute intervention.

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