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HYPOTHESIS

Beyond the usual suspects: a cholinergic route for panic attacks

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For unknown reasons and through poorly understood mechanisms, people at risk of panic attacks are hypersensitive to suffocative stimuli and experience hyperventilation and anxiety after exposure to heightened concentrations of carbon dioxide. Similarly to the physiological reflex response to hypercapnia in animals and man, the anxious response to carbon dioxide in people with panic disorder is at least partially controlled by the central muscarinic receptors. It is suggested here that some modifications of the cholinergic functions could underlie human individual differences in carbon dioxide sensitivity and proneness to experience panic attacks. The hypothesis is based upon experimental evidence that stressful and potentially harmful stimuli prime relatively long-lasting changes in cholinergic genes expression and cholinergic receptors' regulation. The adaptive sequels of these modifications include protection of the brain from overstimulation, and, at the level of the corticolimbic circuitries, promotion of passive avoidance and learning after stress. The extension of the same modifications to the cholinergic receptors involved in chemoception, however, could lower the threshold for reaction to suffocative stimuli, including carbon dioxide. The exaggerated sensitivity to carbon dioxide observed in humans suffering from panic attacks could then be thought of as an evolutionary cost of the involvement of the cholinergic system in shaping otherwise adaptive responses to stress and threatening stimuli.
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Introduction

About 40 years ago DF Klein primed a process, initially based on careful observation of symptoms and pharmacological dissection, that led to the identification of panic disorder (PD) as a nosological entity separate from other anxiety disorders.[1] The relative specificity of respiratory symptoms during spontaneous panic attacks, and the evidence that patients with PD are especially sensitive to carbon dioxide (CO_2) inhalation, led Klein to hypothesize that PD is a disturbance in a suffocation alarm system,[2] in which a physiological misinterpretation of suffocative stimuli produces respiratory distress, hyperventilation and panic. Today we know that panic attacks can be reproduced in the laboratory by means of several agents[3] beyond inhaled CO_2. Yet, CO_2 is considered by some as the most specific panicogenic agent for the rapid onset of effects,[4] the pertinence to Klein's false suffocation hypothesis,[5] and the ability to provoke symptoms similar to those occurring in spontaneous attacks in a sizable percentage of patients with PD and their well relatives.[6–8] Consistent with Klein's theory, it has been hypothesized that individual sensitivity to increasing concentrations of inhaled CO_2 may reflect a continuously distributed, possibly evolutionarily-derived, developmental trait.[9,10] The magnitude of the anxious response to CO_2 administration among individuals may parallel such distribution, with PD patients at one extreme.[2,6] Moreover, a neuroanatomical model connecting different components of PD—acute attack, anticipatory anxiety, phobic avoidance—to the brainstem, limbic system and prefrontal cortex respectively, has been offered[11] and recently revised.[12] It is likely that recent advances in the physiology of conditioned fear will provide substantial insights for PD too. The behavioral, autonomic, and neuroendocrine activation observed in PD may well result from impaired coordination of information to the amygdala from both 'upstream' (or cortical, from primary viscerosensory cortices through corticothalamic relays) and 'downstream' (or brainstem, from the nucleus of the solitary tract through the parabrachial nucleus or the sensory thalamus) pathways and circuits.[12]

Several issues remain unclear, however. These include the identification of the central mechanisms

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underlying the responses to CO_2 and suffocative stimuli in PD, the possible selective advantage of a trait of heightened sensitivity to CO_2, and what would be its relationship, if any, with physiological anxiety.

A model that aims at clarifying some aspects of panic attacks by focusing on cholinergic functions is offered here, together with the indication of some research strategies that would facilitate its empirical exploration.

Why the cholinergic system can be important in panic attacks

It is still unclear why some people—most typically patients with PD—are especially sensitive to suffocative stimuli and develop symptoms of anxiety in response to the inhalation of air mixtures enriched with CO_2: it appears very likely that several neurochemical pathways, as well as various neurotransmitter systems, including norepinephrine, serotonin and gamma-aminobutyric acid,[13,14] and brain structures such as the locus coeruleus, the amygdala, or the hippocampus, are all involved at some time and to some extent in both spontaneous and provoked panic attacks.[11–13] Inasmuch as these neurotransmitters and brain structures are implicated in the etio(patho)logy of emotions including anxiety and fear, they are all very good candidates for a better understanding of PD. But while investigation in these 'usual suspect' pathways is important, concentrating on dyspnea, a feature rather specific of panic attacks at least partially independent from subjective sensations of fear,[2] seems a worthwhile task.

To study CO_2 hypersensitivity as an endophenotype of spontaneous dyspnea in real-world panic, we focused on the regulatory mechanisms of an adaptive phenomenon readily reproducible in the laboratory: the normal reflex response to hypercapnia. The physiological reflex response to hypercapnia in mammals and man is an increase in ventilatory rate and in arousal and vigilance.[15] This is a response controlled at least in part by cholinergic mechanisms, with the muscarinic receptors of the ventral medulla playing a key role.[16–20] Direct stimulation of the ventral medulla by CO_2 in experimental settings elicits hyperventilation, which can be in turn dramatically decreased by the topical application of muscarinic antagonists.[17,18] In man, functional brain imaging studies of volunteers breathing a 5% CO_2 gas mixture showed significant metabolic activation and cerebral flow changes localized primarily to the ventral medulla.[21] The effects of centrally acting antimuscarinic agents compared to peripherally acting antimuscarinic agents and placebo in blocking the ventilatory response to CO_2 (measured as the delta minute ventilation to the delta end-tidal CO_2 pressure ratio) in normal human subjects are usually moderate, but interestingly they correlate significantly with the degree of hypercapnic ventilatory response at baseline.[22] This suggests that the effect attributable to central muscarinic receptors in modulating the physiologic response to specific suffocative stimuli—such as CO_2—varies among individuals, and can be predicted on the basis of individual differences in the degree of chemosensitivity to hypercapnia. The prediction that central muscarinic receptors control the modulation of the response to specific suffocative stimuli seems most consistent in those subjects who have a heightened sensitivity to hypercapnia, ie, in patients suffering from PD. By exploring the putative role of cholinergic receptors and pathways in modulating the response to CO_2 in PD, we[9] have recently shown that the typical anxious response to CO_2 can be blocked by pretreatment with a single dose of an antimuscarinic agent which crosses the blood–brain barrier (biperiden), while a peripherally acting antimuscarinic agent (pirenzepine) has the same lack of effect as placebo. Five different types of muscarinic receptors distributed differently in the CNS[23] have been identified so far, and we are presently unable to say if any such subtypes may have influenced our results more than the other. However, there are at least two potentially important implications that stem from the findings discussed hitherto. First, the degree of individual chemosensitivity and reactivity to CO_2 stimulation varies among individuals, with people with PD at one extreme. Second, the central mechanisms of sensing and reacting to hypercapnia—a suffocative, potentially harmful and unconditioned noxious stimulus—are at least partially controlled by central cholinergic muscarinic receptors.

Is there a way to put into a relationship this information with any known, natural adaptive mechanism meaningful for survival and fitness?

The role of muscarinic receptors in face of harmful stimuli and stress

In addition to subserving impulse at the neuromuscular junction and the visceral stimulation at the base of vegetative function, Acetylcholine (ACh) is acting both in the brainstem and the telencephalon of mammals, as it is implicated in arousal and cognition. Some of the mechanisms that regulate the expression of choline acetyltransferase (ChAT), the biosynthetic enzyme for acetylcholine production, have been clarified recently. The ChAT gene is part of a more complex genetic locus also coding for the vesicular acetylcholine transporter (VAChT) which packages transmitter into synaptic vesicles.[24] The genomic organization of these two distinct but related genetic functions is unique, and has been conserved from Drosophila to humans.[24] Recent animal studies clearly show that through a cascade of events stressful experiences can mediate neuronal plasticity by direct action upon the genetic expression of cholinergic muscarinic receptors in the relative long-term. Kaufer and colleagues[25] showed that acute stress of even moderate intensity (eg, a single session of 4 min forced swimming in mice)—as well as inhibitors of acetylcholinesterase (AChE)—produce a transient increase in the amount of released ACh in corticohippocampal areas, independent of the pituitary-adrenocortical axis.[26] Consequences include an early phase of enhanced neuronal excitability,[27] and most interest-

ingly, long-lasting (up to 3 h *in vitro*) modulation of the genetic regulation of ACh availability[25] in the CNS. More specifically (see Figure 1), following acute stress and ACh release, the central muscarinic receptors mediate the induction of the gene encoding the early immediate transcription factor c-Fos.[27] This produces, by a Ca^{2+}-dependent mechanism, enhanced c-Fos binding to a site close to the promoter regions of genes with key roles for acetylcholine function, namely the gene encoding acetylcholinesterase (AchE), the gene for the acetylcholine-synthetizing enzyme choline acetyltransferase (ChAT), and the vescicular acetylcholine transporter (VAChT). RNA analyses clearly show[25] that more AChE is then produced, while synthesis of both ChAT and VAChT decreases. As a consequence of such changes in cholinergic genes expression, brain cholinergic transmission decreases temporarily, and a later phase of depressed neuronal activity follows. Similarly, mild prenatal stress (a saline injection) increases hippocampal ACh release in rats.[28]

This chain of molecular compensations is thought to have three potentially adaptive consequences. The first is in the short-term, and consists of quietening of brain activity after a traumatic experience.[25] The second implicates the preservation of the brain from neurodegeneration, since the 'readthrough' variant of acetylcholinesterase, the one that is released in stressful conditions in the alternative to the synaptic variant, is protective against neurodeterioration.[29] The third, and the most relevant to this hypothesis, consists of the temporary upregulation of the muscarinic cholinergic receptors.[30] While the five different subtypes of muscarinic receptors could be affected differentially by the mechanisms of compensatory upregulation,[31,32] stressful experiences that induce passive avoidance are consistently paralleled by augmented expression of muscarinic cholinergic receptors widely throughout the CNS, including the areas of the neocortex, amygdala and the hippocampus.[33] Likewise, relatively long-lasting upregulation and hypersensitivity of muscarinic receptors after stress is well documented in the cholinergic areas of the septohippocampal system.[30]

The upregulation of muscarinic—and also nicotinic—receptors after stress is then followed by augmented cholinergic transmission in the amygdala,[34,35] the hippocampus[36] and the corticohippocampal circuitries,[37–40] all structures that promote learning and passive avoidance in response to aversive novel, but not familiar, conditioned stimuli. Animal studies using various knock-down designs confirm that muscarinc receptors—in addition to the nicotinic receptors—play a crucial role at this level.[41]

The same temporary compensatory upregulation after stress may not be limited to the cholinergic neurons of the limbic and cortical structures mentioned above, but may also affect the muscarinic receptors in other districts of the CNS, like those placed on the surface of the ventral medulla, which control chemoception and sensitivity to CO_2. These neurons would then become temporarily more sensitive to hypercapnic stimulation and the individual become more sensitive to suffocative stimuli.

Pertinence to human panic

While the trait of exaggerated sensitivity to CO_2 and clinical PD overlap to some extent, they are clearly not identical, the former being a viable means to explore

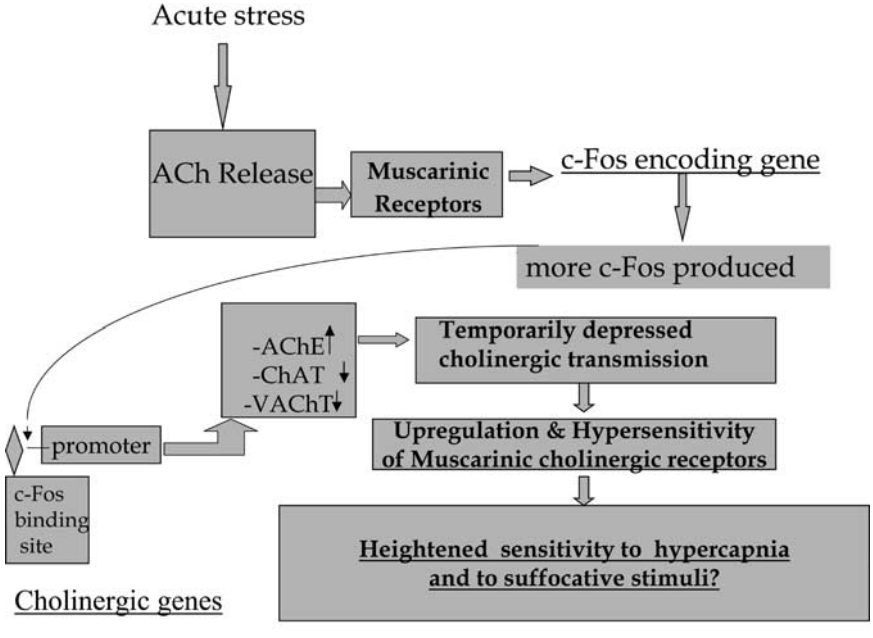

Figure 1 Cascade of events following stress in experimental conditions. It is suggested that the same cholinergic mechanism depicted in the figure can affect individual susceptibility to suffocative stimuli and panic attacks. ACh, acetylcholine; AchE, acetylcholinesterase; ChAT, choline acetyltransferase; VAChT, vesicular acetylcholine transporter.

the latter.[6,42] With this limitation in mind, it is suggested here that the model described in paragraph 2 and depicted in Figure 1 could help understand human panic and related conditions better.

It is conceivable that after one or more stressful events, the temporary functional rearrangements described in paragraph 2 take place in the form of upregulation and/or augmented expression of muscarinic receptors, so that a subject can become more sensitive to subthreshold suffocative stimuli, including variations in CO_2 concentration in the environment. With a lowered chemoceptive threshold there would be higher probability of a rise in ventilatory rate and arousal, and heightened awareness of air hunger in response to mild suffocative stimuli (like a modest increase of CO_2 concentration in a crowded subway), which would in turn start a full panic attack in a predisposed subject.

Recent brain imaging studies of normal human subjects inhaling CO_2-enriched air mixtures provide further evidence in support of a common neurobiological route that connects the physiological responses to hypercapnia with the exaggerated CO_2 sensitivity typical of PD. The inhalation of CO_2 in man activates a pathway of responses that originate in the ventral medulla[21,43,44] and extend to the pons, midbrain, limbic and paralimbic areas, the parahippocampal and fusiform gyrus, and the anterior insula.[43,44] This physiological response network connects an element of basic respiratory control to the affective states of air hunger and fear that are promoted when respiratory disturbance becomes a salient element of consciousness.[43] However, functional imaging data show that activation of the limbic and cortical regions in man also depend upon subjective sensations of respiratory constraint and air hunger, as obtainable by the application to volunteers of a facemask for breathing.[43,44] There thus seem to be partially independent pathways that lead to limbic activation—and the corresponding emotions of anxiety or fear—in response to objective and perceived suffocation. Also, the fact that respiratory physiological measures of reaction to CO_2 inhalation predict a panic response[45] better than the presence/absence of a clinical diagnosis of PD, seems in harmony with the notion that sensitivity to CO_2 can be better viewed as a quantitative trait that incorporates non-patient subjects and extends beyond clinical PD.

Discussion and limitations

Genetic and environmental determinants

One implication of this model is that stressful experiences can heighten the sensitivity to CO_2 and render the onset of a spontaneous panic attack more likely. How does this harmonize with current knowledge about the determinants of recurrent panic attacks and PD?

Liability to spontaneous panic attacks and PD is influenced by both genetic and non-genetic factors[46] and the same seems true for hypersensitivity to CO_2[47] in PD patients. The identification of genes conferring heightened individual liability to PD is still under way, while the role of environmental factors in influencing the liability to psychiatric disorders is often described as transient, but powerful in the short-term action of precipitating the onset of a phase of illness for most phenotypes.[48] Likewise, the onset of PD can be influenced/precipitated by life events[49,50] and stressors—some of which are suffocative in nature[51]—during development and early adulthood, while childhood separation anxiety disorder and familial history of PD-agoraphobia predict earlier age at onset of PD[52,53] and heightened responsiveness to CO_2 administration[7,8] respectively. Inasmuch as moderately stressful experiences can trigger functional rearrangements of the cholinergic system and the muscarinic receptors[25] in experimental conditions, it is suggested that life events can similarly influence intra- and interindividual variations in CO_2 sensitivity in humans, thus acting as revealers of partially heritable differences in liability to spontaneous panic attacks and PD-agoraphobia.

It is presently unclear which subtypes of muscarinic receptors are more prominent in the role of mediating chemoceptive mechanisms in man, but it could be expected that distinctive polymorphisms for the five known subtypes of muscarinic receptors, or polymorphisms at the promoter regions of key cholinergic genes such as those encoding AchE, ChAT and VAChT, may contribute differentially to shape individual differences in chemoception. Some such polymorphisms could be ultimately shown to belong in a polygenic/oligogenic background that explains a significant proportion of the genetic variance in liability to spontaneous panic attacks, PD and CO_2 hypersensitivity (see also 'Perspectives for research').

Relationship with other forms of anxiety

One could argue that another clinical condition that would fit this model is acute stress disorder, whereby subjects are exposed to severe stressors. It would be reasonable to expect muscarinic receptors in the CNS to undergo a similar chain of molecular compensations leading to upregulation and hypersensitivity. While increased risk for panic attacks and PD is described in acute stress disorder,[54] the relationship between reactions to stress and some physical symptoms of anxiety—such as dyspnea—has been studied little in terms of causal chains. There is, however, one interesting observation in this regard: the administration of sodium lactate provokes flashbacks in subjects with post-traumatic stress disorder.[55] If one takes into account that sodium lactate is metabolized to bicarbonate, and then into CO_2 when it passes the blood–brain barrier,[11] the reaction could be partially attributed to the stimulation of upregulated cholinergic receptors of the hippocampal and amygdalar circuits.

Time stability

The dynamic changes affecting the cholinergic system and the muscarinic receptors by the 'gene–environment ' interactions described hitherto predict intra-individual variations in proneness to react anxiously to suffocative stimuli, including CO_2. Therefore, this

model may have some value in explaining how stressful events, perhaps including loss or separation, contribute to precipitating the onset of spontaneous panic attacks, but may not prove helpful in explaining the *maintenance* of PD/CO_2 hypersensitivity as the at least partial result of a deranged suffocative alarm system. During the active phases of PD, patients are more likely to be hypersensitive to suffocative stimuli for a *prolonged* time, as suggested by chronic hyperventilation,[56] supposedly to maintain pCO_2 below the suffocation alarm threshold.[2] On the other hand, it is clear that individual sensitivity to CO_2 stimulation in subjects at risk for PD is far from stable in time, partially due to desensitization,[4,7] and the anxious response to CO_2 inhalation has been reported in up to 20% of normal control subjects,[5] in keeping with the notion that CO_2 sensitivity is a psychobiological trait expected to vary in time, and common also to non-patient subjects, and to subjects having mental disorders other than PD.

Relationships to other neurotransmitters and neuroanatomical structures

Investigations of the cholinergic system in the etiopathogenesis of PD have been rare so far, as there are only few hints that patients with PD may have hypersensitive muscarinic receptors,[57] while several studies failed to show major sleep alterations in PD, and showed the implication of neurotransmitter systems[11,12] other than ACh, and neuroanatomical structures other than the medulla oblongata, in PD.

The intensity of the response to the CO_2 challenge is obviously not the simple and direct result of the degree of sensitivity of muscarinic receptors: while the contribution of the total hypercapnic ventilatory response in humans is largely controlled by structures in the medulla oblongata, a smaller contribution is attributable to the carotid body.[16] Also, several agents including cholecystokinin (CCK) are able to provoke anxiety in patients with PD. Limited data suggest that CO_2 and CCK may act on the same neuronal pathways, but they seem to inhibit, rather than potentiate, each other's effects,[58] while the response to the CO_2 challenge can be enhanced by 5-HT depletion.[59] As a consequence, it is at present unclear how the model presented here may have relevance for CCK-provoked symptoms.

The mechanisms of modulation of the cholinergic genes and the muscarinic receptors upon which this hypothesis is based could be extended to other neurotransmitter systems, and to ACh-mediated interrelationships between circuits relevant for anxiety and fear elicitation. These could include the effects of cholinergic mediation upon other neurotransmitters' circuits in core limbic structures such as the amygdala and the hippocampus.[60] There is experimental evidence that exposure to CO_2 breathing provokes the expression of c-Fos gene encoded protein in several subsets of noradrenergic and adrenergic (but not dopaminergic) neurons of the ventrolateral medulla, locus coeruleus, and the cingulate cortex.[61] The facts that heightened c-Fos expression of catecholaminergic cells can be the result of the excitement of other neuronal populations, and that catecholaminergic cells in the chemoceptor area are located relatively deep (1 mm from the ventral surface) within the medulla, leave the possibility of a primarily cholinergic chemoception, with part of the arousal and anxious responses also mediated by catecholaminergic systems.

Data on Fos expression and upregulation—indeed a mechanism common to several neurotransmitters and neuropeptides—in response to stress may help further connecting the molecular changes shown in Figure 1 to the activation of other neurotransmitters' systems, and perhaps of the hypothalamic-pituitary-adrenal (HPA) axis. Mappings of c-Fos expression in both intact and prepared animals exposed to emotional stress show[62] the recruitment of medullary neurons, likely of the noradrenergic type, that stimulate directly the medial amygdala, a major input to the corticotropin releasing factor (CRF) cells of the paraventricular nucleus. So far, it was generally assumed that HPA activation in response to emotionally stressful events is routed from the association cortex through the medial amygdala, and then to the cells of the paraventricular nucleus independently of the brainstem. These animal data[62] challenge this view by showing evidence in favor of medullary, indirect regulation of the release of hypothalamic CRF in response to an emotional stressor. The relationship of naturally-occurring panic—as opposed to laboratory-induced panic—to the activation of the HPA is far from clear (see Klein[2] for a discussion of the issue). Heightened levels of cortisol are more likely to occur whenever panic disorder is characterized by recurrent situational attacks, anticipatory anxiety and avoidance,[2,63] but these new data will help appreciate the substantial complexity of the picture at this level.

Perspectives for research

Genetics

Inasmuch as reactivity to the CO_2 challenge is a viable endophenotype of PD, and its outcomes—*viz* the indices of subjective anxiety and/or changes in measures of respiratory physiological indices after CO_2 inhalation—are amenable to continuous measuring, quantitative genetic investigations become feasible. Earlier twin studies based on small samples indicate a heritability estimate between 30–62%[64,65] for PD, while recent data obtained on epidemiological samples of twin pairs show moderate heritability: 33–43%.[46] With regard to responsiveness to CO_2 as a panic-provoking agent, there is only a small twin study of categorically-defined, CO_2-induced panic, in which MZ twins have significantly higher concordance than DZ twins.[47] A bivariate twin population study of PD symptoms and responsiveness to CO_2 stimulation could easily estimate to what extent the two phenotypes share genetic and non-genetic determinants, thus providing a first, methodologically robust exploration of the hypothesis that genes influencing the detection of, and response to, suffocative stimuli also partially control liability to spontaneous attacks and PD. Further extensions of the

bivariate twin model, including the application of structural equations modeling to longitudinal assessments of twins, could clarify the proportion of variance in liability accounted for by life events, the degree of time stability of their effects, and possible variations of heritability in time[48] for both the phenotype of PD and CO_2 reactivity.

Inasmuch as heightened responsiveness to CO_2 inhalation is a trait that runs in families, familial association and sib pairs analyses[66,67] could be applied to responses after the challenge considered as endophenotypes of PD influenced by QTLs. Polymorphisms at the muscarinic receptors genes, at the 7q22 AchE locus, and recently individuated polymorphisms at the AChE promoter regions[68] could be reasonable candidates for such approaches. Interestingly, a recent genome-wide screen[69] for PD identified 16 markers suggestive (LOD scores >1) of linkage, six of which are compatible with the location of the genes that codify for the five subtypes of muscarinic receptors. Likewise, a susceptibility factor for PD and phobias has been identified[70] very recently in a polymorphic genomic duplication of the human Chromosome 15 (15q24–26), where the gene coding for human muscarinic receptor M5 (located at 15q25) is situated.

Inasmuch as the stress-induced functional rearrangements described in paragraph 2 take place also in response to inhibitors of AChE,[25] potentially useful hints may derive from the pharmacogenetics of individual sensitivity to organophosphates. Paraoxonase (PON1) is a protein protective against oxidative damage to low-density lipoproteins, and able to detoxify organophosphates.[71] Subjects with low-activity polymorphism at the PON1 promoter gene[72] are more prone to develop neurological symptoms related to wartime exposure to combinations of toxic agents and treatment with pyridostigmine,[73] and under the model outlined here the same polymorphism might predict heightened proneness to panic attacks under similar conditions.

Imaging and animal models

Further PET studies could control whether augmented expression of muscarinic cholinergic receptors already well documented in limbic areas after stress[30,33] is detectable also in the brainstem.

Muscarinic (but also nicotinic) receptors promote passively avoidant behaviors in response to *conditioned* stimuli. One implication of the findings discussed above, however, is that muscarinic receptors can be important also for the detection of—and the priming of the affective sequelae related to—one aversive, *unconditioned* stimulus, namely the suffocative stimulus of hypercapnia. Indeed, brain imaging data show that even normal subjects who were selected for the *absence* of overt anxious responses to hypercapnia, have robust signals in primarily respiratory structures in the brain[44] followed by intense activation in the amygdalar and hippocampal regions, which are usually considered central structures in conditioned fear.

Animal models could assess whether explorative behaviors decrease after stressful stimuli in the presence of non-conditioned, heightened environmental CO_2 concentrations, and whether anticholinergic treatments and the application of knock-down procedures can modulate such behaviors.

Further hints for potentially fruitful animal models are finally offered by recent insights on the genetic regulation of the neuronal cholinergic phenotype by bone morphogenetic proteins during development,[74] the identification of the genetic regulation of the potassium channels responsible for breathing responses to some suffocative stimuli[75] and the patterns of transcription for the cholinergic gene locus.[76]

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