EFFECTS OF TRYPTOPHAN DEPLETION ON CENTRAL AND PERIPHERAL CHEMOREFLEXES IN MAN

by

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ABSTRACT

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Background: Klein (1993) suggests that panic attacks are the result of a defective "suffocation alarm" threshold that presents with carbon dioxide (CO_2) hypersensitivity, exaggerated ventilatory response and panic in panic disorder (PD) patients. Serotonin is expected to normalize the "suffocation alarm" threshold and associated CO_2 hypersensitivity. Current research supports both 5-HT-mediated increases and decreases of ventilatory output. Knowledge of serotonin's role on ventilatory output and its neuroanatomical sources impacts on the "suffocation alarm" theory validity and predictive value. Method: We used tryptophan depletion (TRP-) in concert with a modified Read rebreathing test to determine the effect of deficient serotonergic modulation on the central and peripheral chemoreflex threshold and sensitivity of response to CO_2 in eleven healthy men. Results: TRP- did not affect central or peripheral chemoreflex threshold or sensitivity of response to CO_2. However, basal ventilation was significantly elevated during TRP-. Conclusions: In contrast to "suffocation alarm" theory predictions, deficient 5-HT neurotransmission does not significantly affect the respiratory chemoreflex response to CO_2, instead increasing non-chemoreflex drives to breathe. Panic associated respiratory abnormalities may be related to deranged 5HT modulation of non-chemoreflex drives to breathe, unrelated to any respiratory chemoreflex abnormality.

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LIST OF ABBREVIATIONS

PD	Panic Disorder
PAs	Panic attacks
NA	Noradrenergic
BTPS	Body temperature, ambient pressure, saturated with water vapour
CCK	Cholecystokinin
5-HT	Serotonin
CO_2	Carbon dioxide
PCO_2	Partial pressure of carbon dioxide
SSRI's	Selective Serotonin Reuptake Inhibitors
5-HTP	5-Hydroxytryptophan
PCPA	Parachlorophenylpiperazine
TRP	Tryptophan
CNS	Central Nervous System
ROb	Nucleus raphe obscurus
RMg	Nucleus raphe magnus
RPa	Nucleus raphe pallidus
$[H^+]$	Hydrogen ion concentration
O_2	Oxygen
PO_2	Partial pressure of oxygen
BBB	Blood-brain barrier
$P_{ET}CO_2$	End-tidal partial pressure of carbon dioxide
DRN	Dorsal raphe nuclei
MRN	Medial raphe nuclei
HV	Healthy volunteers

SCID	Semi-structured Clinical Interview per Diagnostic Criteria
EKG	Electrocardiogram
TRP-	Tryptophan-depleted amino acid drink
TRP+	Tryptophan non-depleted/placebo amino acid drink
HPLC	High pressure liquid chromatography
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders – 4th Edition
PSS	Panic Symptom Scale
$P_{ET}O_2$	End-tidal partial pressure of oxygen
V_E	Ventilation
V_T	Tidal volume
f	Breathing frequency
B	Baseline
T	Chemoreflex threshold response to carbon dioxide
S	Chemoreflex sensitivity response to carbon dioxide
V_{Eb}	Basal ventilation
V_{Tb}	Basal tidal volume
f_b	Basal breathing frequency
V_{ET}	Ventilatory threshold
V_{TT}	Tidal volume threshold
f_T	Breathing frequency threshold
V_{ES}	Ventilation sensitivity
V_{TS}	Tidal volume sensitivity
f_S	Breathing frequency sensitivity
RM-ANOVA	Repeated-measures analysis of variance

Onset	Time of onset of symptoms
Duration	Duration of symptoms
#Sx	Number of symptoms
ΣSx	Severity of symptoms
SEM	Standard error of the mean
SD	Standard deviation
Baseline	Baseline tryptophan levels
Amg	Amygdala
PAG	Dorsal periaquaductal gray matter

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INTRODUCTION

Panic Disorder

Panic disorder (PD) is a common and debilitating disease characterized by the presence of spontaneous panic attacks (Wittchen and Essan, 1993; American Psychiatric Association, 1994). Panic attacks (PAs) are discrete episodes of intense anxiety that are perceived to be uncontrollable and are accompanied by unpleasant physical sensations such as palpitations, chest pain, dyspnea, choking, sweating, tremors, faintness and paraesthesia. PAs are also accompanied by psychosensorial symptoms such as depersonalization and cognitive symptoms such as fear of losing control, going crazy or dying. A PA can last from a few minutes to more than an hour, and intense fatigue is frequently reported at the conclusion of the episode (Bradwejn and Koszycki, 1994). Although successful treatments for this condition are now available, the underlying pathophysiology of the illness remains elusive.

Etiological Theories of Panic

Etiological considerations of panic broadly fit into those supported by "cognitive theorists" (ex. Clark 1986, 1988; Barlow 1985; Cox 1996) and "biological theorists" (ex. Klein 1993, Charney and Redmond, 1983; Ley 1989; Gorman et al., 2000). Proponents of the former consider panic to be the result of catastrophic cognitive misinterpretation of interoceptive sensations or thoughts. A PD patient, on perceiving sensations either peripheral (e.g. dyspnea, sweating, tachycardia) or central (e.g. derealisation, loss of control), is believed to interpret their significance as catastrophic (i.e. a disastrous event like a heart attack or faint is forthcoming). This elicits further apprehension and sensation focus, resulting in positive feedback of catastrophic cognitions culminating in a

panic attack. Major objections to the theory often raised by "biological theorists" include: nocturnal panic during the non-rapid eye movement (non-REM, dream) phase of sleep (ex. Mellman and Uhde, 1990; Hauri et al., 1989) where danger cues cognitions are absent; absence of panic in PD patients during states that generate numerous uncomfortable physical sensations (ex. pregnancy and parturition, Klein, 1993) and the absence of panic in pharmacologically-induced states of somatic panic symptoms (see Klein, 1993). Panic is considered the result of a physiological flaw. While catastrophic cognitions may be part of a panic attack, they are not thought to be a causal element in panic initiation (see Coplan and Klein, 1996; Nutt and Lawson 1992 for review).

Panicogenic Challenges

The spontaneous nature of PAs necessitated the introduction of a number of laboratory panic inducing agents (panicogens) in an attempt to study the phenomenon. More extensively studied physiological and pharmacological challenges include: noradrenergic (NA) agents (ex. yohimbine and noradrenaline), cholecystokinin (CCK), lactate, bicarbonate, hypercapnia, serotonergic agents (ex. 5-HT agonists and antagonists) and hyperventilation (see Nutt and Lawson, 1992 for review). Of the numerous agents identified, lactate, bicarbonate and hypercapnic challenges have focused on the ventilatory response in PD patients (see Coplan and Klein, 1996 for review).

Challenge studies using intravenous sodium lactate have long shown its effectiveness in inducing PA's in PD patients but not in controls (Pitts and McClure, 1967). Despite years of research, current literature provides no mechanism on its mode of action. Because sodium lactate and sodium bicarbonate are both panicogenic and

metabolized to carbon dioxide (CO_2; see review, Coplan and Klein, 1996), this elevation in CO_2 has been proposed as the mechanism of panic induction. However, this explanation is now not accepted since a non-metalisable dextro-rotary form of lactate also induced PAs (Gorman et al., 1990). Nevertheless, the success of CO_2 in inducing panic in a number of studies (Lousberg et al., 1988; Gorman et al., 1993) gave rise to the "CO_2 hypersensitivity theory" of panic. Upon inhalation of a hypercapnic gas mixture (5% or 35% CO_2) PD patients are believed to experience a subjective sense of dyspnea, resulting in a panic attack. A number of other reports also confirm the presence and relevance of respiratory abnormalities in patients with PD (Gorman et al., 1988; Woods et al., 1986; Griez et al., 1987; Carr et al., 1987; Sanderson et al., 1989.) Based on this research, Klein (1993) formulated an integrative "false suffocation alarm" theory of panic.

Klein's (1993) Hypothesis

Klein (1993) argues that panic patients are hypersensitive to CO_2, with acceptable blood CO_2 levels lower than those of healthy controls, so that when this threshold level is exceeded, "the brain's suffocation monitor erroneously signals a lack of useful air, thereby maladaptively triggering an evolved suffocation alarm system" which manifests itself as a panic attack (PA). The monitor of the partial pressure of CO_2 (PCO_2) is thus considered the physiological mechanism for detecting potential suffocation. According to the theory everyone, including healthy subjects, possesses a "suffocation alarm," but in PD this "evolutionarily derived set-point has become dysfunctional," and the resultant hypersensitivity becomes the instigator of spontaneous panic attacks in a variety of common situations in life where one's CO_2 levels would slightly rise. A major limitation of this theory has been the lack of an anatomical system for the "suffocation alarm."

In an attempt to define the neuroanatomical site of the "suffocation alarm," we examined the respiratory chemoreflexes, the respiratory sensors of suffocation, in PD patients and controls (Katzman et al., 2001, in submission.) We found no differences in the chemoreflexes in terms of either the threshold or sensitivity of response to CO_2 between the two groups. However, Klein also states that PD patients go through periods when their condition worsens, that is through "bad periods" and through less morbid or "good periods" when they have fewer PAs. It is believed that "during bad spells the suffocation alarm threshold is pathologically depressed." It is therefore possible that differences were masked due to a temporary reset of suffocation alarm in all or a subset of patients during a "good spell" of disease pathology. This, and the various challenge studies cited, have in part, been undertaken to elucidate the specific brain system involved in panic. One system that has shown a good deal of promise in terms of being a player in the anxiogenic/panicogenic system is the serotonergic system.

Serotonin, Panic and Respiratory Effects

Many lines of evidence have now linked a deficiency in serotonin (5-hydroxytryptamine; 5-HT) with anxiety and specifically with panic disorder (see Anderson and Mortimore, 1999; Coplan et al., 1992 for review), but its role remains unclear. Clinical trials indicate that drugs that putatively increase 5-HT neurotransmission (Blier et al., 1987), the selective serotonin reuptake inhibitors (SSRI's), are effective anti-panic agents (Evans et al., 1986; Gorman et al., 1987; Den Boer and Westenberg, 1988; Schneier et al., 1990). The success of the SSRI's provides the bulk of evidence for the involvement of the 5HT system in panic.

Early preclinical studies provided the link between the hypothesis of deficient 5-HT neurotransmission and associated respiratory hyperactivity in panic disorder (Olson et al., 1979; Lundberg et al., 1980; Mitchell et al., 1983). Several studies showed that ventilation decreases after both peripheral and central administration of 5-HT agonists (Armijo and Florez, 1974; Mueller et al., 1980). Lundberg et al. (1978) has also shown that a precursor of the substance (5-hydroxytryptophan; 5-HTP) decreased ventilation and CO_2 sensitivity in rats in a dose-dependent fashion. On the other hand, the tryptophan hydroxylase inhibitor parachlorophenylpiperazine (PCPA) caused hyperventilation in goats (Mitchell et al., 1983) and rats (Olson et al., 1979) with repletion of 5-HTP normalizing ventilation and CO_2 levels. From these animal studies, it was generally agreed that the role of 5-HT was to attenuate ventilation. This has led a number of researchers to suggest and test the theory of deficient 5-HT modulation as a cause of respiratory hypersensitivity in PD.

Recent human research into the etiology of panic has employed manipulation of brain 5-HT levels using a tryptophan (TRP) depletion intervention in concert with a CO_2 challenge (Kent et al., 1996; Miller et al., 1996; Klaassen et al., 1998; Miller et al., 2000). Tryptophan being the pre-cursor amino acid to 5-HT synthesis, its depletion causes a short-term global reduction in brain 5-HT function in human subjects as a consequence of ingesting a tryptophan free amino acid drink (Reilly et al., 1997). In humans, there is evidence of reduced 5-HT synthesis (Nishizawa et al., 1997) and reduced cerebrospinal fluid tryptophan and 5-hydroxyindoleacetic acid (5-HT metabolite) after TRP depletion (Carpenter et al., 1998; Williams et al., 1999) with technique specificity verified by

Gessa et al. (1975) and more recently by Klaassen et al. (1999.) The general consensus of these studies is that TRP depletion enhances the effect of a CO_2 panic challenge by elevating tidal volume, frequency and minute ventilation, with little effect on general or anticipatory anxiety levels in PD patients (Kent et al., 1996; Miller et al., 1996; Klaassen et al., 1998; Miller et al., 2000). This suggestion also fits with the Klein theory in the context of defective 5-HT neurotransmission sensitizing the respiratory control centers/chemoreflexes (during low CNS 5-HT periods, or during "bad spells" of panic), which misfire in response to slight elevations in CO_2.

However, recent *in vitro* studies of 5-HT involvement in control of breathing contradict early preclinical studies and therefore negate the premise of serotonin's role as a depressant of ventilation. The caudal raphe nuclei have been shown to have putative chemoreceptive properties (Bernard et al., 1996; Richerson et al., 1995; Wang et al., 1998) and to have stimulatory effects on ventilatory output via increased phrenic/hypoglossal drive (Hilarie et al., 1997; Dreshnaj et al., 1997). This is further complicated by the presence of CO_2-stimulated (nucleus raphe obscurus, ROb; nucleus raphe magnus, RMg) and CO_2-inhibited (nucleus raphe pallidus, RPa; Lalley, 1986) regions within the caudal raphe.

The role of 5-HT on ventilatory output needs to reassessed before we are able to accept previous findings of 5-HT deficiency mediating the respiratory abnormality in PD and consequently the role of deficient 5-HT in depressing/sensitizing the "suffocation alarm" threshold. The present study employed the TRP depletion technique together with a modified Read (1967) rebreathing method, an accurate measure of the chemoreflex and

basal drives to breathe, to answer the following questions: What is the role of 5-HT neuromodulation on the central and peripheral chemoreflex threshold and sensitivity of response to CO_2 and, as a corollary, what is the implication of that outcome on Klein's "suffocation alarm" theory of panic. Study of any neurochemical system's modulation of respiratory control demands a basic understanding of both the former and the latter. The following discussion presents an overview of control of breathing and the modulating neurochemical system in question, the serotonergic system.

Respiratory Control Overview

Unlike PD, the respiratory system has been studied for over a century. This has meant that many of its major characterizations were made in the early stages of research in this field. Early on it was determined that there are three main stimuli that drive ventilation: CO_2 (Miescher-Rusch, 1885) and its effective vascular equilibrium biproduct, hydrogen ion (H^+; Walter, 1868) (i.e. increases in hydrogen ion concentration, $[H^+]$) were identified as the primary stimuli, with a role for oxygen (O_2; Pfluger, 1868) as a contributing stimulus. Haldane and Priestley's (1905) groundbreaking work established that alveolar partial pressures of CO_2 are regulated by pulmonary ventilation. With this work, they essentially described a respiratory feedback control system. That is, CO_2 is both regulated by lung ventilation (feed-forward control) and it stimulates lung ventilation (feedback control). To detect changes in these drive stimuli and effect a feedback role to this control system necessitates a set of signal sensors or receptors, these are the central and peripheral chemoreceptors.

Aside from the basic chemical drives to breathe (CO_2, H^+, O_2) from the chemoreceptors, there are other feed forward stimuli from muscle afferents (Freyschuss, 1970; Goodwin et al., 1972) as well as widespread cortical and subcortical centers (see reviews by Plum & Leigh, 1981; Hugelin, 1986; and Davenport & Reep, 1995, among others). The latter may play an important role in the expression of ventilatory pattern in PD.

Knowledge of the nature of the stimuli to breathe and the location of their receptors, allows a conceptualisation of a respiratory feedback control system (Grodins, 1950; Grodins et al., 1954). This system has both forward and feedback components, briefly described below.

Metabolic Hyperbola

The metabolic hyperbola (also known as the ventilation equation, Gray, 1946, 1950; Rahn & Fenn, 1955) describes the forward part of the feedback loop. It describes the effect of ventilation on carbon dioxide and oxygen (Stewart, 1897; Douglas, 1911). At zero ventilation, the partial pressure of oxygen approaches zero and as ventilation increases, the partial pressure of oxygen asymptotically approaches the inspired level (Figure 1, solid red line). On the other hand, the partial pressure of carbon dioxide approaches zero as ventilation increases and increases as ventilation tends towards zero (Figure 1, solid blue line).

The shape of the metabolic hyperbola is dictated by the metabolic rate. As the metabolic rate increases, the hyperbolas described by ventilation vs. carbon dioxide and oxygen respectively move outward as shown in Figure 1 (dashed blue and red lines

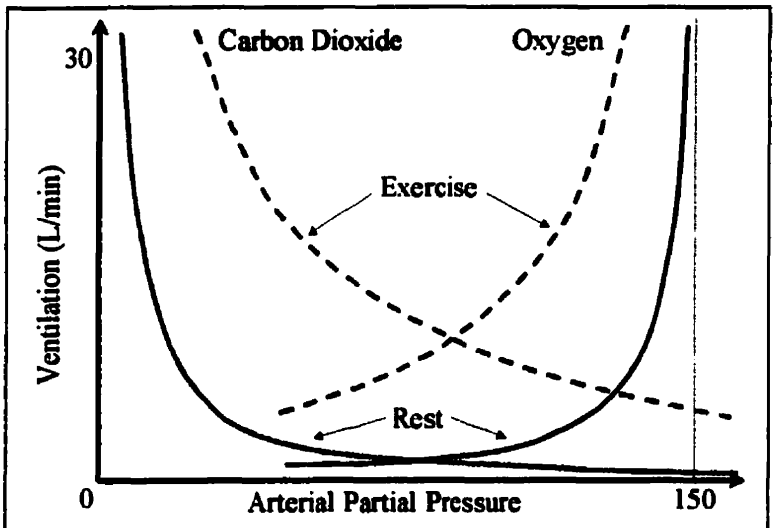

Figure 1: Metabolic hyperbolas at rest and during exercise.

respectively). In the range of normal ventilation at rest (5 L/min and 40 mmHg CO_2), the hyperbolas are relatively flat, demonstrating the fact that small changes in ventilation result in profound changes in PO_2 and PCO_2. In addition, the mathematical intersection of the forward part of the controller loop (metabolic hyperbola), with the feedback (chemoreflexes) and other feed-forward parts (behavioural drives) explained below, describes the equilibrium point of the chemoreflex control system (see Figure 2).

Central Chemoreflex

The central chemoreceptors are the basis of the central chemoreflex, forming part of the feedback of the respiratory control loop that describes the relationship between carbon dioxide and ventilation. The central chemoreceptors are localized mainly, but not exclusively (Nattie 1999) on a well-perfused ventral medullary surface (Loeschcke & Koepchen, 1958; Loeschcke & Mitchell, 1963; Mitchell et al., 1963; Schlaefke et al., 1970). Unlike their peipheral counterparts, these chemoreceptors are unaffected by different levels of iso-oxia, responding only to hypercapnic and acidic ventilatory drive stimuli (Leusen, 1954; Mitchell et al., 1963; Pappenheimer et al., 1965; Fencl et al.,

1966; Nattie, 1983; Nattie, 1999). Due to their location within the blood brain barrier (BBB; Pappenheimer, 1970; Brightman, 1977) the central chemoreceptors are isolated from polar solutes and are thus expected to have a damped and delayed response to these drive stimuli.

The central chemoreflex responds to changes in CO_2 and compensates for these changes by adjusting ventilation. Below a certain threshold level of CO_2, changes in CO_2 level have no effect on ventilation; above this CO_2 threshold, ventilation raises with a linear slope in proportion to CO_2. These same threshold and sensitivity response properties govern the peripheral chemoreflex response.

Peripheral Chemoreflex

The peripheral (carotid body) chemoreceptors (Heymans and Heymans, 1927) form the basis of the peripheral chemoreflex feedback drive of the respiratory control loop, additive to the central chemoreflex (Gray, 1950). Located at the bifurcation of the carotid artery (Comroe & Schmidt, 1938; Biscoe, 1971), the peripheral chemoreceptors consist of glomeruli well encapsulated by dense capillary beds (de Castro & Rubio, 1968) with a high rate of perfusion (Gonzalez et al., 1995), thus they are ideally situated and equipped to sample blood approaching the brain. Like the central chemoreceptors, the peripheral chemoreceptors respond to hypercapnic and acidic ventilatory drive stimuli. However, their response is to a given level of CO_2 stimulus is uniquely affected by the accompanying level of oxygen tension.

The peripheral chemoreflex is described by the reflex arc formed by the afferent and efferent connections to the peripheral chemoreceptors. Sensory information from the

peripheral chemoreceptors leaves through the cranial sinus nerves (Gonzalez et al., 1992) where they meet at the glossopharyngeal nerve, and continue through the nucleus tractus solitarius, terminating in the ventral respiratory group of the medulla (Torrance, 1996).

Wakefulness Drive

Aside from basic chemical drives to breathe (CO_2, acidity, O_2) mediated by the chemoreflexes, many behavioural drives to breathe (Shea, 1996) contribute to the feed-forward part of the respiratory control loop. These drives originate from cortical, sub-cortical or general arousal drives of reticular system origin (see reviews by Plum and Leigh, 1981; Hugelin, 1986; Davenport & Reep, 1995; Philipson and Bowes, 1986). The latter of these may represent what Fink (1961) called the "wakefulness drive," since it disappears in sleep and anasthesia (Skatrud & Dempsey, 1983) but is maintained during hypocapnia in awake humans. These drives are thus state dependent, but independent of CO_2 or O_2 levels and therefore independent of chemoreflex drives to breathe.

Integrated Model of Respiratory Control

The modern respiratory control system integrates the "wakefulness drive," the feed-forward and feedback components of the respiratory control loop with the metabolic hyperbola on the same set of axes for the purpose of making predictions (Duffin et al., 2000). The ordinate represents minute ventilation with arterial PCO_2 on the abscissa. End-tidal PCO_2 ($P_{ET}CO_2$) values are taken as a close approximation of arterial values (Read & Leigh, 1967). A fan of lines with increasing slopes represents the effects of different levels of PO_2 on the ventilatory response to increasing $P_{ET}CO_2$ levels. The central and peripheral chemoreflex responses add together to give a common chemoreflex drive threshold and chemoreflex sensitivity of response to CO_2 – the

feedback part of the respiratory controller. Sensitivity of the peripheral chemoreflex to different levels of PO_2 can be diminished to zero or near zero (Pedersen et al., 1999) with a PO_2 level of 150 mmHg or higher. At this hyperoxic level, the chemoreflex contribution to the ventilatory response derives primarily from the central chemoreflex drive (Dejours, 1962; Duffin, 1971; Mohan and Duffin, 1997). Below the chemoreflex threshold response to CO_2, ventilation is independent of PCO_2 or PO_2 levels and is driven by "wakefulness drives" and other behavioural feed-forward drives (Shea, 1996). A superimposition of the metabolic hyperbola on the chemoreflex response fan of lines gives the position of ventilatory equilibrium. Furthermore, an isocapnic line shows intercepts along the chemoreflex response line that predict the system's response. This integrated model of respiratory control is summarized graphically in Figure 2.

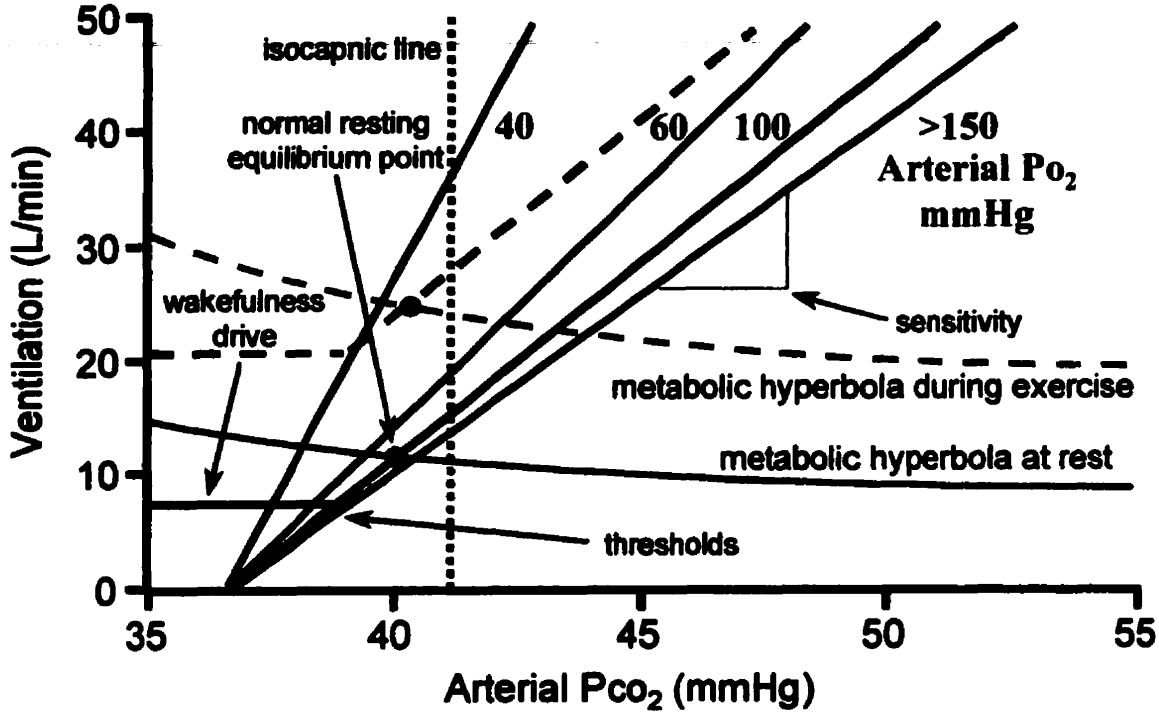

Figure 2: Current model of the chemoreflex control system. The combined effect of the central and peripheral chemoreflex responses and their resultant threshold and a slope are shown as a fan of lines (black; 100, green; 60, and blue; 40 lines). At levels of hyperoxia greater than 150 mmHg, the chemoreflex contribution is derived principally from the central chemoreflex (red line). Below the threshold to carbon dioxide, neither oxygen nor carbon dioxide affect ventilation (purple line). Superimposing the metabolic hyperbola (grey line) on the chemoreflex response lines shows the position of equilibrium. Varying the metabolic demand (grey dashed line) shifts this equilibrium point keeping in mind the vertical shift of the chemoreflex response line and the wakefulness drive (purple dashed line and black dashed line). In addition, maintaining isocapnia (black dotted line) shows intercepts along the chemoreflex response line that predict the system's response.

Serotonergic System

The majority of the brain's serotonergic neurons are clustered within two regions: the rostral raphe nuclei and the caudal raphe nuclei. Afferents from the serotonergic neurons located in these nuclei project to virtually all regions of the human brain. The rostral raphe consist primarily of the dorsal raphe nuclei (DRN) and the medial raphe nuclei (MRN). The caudal raphe comprise the nucleus raphe obscurus (ROb), nucleus raphe magnus (RMg), and the smallest, nucleus raphe pallidus (RPa), in between. The

DRN and MRN send their projections toward the forebrain in two morphologically distinct major bundles: one runs in the central gray matter, while the other is in the ventral part of the tegmentum. The two fibre systems are thoroughly mixed in the medial forebrain bundle, which then distributes axons to the many brain areas, including limbic and cortical structures. The caudal raphe send their projections primarily, but not exclusively, to the brainstem and spinal cord. Organisation of the principal pathways of the human serotonergic system are illustrated in Figure 3.

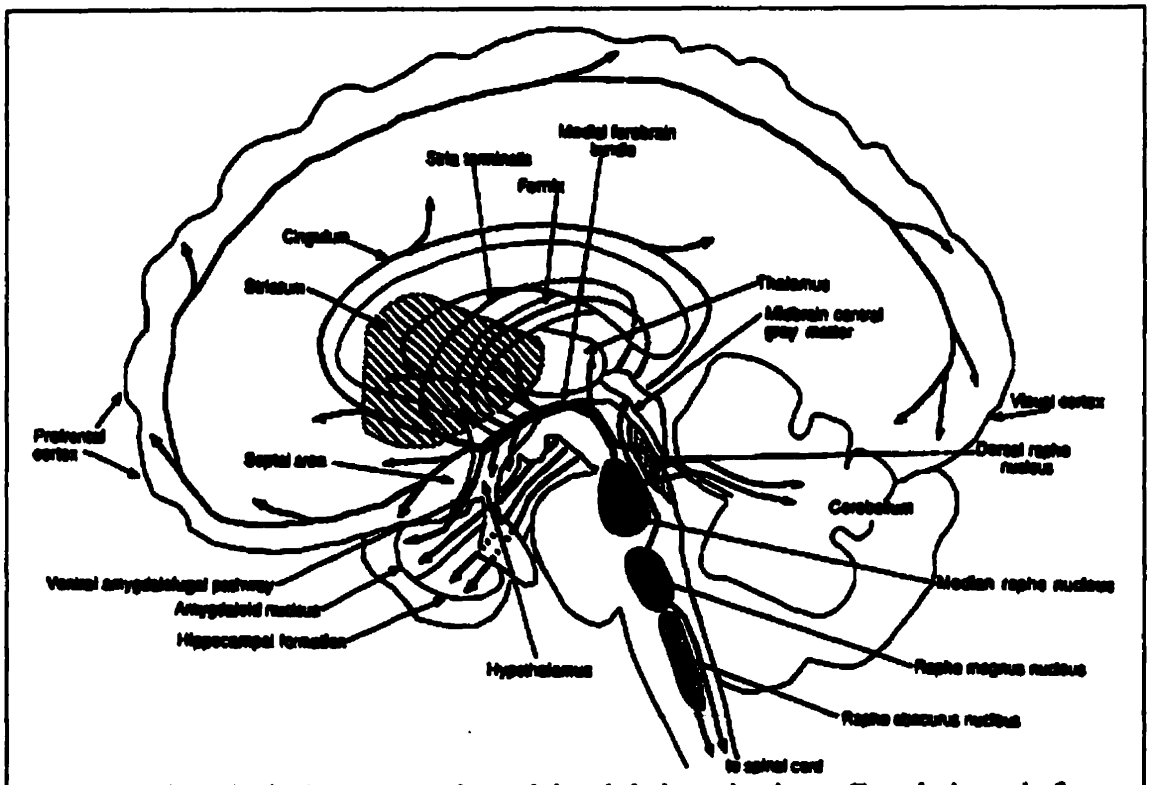

Figure 3: The principal serotonergic nuclei and their projections. For clarity only four raphe nuclei are displayed: dorsal raphe nucleus, median raphe nucleus, raphe magnus nucleus, and raphe obscurus. The two rostral nuclei are the principal sources of projections to the forebrain, while the two caudal nuclei project mainly to the brainstem and spinal cord. (From Tork and Hornung, 1995).

Study Objectives

With an understanding of the essentials of respiratory control and knowledge of the 5-HT system projections, we are ready to address the initial questions posed in this study, namely:

1) What is the role of 5-HT neuromodulation on the central and peripheral chemoreflex threshold and sensitivity of response to CO_2.
2) And, as a corollary, what is the implication of that outcome on Klein's "suffocation alarm" theory of panic.

To answer these questions, I employed the TRP depletion technique together with a modified Read rebreathing method, an accurate measure of the chemoreflex and basal drives to breathe.

Based on our previous measures of chemoreflex thresholds and sensitivities to CO_2 in PD and healthy volunteer (HV) populations (Katzman et al., 2001, in submission), I hypothesized that decreases in 5-HT levels, as induced via TRP depletion, will have no significant effect on the chemoreflex threshold or sensitivity of response to CO_2.

METHODS

Subjects

Eleven healthy male volunteers participated in the study. Female subjects were excluded due to potential interactions of menstrual cycle and 5-HT levels (Menkes et al., 1994). None of the subjects met criteria for any psychiatric diagnoses, as established by a semi-structured clinical interview (SCID) per DSM-IV criteria (APA, 1994). All subjects were in good physical health, as determined by history, laboratory hematology and chemistry, urinalysis, EKG and medical examination. None of the participants were taking any medication and all gave informed consent for the experiments after the Centre for Addiction and Mental Health Research Committee for human experimentation approved the protocol. None of the subjects were born at high altitudes or were smokers.

Protocol

Subjects arrived to the clinic on three separate occasions. On the first day (day 1), as part of a physical workup, blood was drawn following an overnight fast for later analysis of plasma TRP levels (baseline plasma TRP). The next two visits were test days (day 2 and 3), consisting of two interventions: a TRP-depleted (TRP-) or non-depleted (TRP+; placebo) drink, consumed between 8:00h-8:30h; modified Read rebreathing tests, under hyperoxic or hypoxic conditions, completed at a respiratory laboratory 4.5-6.5 hours following drink consumption, when brain TRP levels are lowest (Carpenter et al., 1998; Williams et al., 1999). Each subject completed 3 rebreathing tests on each test day: a practice test, a hyperoxic test, and a final hypoxic test, with a 20min inter-test interval. Comparison of hypoxic and hyperoxic measurements determined if changes occurred in

peripheral or central chemoreflexes, making assumptions discussed in Duffin et al. (2000). Each test visit was preceded with a 24hr low-protein or TRP-supplemented diet as appropriate. A blood sample was drawn 2.5 hours after drink consumption for later analysis of plasma free TRP levels to ensure sufficient plasma TRP depletion. Test days 2 and 3 were separated by no less than 3 and no more than 7 days with all interventions administered in a double blind, randomized fashion.

Amino Acid Drink

The 100g tryptophan depleted (TRP-) amino acid drink was prepared following a standard protocol by Young et al. (1985) (Table 1) with L-Lysine replaced by Lysine monohydrochloride to improve drink palatability. The placebo drink (TRP+) contained an additional 2.3g of L-tryptophan. The amino acid content of both drink types is approximately that of a 500g steak, with expected increases in plasma amino acid levels comparable to those following such a meal, except for the TRP- drink, where plasma TRP levels are expected to fall significantly. The amino acids were dissolved in 250mL of bottled water and 60mL of chocolate syrup (Quick, Nestle). Subjects were instructed to swallow the drink as quickly as possible while removing olfactory contributions using noseclips. A sugar-free mint gum was provided to all subjects after drink consumption with 750mL water for consumption until end of test day.

Table 1: Composition of the amino acid drink.

Amino Acid	Composition (g)
L-Alanine	5.5
L-Arginine	4.9
L-Cysteine	2.7
Glycine	3.2
L-Histidine	3.2
L-Isoleucine	8.0
L-Leucine	13.5
Lysine monohydrochloride	8.9
L-Methionine	3
L-Phenylalanine	5.7
L-Proline	12.2
L-Serine	6.9
L-Threonine	6.5
L-Tyrosine	6.9
L-Valine	8.9
L-Tryptophan	2.3

In the tryptophan depleted drink, L-Tryptophan was excluded.

Low Protein Diet

All subjects ate a low protein diet as per Delgado et al. (1990) for 24hrs prior to days 2 and 3 of testing. The diet consisted of a minimum possible amount of tryptophan (160mg/day), while supplying adequate protein (48g/day) and energy (10 500kJ/day). Subjects were permitted to consume water without restrictions but were asked to refrain from caffeine or alcohol consumption for 48 hours prior to each test day.

Plasma Amino Acid Measurements

Following venipuncture, blood was immediately placed on ice and centrifuged at 3000 rpm for 10 minutes at 4 °C. Subsequently, plasma was stored at -80 °C and analysed for levels of free tryptophan, using high pressure liquid chromatography (HPLC).

Rebreathing

Before rebreathing, subjects voluntarily hyperventilated room air for 5 minutes, while coached to maintain end-tidal partial pressure of carbon dioxide ($P_{ET}CO_2$) between 19 and 25 mmHg. Following an expiration, subjects were switched to the rebreathing bag and took three deep breaths to ensure the PCO_2 in the bag, lungs and arterial blood quickly equilibrated to the mixed venous PCO_2. The latter estimates the PCO_2 at the central chemoreceptor. A plateau in $P_{ET}CO_2$ at the start of rebreathing evidenced adequate equilibration, and was a prerequisite for continuing the test.

The rebreathing bag initially contained a PCO_2 of 42 mmHg and a partial pressure of oxygen (PO_2) of 150 mmHg for the hyperoxic rebreathing test, or a PO_2 of 50 mmHg for the hypoxic rebreathing test. The hyperoxic rebreathing test diminishes the peripheral chemoreflex drive (Torrence, 1996) to breathe, generating a net central chemoreflex response to hypercapnia. The hypoxic test sensitizes the peripheral chemoreflex drive to breathe, with any changes in ventilatory response the result of peripheral chemoreflex drive (Mohan et al., 1997). During rebreathing, PO_2 was maintained constant (iso-oxic) by the addition of oxygen to the rebreathing bag under computer control. Rebreathing was terminated either when ventilation exceeded 100 L/min. or $P_{ET}CO_2$ exceeded 65 mmHg.

During each rebreathing test subjects indicated the onset of any symptoms experienced by an upward hand-swing. Cessation of symptoms was indicated by a downward hand-swing, if it occurred prior to completion of rebreathing. Following each rebreathing test, subjects' symptoms were retrospectively assessed with the use of a

DSM-IV derived Panic Symptom Scale (PSS; Koszycki et al., 1991) with severity of symptoms ranging from 0 (not present) to 5 (extremely severe). A panic attack was defined as the presence of four symptoms with a score of greater than 1 including the "anxiety, fear and/or apprehension" measure.

Apparatus

The rebreathing apparatus was described previously by Mohan and Duffin (1997) and is illustrated in Figure 4. Briefly, subjects wore noseclips throughout the experiment and breathed through a mouthpiece connected to one side of a wide-bore Y valve (Collins P-319, 80-ml dead space) that allowed them to switch from room air to the rebreathing bag. The 5-L rebreathing bag was enclosed in a rigid container with a 50-mm diameter tube connected to a dry rolling-seal spirometer (Morgan, Spiroflow Model 130). A sample flow of 90 ml/minute from the mouthpiece side of the Y valve (gas sample tube UD 5037, Bruel and Kjaer) permitted continuous analysis of carbon dioxide and oxygen.

Carbon dioxide and oxygen were analyzed with an anesthetic gas monitor with a resolution of 1 mmHg and 5 mmHg respectively (Bruel and Kjaer, type 1304). The sampled gas was returned to the rebreathing bag. Iso-oxia was maintained by a flow of oxygen to the mouthpiece side of the Y valve, under computer control. A 16-bit analog to digital converter (National Instruments, AT-MIO-16XE-50) digitized the analog signals for on-line computer analysis using specially written software (National Instruments, LabVIEW) available on request.

The software calculated tidal volumes, inspiratory and expiratory times, ventilation, $P_{ET}CO_2$ and $P_{ET}O_2$ on a breath-by-breath basis. The volume changes and

$P_{ET}CO_2$ and $P_{ET}O_2$ were also written on a chart recorder (Graphtec, Lineacorder Mark VII WR 3101) to monitor the initial rebreathing equilibration. The measurement system was calibrated before each experimental session using gases of known concentrations (room air and commercial gas mixture of 5% CO_2, 10% O_2.

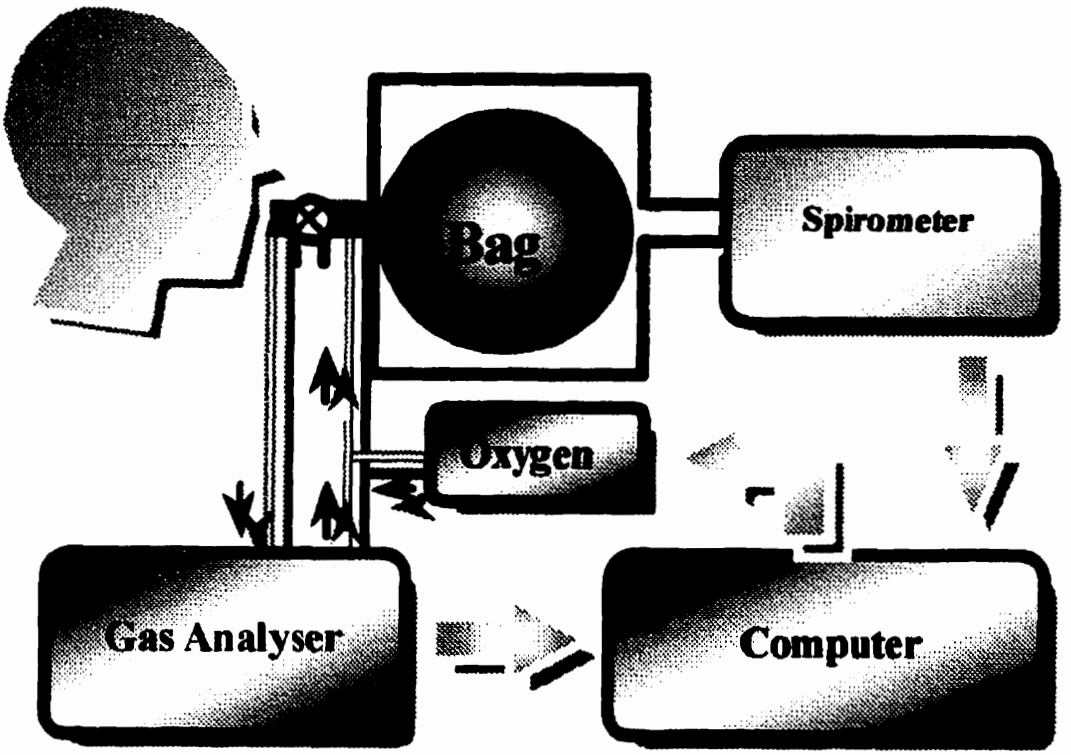

Figure 4: Setup of the rebreathing apparatus.

Data Analysis

Analysis of the breath-by-breath data was accomplished using a spreadsheet (Microsoft, Excel) specially designed for this purpose. Data from the initial equilibration at the start of rebreathing, as well as sighs, swallows and breaths incorrectly detected by the software were excluded from further analysis. Then, plots of ventilation (V_E, L/min.) and its components, tidal volume (V_T, ml BTPS) and breathing frequency (f,

breaths/min.) were plotted against end-tidal P_{CO_2} (mmHg) to generate three respiratory outcome variables (ventilation, V_E; tidal volume, V_T; breathing frequency, f).

These plots were analyzed by fitting a model for the ventilatory response made up of a sum of three possible segments to each of the respiratory variables: ventilation (V_E), tidal volume (V_T), and breathing frequency (f) according to assumptions detailed in Duffin et al. (2000). The first segment representing basal values of the respiratory parameters was fitted with an exponential decline to account for any short-term potentiation secondary to the voluntary hyperventilation prior to rebreathing. The second segment was a straight-line segment from the first breakpoint to the second. If a second breakpoint was evident, a third segment was fitted above the second breakpoint. The fits were generated using a commercial fitting program (Sigmaplot, SPSS) that simultaneously calculated the breakpoints, slopes, basal values and exponential constants. The first breakpoint for V_E measures the chemoreflex threshold and the slope above the first breakpoint measures the chemoreflex sensitivity. The basal values measure the drives to breathe other than that from the chemoreflexes, including state arousal and behavioral drives.

Statistical Analyses

Three respiratory outcome variables were collected (ventilation, V_E; tidal volume, V_T; breathing frequency, f) for each of three response characteristics (baseline, B; threshold, T; and sensitivity, S). Thus, nine respiratory outcome variables were generated: basal values (B) for ventilation (V_{Eb}), tidal volume (V_{Tb}), and breathing frequency (f_b); threshold values (T) for ventilation (V_{ET}), tidal volume (V_{TT}) and breathing frequency

(f_T); and sensitivity values (S) for ventilation (V_{ES}), tidal volume (V_{TS}) and breathing frequency (f_S). These variables were compared for each response characteristic using repeated-measures analysis of variance (RM-ANOVA) in a 2 (drink type: TRP-, TRP+) x 2 (PO_2: hyperoxia, hypoxia) design.

Effects of the different interventions (drink type and PO_2) on variability in ventilation (V_E) were also assessed by determining individual subject data standard deviations (SD) from fit, followed by a RM-ANOVA analysis of SD using the same 2 (drink type: TRP-, TRP+) x 2 (PO2: hyperoxia, hypoxia) design.

The same analysis was made for PA outcome variables: PA presence (dichotomous outcome variable), time of onset of symptoms (Onset), duration of symptoms (Duration), number (#Sx) and severity (ΣSx) of symptoms using a 2 (drink type: TRP-, TRP+) x 2 (PO_2: hyperoxia, hypoxia) design (SigmaStat 2.0, SPSS). Effects of amino acid drink on free tryptophan levels were analyzed using one-way RM-ANOVA on ranks (condition: baseline, TRP-, TRP) followed by multiple-pairwise comparisons (Student-Neuman-Keuls test) where appropriate. The significance level for all analyses was set at 5%. Unless otherwise indicated, all results are reported as mean +/- SEM.

RESULTS

Plasma Tryptophan Depletion

Table 2 presents the TRP-, TRP+ and baseline measures for free plasma tryptophan. There was a significant fall ($P<0.05$) in free tryptophan levels between the TRP- and baseline conditions, with median percent fall of 71%. Conversely, a significant increase ($P<0.05$) in free tryptophan levels was observed between the TRP and baseline conditions, with median percent increase of 147%. Tryptophan levels between TRP- and TRP+ conditions were also significantly different ($P<0.05$), with median percent difference of 747%.

Table 2: Plasma free tryptophan levels during various treatment interventions (baseline, TRP- and TRP+ with associated medians and percentiles).

	Baseline (Median, 25% & 75%)	**TRP-** (Median, 25% & 75%)	**TRP +** (Median, 25%&75%)	**Significance**
Free Tryptophan (μg/ml)	8.977 25% = 7.411 75% = 9.779	2.630 25% = 1.677 75% = 4.119	22.273 25% = 17.350 75% = 22.778	$p<0.05$ for all pair-wise comparisons

Rebreathing

Figure 5 presents a typical rebreathing test on a ventilation (V_E) vs. time graph as it appears under experimental conditions during data acquisition. Representative rebreathing test responses for a typical subject are shown in Figure 6, representing a hyperoxic rebreathing test under different conditions of drink type (TRP- and TRP+); and Figure 7 representing a hypoxic test under the different conditions of drink type (TRP- and TRP+). The different rebreathing response characteristics of the respiratory variables studied (baseline (B), chemoreflex threshold (T) and sensitivity (S)) are indicated in each

of Figures 6 and 7. Tables 3 and 4 present a summary of the effect of TRP- and PO_2 respectively on all the respiratory variables studied during rebreathing.

There was a significant effect on baseline ventilation (V_{Eb}), for drink type (P=0.043), but not for PO_2 (P=0.09) with no drink x PO_2 interaction effects. Baseline tidal volume (V_{Tb}) and breathing frequency (f_b) showed no significant differences for drink type or PO_2 with no drink x PO_2 interaction effects.

Similarly, there was no significant effect of drink type on chemoreflex threshold response to CO_2 for ventilation (V_{ET},) tidal volume (V_{TT}) or breathing frequency (f_T). Expectedly, there was a significant effect of PO_2 on all of V_{ET} (P<0.001), V_{TT} (P<0.001), and f_T (P=0.014) with no drink type x PO_2 interaction effects.

Chemoreflex sensitivity of response to CO_2 was also not significantly affected by drink type with no significant effect on ventilation sensitivity (V_{ES}), tidal volume sensitivity (V_{TS}), or breathing frequency sensitivity (f_S). Expectedly, PO_2 had a significant effect on V_{ES} (P=0.015), and V_{TT} (P=0.044), but no significant effect on f_S. No significant drink type x PO_2 interaction effects were observed for V_{ES}, V_{TS} or f_S.

Consistent with the other data, drink type had no significant effects on variability in V_E (P=0.423). Expectedly, PO_2 had a significant effect on V_E variability, with significantly higher variability during the hypoxic rebreathing tests (P<0.01). There were no drink type x PO_2 interaction effects on V_E variability.

Panic Measures

There were 2 rebreathing tests (hyperoxic and hypoxic test; practice test excluded) performed on each of the 11 subjects for each drink type, for a final total of 44 rebreathing tests. There were a total of 4 PAs experienced in 3 different subjects (2 PAs per each drink type) in all these rebreathing tests. There was no significant effect of drink type or PO_2 on the presence of panic, with no drink type x PO_2 interaction effects.

The remaining panic variables studied, Onset of Sx, Duration of Sx, #Sx, and ΣSx, were unaffected by drink type or PO_2, with no drink type x PO_2 interaction effects.

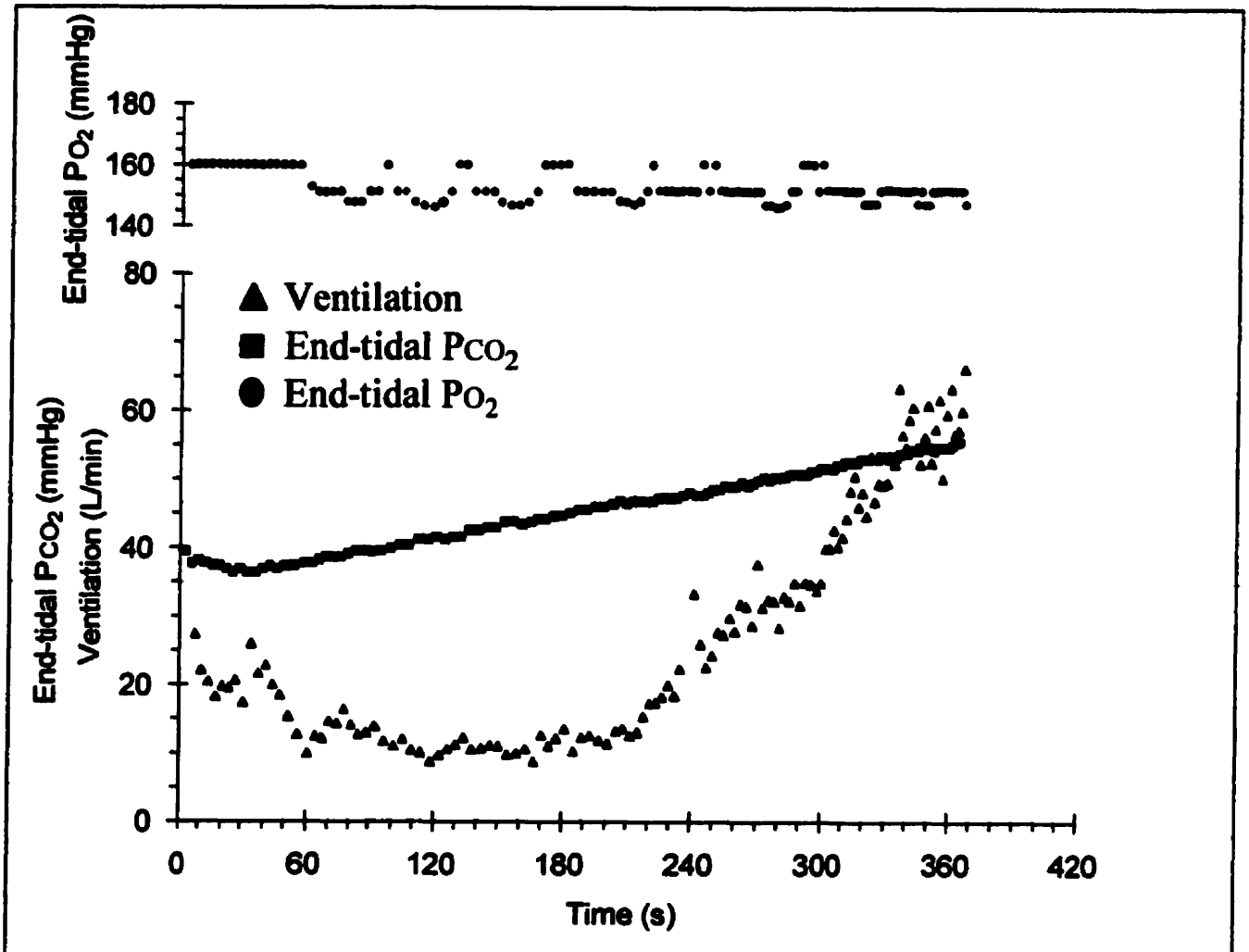

Figure 5: On line rebreathing test response for ventilation, end-tidal PCO_2 and PO_2.

Table 3: Effect of tryptophan depletion on respiratory variables.

Variable	Drink Type (Mean +/- SEM)		Significance
	TRP-	TRP+	
V_{Eb} (L/min)	11.05 +/- 1.22	9.44 +/- 1.22	p<0.05
V_{Tb} (mL)	621.62 +/- 79.24	548.51 +/- 79.24	NS
f_b (b/min)	12.74 +/- 1.78	14.23 +/- 1.82	NS
V_{ET} (mmHg)	39.76 +/- 0.85	40.15 +/- 0.85	NS
V_{TT} (mmHg)	38.23 +/- 0.88	38.63 +/- 0.85	NS
f_T (mmHg)	40.92 +/- 1.83	40.40 +/- 0.39	NS
V_{ES} (L/min/mmHg)	8.57 +/- 1.18	8.53 +/- 1.18	NS
V_{TS} (mL/mmHg)	237.56 +/- 33.56	222.51 +/- 33.56	NS
f_S (b/min/mmHg)	2.82 +/- 0.55	3.01 +/- 0.55	NS

Table 4: Effect of different levels of PO_2 on respiratory variables.

Variable	PO_2 (Mean +/- SEM)		Significance
	Hyperoxia	Hypoxia	
V_{Eb} (L/min)	9.71 +/- 1.25	10.78 +/- 1.25	NS
V_{Tb} (mL)	558.70 +/- 72.85	611.43 +/- 93.70	NS
f_b (b/min)	13.62 +/- 1.73	13.41 +/- 1.69	NS
V_{ET} (mmHg)	42.06 +/- 0.81	37.85 +/- 0.81	p<0.001
V_{TT} (mmHg)	40.84 +/- 0.81	36.03 +/- 0.87	p<0.001
f_T (mmHg)	43.14 +/- 1.14	38.18 +/- 1.64	p=0.015
V_{ES} (L/min/mmHg)	5.90 +/- 1.43	11.20 +/- 1.43	p=0.015
V_{TS} (mL/mmHg)	186.32 +/- 36.30	273.75 +/- 36.30	p<0.05
f_S (b/min/mmHg)	2.75 +/- 0.57	3.08 +/- 0.57	NS

NS=non-significant statistical result (p>0.05.)

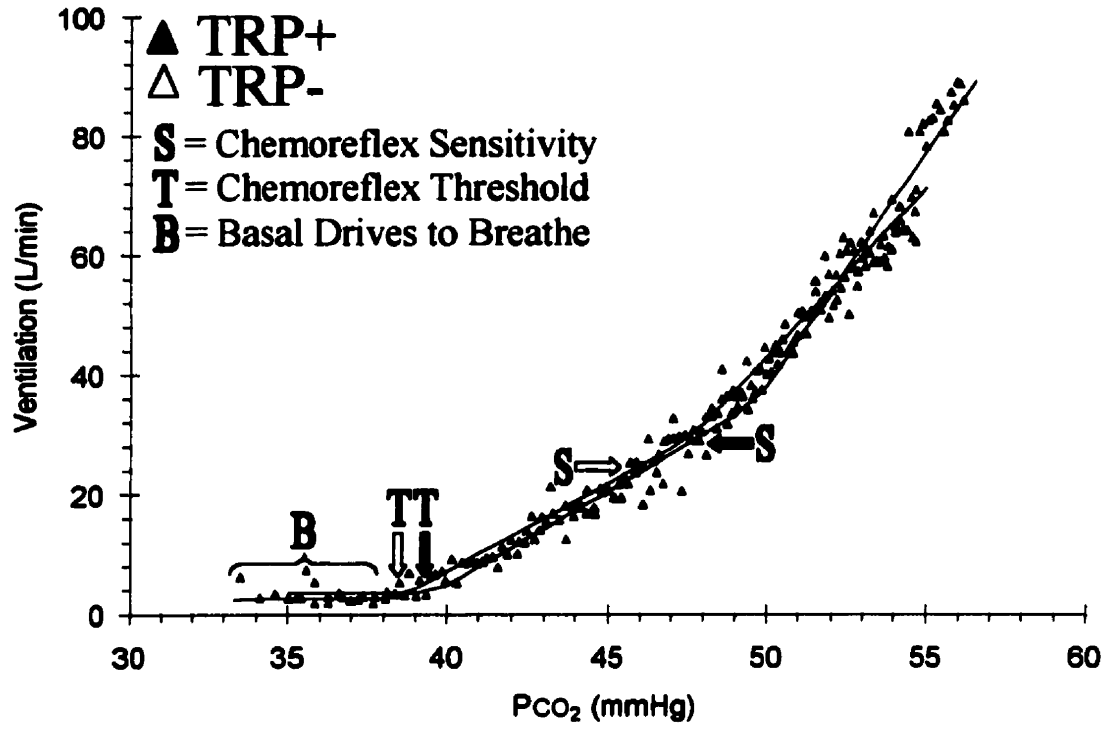

Figure 6: Representative ventilatory responses during a hyperoxic rebreathing test.

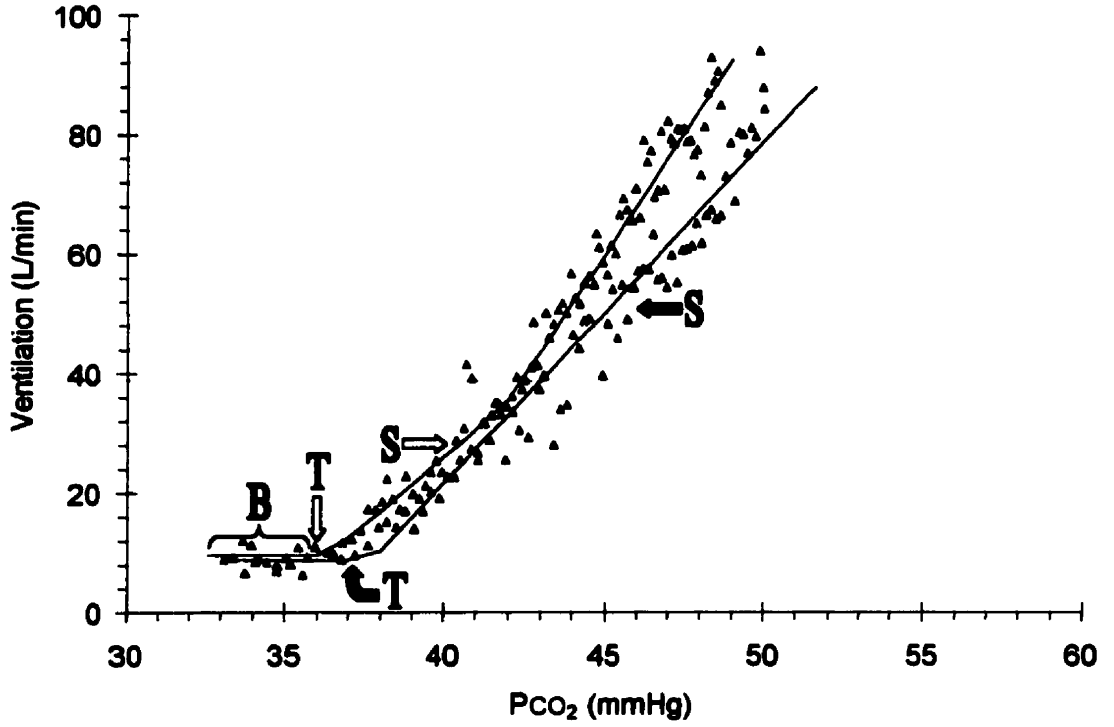

Figure 7: Representative ventilatory responses during a hypoxic rebreathing test

DISCUSSION

I measured the effects of tryptophan depletion on the central and peripheral chemoreflex threshold and sensitivity of response to CO_2 and found no effect on these chemoreflex parameters, as measured by ventilation, tidal volume and breathing frequency responses. These negative findings were observed in presence of sufficient CNS tryptophan depletion as indicated by significant fall in free blood tryptophan levels (71% vs. 83% Delgado et al., (1994) and 70% Price et al., (1997)), as measured 2.5 hours after drink consumption. As with food intake, the placebo drink caused a significant increase in free tryptophan (147%), comparable to others' results (360% Delgado et al., (1990); 184% Young et al., (1985)) – an issue of potential concern for positive findings. Research has shown that plasma nadirs of tryptophan are reached between 2-6 hours after drink consumption with CNS depletion reaching comparable levels 3-4 hours later (ex. Carpenter et al., 1998; Williams et al., 1999). As the fall in tryptophan observed here is comparable with that in other studies, temporal effects considered (rebreathing tests were done at time of both CNS and plasma nadirs, 4.5-6 hours after drink consumption), I reject the possibility of insufficient tryptophan depletion as the cause of these negative findings. It therefore appears that the central and peripheral chemoreflexes, the only respiratory sensors of suffocation, are not significantly affected in their threshold or sensitivity of response to CO_2 by diminished 5-HT function.

An alternate explanation for this result is that the method I employed is not sensitive enough to measure the effect of the intervention. Though this is a possibility, I do not think it is a likely one. Our group has employed this technique to successfully

determine effects of other interventions on these chemoreflex parameters in many studies (ex. Duffin and McAvoy, 1988; Mohan and Duffin, 1997; Mahamed et al., 2001; Mahamed and Duffin, 2001). Also, our finding of elevated V_{Eb} with TRP- suggests that the technique is sensitive enough to detect significant chemoreflex changes imparted by this intervention. This last observation also suggests that the power of the test was sufficient, with unlikely possibility of a Type II error.

Expectedly, hypoxia decreased the chemoreflex threshold of response to CO_2 for all of ventilation, tidal volume and breathing frequency, while increasing sensitivity of the chemoreflex response for ventilation, tidal volume but not for breathing frequency. This is not a surprising result as it follows the model response to rebreathing (Duffin, 2000). That is, most subjects increase their ventilation during rebreathing primarily by elevating their tidal volume response and subsequently increasing breathing frequency (second breakpoint), once tidal volume reaches a plateau. At that point increases in ventilation are driven by increases in breathing frequency. This usually happens late during the rebreathing test, shortly before its termination, with few data points between this increase in frequency (second breakpoint) and end of rebreathing. More importantly, there were no drink type x PO_2 effects for this respiratory parameter (f_S), indicating that tryptophan depletion did not contribute to this effect.

Our measures for basal tidal volume or breathing frequency were not significantly affected by drink type, but basal ventilation was significantly higher during the TRP- condition. This is an interesting result considering the role of 5-HT in arousal. Our baseline measures consisted of the rebreathing period from the end of hyperventilation to

just before chemoreflex threshold (see Figures 6 and 7), accounting for any short-term potentiation effects due to the hyperventilation. As this is a hypocapnic phase, ventilatory output during baseline measures is the result of arousal (or "wakefulness" Fink, 1961) drive to breathe, without any chemoreflex contribution (Philipson and Bowes, 1986; Mahamed, et al., 2001.) Indeed, using ventilation as an index of arousal state, it would appear that serotonin has a "calming" effect on arousal, since a 5HT deficient state elevates ventilation. A compromised arousal system (ascending reticular activating system) may play an important role in panic and associated respiratory abnormality, with 5-HT a potentially important modulator of this system.

From the above discussion, it is also expected that PO_2 should not affect basal ventilation (V_{Eb}), basal tidal volume (V_{Tb}), or basal breathing frequency (f_b), as indeed, it did not, irrespective of drink type (no drink type x PO_2 interactions).

The negative results of TRP- on chemoreflex threshold and sensitivity parameters does not resolve the conflicting pre-clinical animal findings concerning the role of 5HT as a stimulatory (Bernard et al., 1996; Richerson et al., 1995; Wang et al., 1998; Hilarie et al., 1997; Dreshnaj et al., 1997) vs. an inhibitory (Armijo and Florez, 1974; Mueller et al., 1980; Lundberg et al. (1978); Mitchell et al., 1983; Olson et al., 1979) modulator of ventilatory output. We can only conclude that global TRP- does not sufficiently affect the in vivo central and peripheral chemoreflex response to CO_2.

What is the implication of this overall lack of effect of TRP- on chemoreflex parameters on the "suffocation alarm" theory of panic? The efficacy of SSRI's in relieving panic suggests that increased 5HT function should normalize any associated

ventilatory abnormality and implicated "suffocation alarm" dysfunction in PD. Conversely, deficient 5-HT function is expected to sensitize/depress the "suffocation alarm" threshold. The early in vivo animal studies were supportive of this notion (Armijo and Florez, 1974; Mueller et al., 1980; Lundberg et al. (1978); Mitchell et al., 1983; Olson et al., 1979), implicating 5-HT as an attenuating modulator of ventilatory output. This work has stimulated several studies showing respiratory hyperactivity to rebreathing during TRP- in PD but not in HV (Kent et al., 1996; Miller et al., 1996, 2000; Klassen et al., 1998). However, methodological limitations of these studies did not permit assessment of low 5-HT on specific sites involved in suffocation sensing (ex. respiratory chemoreflexes), specifically on the chemoreflex threshold and sensitivity of response to CO_2. Our negative results of TRP- on these chemoreflex parameters in the healthy volunteers concur with results from these studies.

Our study was carried out in a non-clinical population. It has been previously suggested that the response of panic patients to the intervention used here could be different than that of controls, indeed that a population with an "anxiety/panic system" pathology would respond differently to a combination TRP-/CO_2 challenge than that with a non-compromised "anxiety/panic system." This is a valid objection from the point of view of anxiety or panic etiology as a whole, though it does not undermine our paradigm as a test of the "suffocation alarm theory," which states that all subjects, including healthy volunteers, have a suffocation alarm, as evidenced by the fact that "suffocation is an extremely intense, remarkably common fear reported in normal subjects" (Klein, 1993). The negative results of this study thus speak against any chemoreflex mediated suffocation alarm, since such a major disruption of 5-HT function (71% fall in free

plasma TRP) would be expected to seriously derange the alarm, even in healthy volunteers.

It is worth noting that TRP- affects global CNS 5-HT function, affecting all serotonergic brain nuclei. While *in vitro* work has focused on the role of the caudal raphe, more traditionally associated with 5HT-modulated control of breathing, it is interesting to speculate on the role of the rostral raphe nuclei (dorsal raphe nucleus, DRN; medial raphe nucleus, MRN) in ventilatory pattern expression. It is the rostral raphe nuclei that appear to be involved in anxiety and panic in general (Grove et al., 1997). If the problem with anxiety and panic is flawed rostral raphe (especially MRN, Grove et al. 1997) modulation of their target structures (ex. amygdala (Amg) and dorsal periaquaductal gray matter (PGA); Deakin and Graeff, 1991) as many researchers suggest (Kent et al. 1998; Shruers et al., 2000; Grove et al., 1997), then such malfunctional neuromodulation would affect structures implicated in expression of panic (ex. PAG), generation of anticipatory anxiety (ex. Amg) and cognitions (cortical) that may participate in anticipatory anxiety and their catastrophisation which follows the physical sensations of panic (Clark, 1988). Thus, the respiratory abnormality often cited with PD and panic attacks (Gorman et al., 1988; Woods et al., 1986; Griez et al., 1987; Carr et al., 1987; Sanderson et al., 1989) may be nothing more than an epiphenomenon of the indirect (cortical/behavioral) input to the respiratory system, with the core biological flaw possibly limited to deranged regulation of subcortical structures by the rostral raphe. One might imagine that such 5-HT-compromised regulation of cortical/behavioral drives would be expressed on the ventilatory response where these drives play a key role, that is on V_{Eb} in the hypocapnic phase of rebreathing, as indeed observed in this study. Thus, a

biological flaw in panic (and anxiety) may exist in the form of defective 5-HT modulation of subcortical structures (ex. PGA, panic; Amg, anxiety), and so as such, a "suffocation alarm" may exist, but may have nothing to do with the main areas involved in physiological detection of suffocation/CO_2 – the respiratory chemoreflexes. Associated breathlessness/suffocation, a key feature of panic, as proposed by Klein (1993), may be nothing more than symptom catastrophisation (Clark, 1988) from viscerosensory interoceptive cues of an already triggered panic cascade.

In summary, TRP- does not significantly affect the central and peripheral chemoreflex threshold and sensitivity of response to CO_2 in healthy men. In conjunction with our previous work (Katzman et al., 2001, in submission), I conclude that the respiratory chemoreflexes, the only respiratory sensors of suffocation, are not the trigger of panic in a nominal or 5-HT deficient state. Panic associated respiratory abnormalities may be related to deranged 5-HT function in a general arousal mechanism, or other brain regions that trigger panic, unrelated to any respiratory chemoreflex abnormality.

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APPENDICES

(1) Complete Study Protocol

(2) Study Consent Form

(3) Study Recruitment Advertisement

(4) CAMH Abbreviated Study Protocol

(5) Individual Subjects' Rebreathing Test Responses

Finalized

Research Proposal

EFFECTS OF TRYPTOPHAN DEPLETION ON CENTRAL AND PERIPHERAL CHEMOREFLEXES IN PANIC DISORDER PATIENTS AND HEALTHY VOLUNTEERS

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I. BACKGROUND AND RATIONALE

I.I Panic Attacks and Panic Disorder

Description, prevalence and implications

Panic disorder with or without agoraphobia (PD) is a common psychiatric illness that afflicts 2% to 5% of the general population (Nutt and Lawson, 1992; and Wittchen and Essan, 1993). 35% of the population has reported to have had at least one panic attack (Nutt and Lawson, 1992; and Wittchen and Essan, 1993). The hallmark of PD is the repeated occurrence of spontaneous panic attacks (PA) (Diagnostic and Statistical Manual 4'th edition, DSM-IV). These PAs are discrete episodes of intense anxiety that are perceived to be uncontrollable and are accompanied by unpleasant physical sensations such as palpitations, chest pain, dyspnea, choking, sweating, tremors, faintness and paraesthesia. PAs are also accompanied by psychosensorial symptoms such as depersonalization and cognitive symptoms such as fear of losing control, going crazy or dying. A PA can last from a few minutes to more than an hour, and intense fatigue is frequently reported at the conclusion of the episode (Bradwejn and Koszycki, 1994). Because of these alarming physical and psychosensorial symptoms, many patients interpret their PA as a potentially life threatening event, resulting in emergency room visits and other help-seeking behavior. Although successful treatments for PD are now available, there are still problems with successful diagnosis (Papp et al., 1993a). PAs are usually followed by the development of anticipatory anxiety and agoraphobia. The common complications of PD include depression, substance abuse and significant social and occupational impairment (Bradwejn and Koszycki, 1994). The National Institute of Mental Health has recently ranked PD in the primary care medical setting as a high priority (Gerdes et al., 1995).

Challenge Paradigms and Theories in Panic Disorder

By its very definition, a PA is "spontaneous" in nature (APA, 1994). Therefore, in an attempt to identify an abnormal response in panic patients, a number of panic inducing agents have been introduced in the laboratory. More extensively studied physiological and pharmacological challenges include tests involving noradrenergic (NA) agents (ex. yohimbine, and noradrenaline), cholecystokinin (CCK), lactate/bicarbonate/hypercapnia, serotonergic agents (ex. 5-HT agonists/antagonists) and

hyperventilation (for review see Nutt and Lawson, 1992). Of the numerous agents identified, lactate, bicarbonate and hypercapnic challenges have focused on the ventilatory response and changes in PAs (see Coplan and Klein, 1996 for review).

Challenge studies using intravenous sodium lactate have long shown its effectiveness in inducing PA's in PD patients but not in controls (Pitts and McClure, 1967). Despite the years of research, current literature provides no mechanism on its mode of action. Because sodium lactate and sodium bicarbonate (another proven panicogen, see Coplan and Klein, 1996 for review) are both panicogenic and metabolized to carbon dioxide (CO_2), this elevation of CO_2 has been proposed as the mechanism of panic induction, though this explanation is now not accepted (Gorman et al., 1990). CO_2 panicogenesis has however been confirmed by various studies (Lousberg et al., 1988; Gorman et al., 1994; Katzman et al., 2000, in preparation), giving rise to the "CO2 hypersensitivity theory" of panic. The suggestion here is that PD patients are hypersensitive to increases in their blood CO_2 levels. Upon inhalation of a hypercapnic gas mixture (5% or 35% CO_2), patients with PD are believed to experience a subjective sense of dyspnea, stimulated by hypersensitive brainstem respiratory control nuclei. Following the dyspnea, a triggering of a series of typical autonomic symptoms is expressed (Papp et al., 1995). Introduction of hypercapnia has been performed through either a slow rebreathe of 5-7% CO_2 in air (Bystritsky & Shapiro, 1992), or breathing one or two deep breaths of 35% CO_2 (Verburg et al., 1995; Perna et al., 1994 and 1996). Both steady-state (Bailey et al., 1986; Milic-Emili et al., 1976; and Papp et al., 1989) and rebreathing (Carr et al., 1987; Lousberg et al., 1988; Pain et al., 1988; and Papp et al., 1990a and 1995) techniques have been used to record the chemosensitivity of clinical panickers, to both 5% and 35% hypercapnic tests mentioned above. Attempts to measure the CO_2 sensitivity of both clinical panickers and non-panickers, have produced mixed results that do not unequivocally support the notion of enhanced chemosensitivity to CO_2 in individuals with PD (Papp & Gorman, 1990b; Katzman et al. 2000, in preparation). Nevertheless, a number of reports confirm the presence and relevance of respiratory abnormalities in patients with PD (Gorman et al., 1984; Woods et al., 1986; Griez et al., 1987; Carr et al., 1987; Sanderson et al., 1989). Based on research using lactate, bicarbonate and CO_2 challenges Klein (1993) has formulated an integrative "false suffocation alarm" theory of panic.

Klein (1993) argues that panic patients are hypersensitive to CO_2, their acceptable blood CO_2 levels

are therefore lower than those of healthy controls, and when this threshold level is exceeded, "the brain's suffocation monitor erroneously signals a lack of useful air, thereby maladaptively triggering an evolved suffocation alarm system" which manifests itself as a panic attack. Klein also states that patients go through periods when their condition worsens, that is through "bad periods" and through less morbid or "good periods" when they have fewer PAs. It is believed that "during bad spells the suffocation alarm threshold is pathologically depressed."

Klein's argument may be summarized as follows: Suffocation fears are common in all subjects, and the PCO_2 monitor is the physiological mechanism for detecting potential suffocation. In a PD patient, this "evolutionarily derived set-point has become dysfunctional," and the resultant hypersensitivity becomes the instigator of spontaneous panic attacks in a variety of common situations in life, where one's CO_2 levels would slightly rise. A major limitation of this theory has been the lack of an anatomical system for the "suffocation alarm."

These challenges have in part been undertaken to elucidate the specific brain system involved. One system that has shown a good deal of promise in terms of being a player in the anxiogenic/panicogenic system is the serotonergic system.

Many lines of evidence have now linked a deficiency in serotonin (5-hydroxytryptamine; 5-HT) with anxiety and specifically with panic disorder (see Anderson and Mortimore, 1999; or Coplan et al., 1992 for review,) but its role remains unclear. Clinical trials indicate that drugs that putatively increase 5-HT neurotransmission (Blier et al., 1987), that is, the selective serotonin reuptake inhibitors (SSRI's), such as fluoxetine, fluvoxamine, paroxetine, and sertraline, are effective anti-panic agents (Evans et al., 1986; Gorman et al., 1987; Den Boer and Westenberg, 1988; Schneier et al., 1990). The success of these and other SSRI's provides the bulk of evidence for the involvement of the 5HT system in panic.

Preclinical studies provide the link between the hypothesis of deficient 5-HT neurotransmission and associated respiratory hyperactivity in panic disorder (Olson et al., 1979; Lundberg et al., 1980; Mitchell et al., 1983). Several studies showed that ventilation decreases after both peripheral and central administration of 5-HT agonists (Armijo and Florez, 1974; Mueller et al., 1980). Lundberg et

al. (1980) has also shown that a precursor of the substance (5-hydroxytryptophan; 5-HTP) decreased ventilation and CO_2 sensitivity in rats in a dose-dependent fashion. On the other hand, the tryptophan hydorxylase inhibitor parachlorophenylpiperazine (PCPA) causes hyperventilation in goats (Mitchell et al., 1983) and rats (Olson et al., 1979) with repletion of 5-HTP normalizing ventilation and CO_2 levels. From these animal studies, it is generally agreed that an interaction between 5-HT function and respiration exists.

The picture is somewhat more complicated when one considers research on the involvement of the midline raphe nuclei in respiration and control of breathing. The raphe nuclei, the major sites of 5-HT neurons in the CNS, have been shown to have chemoreceptive properties (Dreshaj et al., 1998; Wang et al., 1998; Bernard et al., 1996), and have been shown to have stimulatory effects on ventilatory output by increasing respiratory frequency and triggering tonic spinal activity (Hilarie et al., 1997). Also, withdrawal of neuronal dorsal raphe activity resulted in decreased peak phrenic and hypoglossal activities at any given end-tidal CO_2 level (Dreshaj et al., 1998). From this evidence, one would conclude that enhanced 5-HT neurotransmission should mediate increases in ventilatory output, be it via intrinsic chemoreceptive function, or via increased phrenic/hypoglossal drive. However, a closer look at the raphe reveals the presence of sensitive CO_2-stimulated neurons and CO_2-inhibited neurons (Richerson, 1995). It seems that specific regions of the raphe decrease respiratory discharges (nucleus raphe obscurus, ROb; nucleus raphe magnus, RMg,) while other regions increase phrenic discharge (nucleus raphe pallidus, RPa; Lalley, 1986). Indeed, Lalley et al. (1997) have shown that ROb stimulation depresses excitability and rhythmic behavior of several types of medullary respiratory neurons. This study implicated the 5-HT -1A receptor subtype in modulating respiratory depth and pattern of breathing. Hilarie et al. (1997) have implicated certain 5-HT subtypes in stimulatory effects on ventilatory output and others in inhibitory contributions. Part of the problem with these studies is that they are either brain slice or in vitro studies with no intact in vivo research on awake mammals, much less so on humans. This approach, while specific overlooks the concerted work of all the 5-HT receptor subtypes, and all of the raphe nuclei in their work at an integrated animal level. The 5-HT is a diffuse projection system extending throughout the CNS with modulatory functions throughout. It is not unlikely then that the raphe's influence on control of breathing acts in a similar fashion. The net effect of the raphe on breathing could be the result of a balance between stimulatory and inhibitory

influences (RMg and ROb vs. RPa activity.) Pathology in this region has been suggested as a possible cause of Congenital Central Hypoventilation Syndrome, Sudden Infant Death Syndrome and sleep apnea (Filiano et al., 1990; Kinney et al., 1995) (lack of stimulatory modulation,) and could be the problem in panic if there is a net excess of modulatory stimulation (by decreased inhibitory raphe activity, possibly due to deficient 5-HT neurotransmission in the ROb.)

Investigation of 5-HT function in humans is made difficult by the lack of selective tools and the complexity of the 5-HT system. A widely used method of investigating global 5-HT function is the technique of acute tryptophan depletion (TRP-depletion). Tryptophan being the pre-cursor amino acid to 5-HT synthesis, the underlying belief here is that tryptophan depletion will cause a short-term global reduction in brain 5-HT function in human subjects as a consequence of ingesting a tryptophan free amino acid drink (Reilly et al., 1997). In humans, there is evidence of reduced 5-HT synthesis (Nishizawa et al., 1997) and reduced cerebrospinal fluid tryptophan and 5-hydroxyindoleacetic acid (5-HT metabolite) after TRP-depletion (Carpenter et al., 1998), with technique specificity recently verified by Klaassen et al. (1999).

Recent human research into the etiology of panic has employed TRP-depletion with a CO_2 challenge approach, further suggesting interactions between the 5-HT and respiratory system (Kent et al., 1996; Miller et al., 1996; Klaassen et al., 1998; Miller et al., 2000). The general consensus of these studies is that TRP-depletion enhances the effect of a CO_2 panic challenge with little effect on general or anticipatory anxiety levels in PD patients. However the results on healthy controls remain unclear. Studies by Kent et al. (1996) and Miller at al. (1996) show that TRP-depletion increases respiration by elevating tidal volume, frequency and minute ventilation. However, to our knowledge no study has looked at how the reported changes in respiration are brought about and what is the site of interaction between the 5-HT and respiratory system. Klein's "false suffocation alarm" theory suggests a flawed CO_2 sensor erroneously signaling suffocation. We looked at the respiratory sensors which detect suffocation in both PD patients and healthy controls and found no differences in central or peripheral chmoreflex responses to CO_2 (Katzman et al. 2000, in preparation). Yet there is abound research showing respiratory anomalies in PD (Gorman et al., 1984; Woods et al., 1986; Griez et al., 1987; Carr et al., 1987; Saunderson et al., 1989) supporting the hypothesis of an abnormal respiratory response, with research showing that reduced serotonergic neurotransmission affects this response

(Olson et al., 1979; Mitchell et al., 1983; Armijo et al., 1974; Mueller et al., 1980; Kent et al., 1996; Miller et al., 1996; Klaassen et al., 1998; Miller et al., 2000). However none of these studies has looked for a possible anatomical site of interaction between the two systems, in particular with consideration of the CO_2 hypersensitivity theory of panic. Kent et al. conclude that their "... findings raise the possibility that the respiratory component of panic may be an appropriate target for selective serotonin reuptake inhibitors, while the anxiety component may, in turn, be diminished as respiration is better regulated." This suggestion also fits with the Klein theory in the context of defective 5-HT neurotransmission sensitizing the respiratory control centres (during low CNS 5-HT periods, or during "bad spells" of panic) which misfire in response to slight elevations in CO_2.

I.II Rebreathing Technique

The purpose of rebreathing is to induce hypercapnia in the subject over a period of several minutes. A progressive increase in the end-tidal partial pressure of carbon dioxide (hypercapnia) is produced when a subject breathes in and out of a bag or spirometer so that normal gas exchange with room air is prevented. The end-tidal partial pressure of carbon dioxide rises solely due to the resting metabolic production of carbon dioxide of the subject. Hypoxia is avoided by filling the rebreathing bag with a high percentage of oxygen (hyperoxic), sufficient for the subject's metabolic needs during the rebreathing.

In 1967 Read developed a rebreathing technique that accurately measured the ventilatory response to carbon dioxide. The subject would rebreathe from a bag containing an initial hyperoxic and hypercapnic gas mixture. Exposure to these conditions allowed for a rapid equilibration between the partial pressures of carbon dioxide in the alveolar gas and the arterial blood, quickly raising their levels to that of mixed venous blood and the tissue (due to hypercapnia) as well as for the withdrawal of the peripheral chemoreceptors response (due to hyperoxia).

Read's modifications of a smaller bag and an initial carbon dioxide level for the bag closer to that of mixed venous blood thereby enabled the establishment of a constant relationship between the end-tidal partial pressure of carbon dioxide and that of the brain tissue and central chemoreceptors.

As the blood recirculates throughout the body, carbon dioxide from the tissue is loaded into the blood

and the partial pressures of carbon dioxide throughout the body, the lungs and the rebreathing bag rises linearly with time due to metabolism, independent of ventilation. The increase in the end-tidal partial pressure of carbon dioxide is taken to be indicative of the increase at the central chemoreceptors and is the underlying principle of the ability of the rebreathing technique to measure the central chemoreceptor ventilatory response to carbon dioxide (central chemoreflex).

While Read's rebreathing method is the best technique for measurement of the central-chemoreflex sensitivity, since the subject begins rebreathing at their resting mixed venous level of the carbon dioxide, there is no opportunity to measure the threshold of the central-chemoreflex. Therefore, in order to measure the threshold of the central-chemoreflex response, Read's technique was modified by our laboratory to include a prior voluntary hyperventilation (Duffin and McAvoy, 1988) to lower carbon dioxide levels below threshold. By starting rebreathing after a period of hyperventilation to lower body stores of carbon dioxide, the initial equilibration of carbon dioxide occurs at levels below the central-chemoreflex threshold. As a result, the central-chemoreflex threshold can be measured. In a further modification these rebreathing tests can be carried out under iso-oxic conditions by providing a small inflow of oxygen under computer control. In this way not only can hyperoxic rebreathing tests be done, that measure the central chemoreflex response, but also hypoxic rebreathing tests, that measure both the central and peripheral chemoreflex responses.

In summary, Read's rebreathing technique as modified in this laboratory allows the determination of the threshold and sensitivity of the chemoreflex ventilatory response to carbon dioxide under both hyperoxic and hypoxic conditions, enabling estimations of both the central and peripheral chemoreflex characteristics.

II. Objectives

We are proposing a study to investigate the possible site of interaction between the 5-HT system and the neural respiratory control centres. According to Klein (1993) the origin of PAs and indeed of PD is in "a physiologic misinterpretation by a suffocation monitor, which misfires as an evolved suffocation alarm." We have looked at the only respiratory suffocation detectors, namely the central and peripheral chemoreflexes in PD patients and healthy controls and found no "misfiring" in terms of

threshold or sensitivity of response to CO_2 during a rebreathing procedure (Katzman et al., 2000, in preparation). To date, no studies have looked at the effect of low brain tryptophan (i.e. 5-HT) on the thresholds and sensitivities of the central and peripheral chemoreflexes, and thus no anatomical localization of respiratory-5-HT system interaction has been proposed. Therefore, we propose to employ the TRP-depletion technique along with the modified Read rebreathing procedure to look at the effect of low brain 5-HT on the thresholds and sensitivities of the central and peripheral chemoreflex responses to rising CO_2 in healthy subjects.

Observation of the subject's response to increased CO_2, and analysis of the data using appropriate software, will yield results. If a difference is shown in the central chemoreflex, this would lend credence to the "false suffocation alarm" in the form of defective 5-HT neurotransmission sensitizing the CO_2 sensor (i.e. the chemoreceptor) which, according to the theory, would erroneously signal a PA with rising CO_2. An anatomical locale for the "suffocation alarm" would also then be found. Alternatively, evidence to the contrary would seriously challenge this theory.

III. Criteria for Eligibility and Exclusion

Eligible subjects will be males between the age 18-45 years, nonsmokers, with the ability to understand the nature of the study and complete a self-rating scale, and answer the evaluator.

Healthy Volunteers will be ineligible if one or more of the following exist(s):

Signs of significant illness upon history, physical examination and laboratory findings
History of compromised respiratory function (ex. COPD, asthma, cardiovascular, disease, etc.)
Weight outside normal limits
Meeting Criteria for any DSM-IV Diagnosis
Not providing informed consent in a manner consistent with nationally approved standards before entering the study.

The responsible investigator will obtain informed consent, after having first offered the patient an information sheet with a full explanation of the study on it. The responsible investigator will provide a

copy of the consent form to the participant.

Panic Disorder patients will be ineligible if one or more of the following exist(s):

Signs of significant illness upon history, physical examination and laboratory findings

History of compromised respiratory function (ex. COPD, asthma, cardiovascular, disease, etc.)

Weight outside normal limits

Meeting Criteria for any DSM-IV Diagnosis other than Panic Disorder with or without Agoraphobia.

Currently use of any medication

Not providing informed consent in a manner consistent with nationally approved standards before entering the study.

The responsible investigator will obtain informed consent, after having first offered the patient an information sheet with a full explanation of the study on it. The responsible investigator will provide a copy of the consent form to the participant.

NOTE: Subjects on Psychotropic or disallowed medication will not be considered eligible unless having undergone a washout corresponding to 5 half-lives of the drug and its active metabolite before the entry into the test day.

IV. Method

Rebreathing

The apparatus used for the rebreathing tests has been previously used in this laboratory (Duffin and McAvoy, 1988; Mohan et al., 1997; Katzman et al., 2000, in preparation) with the addition of a computer controlled feedback system to maintain a hyperoxic or hypoxic condition throughout the experiment. The entire apparatus (Figure 1) will be calibrated before each subject's experimental session.

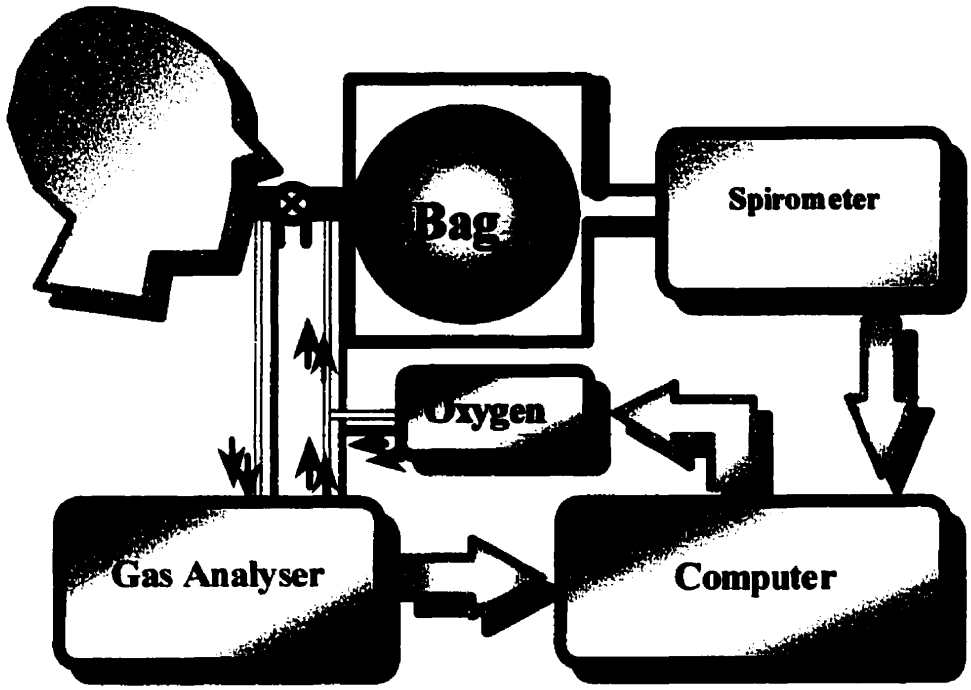

Figure 1: Setup of the rebreathing apparatus.

An oximeter probe (Bruel and Kjaer, model 8852) is placed on the subject's index finger in order to monitor heart rate and oxygen saturation. The subject will wear a nose clip throughout the experiment and breathe via a mouthpiece connected to a Y valve (Collins P-319; 80 ml dead space). This valve allows subjects to switch themselves from room air to the rebreathing bag. A tube attached to the valve will sample the air breathed at the mouth for monitoring values of carbon dioxide and oxygen (Bruel and Kjaer, anaesthetic monitor type 8852). The rebreathing bag, approximately 5 liters, is enclosed in a rigid container and is connected to a dry rolling seal spirometer (Morgan Spiroflow, model 130) by a short length of wide bore (37 mm) tubing so that ventilation can be monitored on a breath-by-breath basis. The bag is filled with a gas mixture in which the partial pressure of carbon dioxide is at 42 mmHg and the iso-oxic oxygen level is at 150 mmHg for the hyperoxic condition and 50 mmHg for the hypoxic condition.

Tryptophan Depletion

Subjects will undergo two days of testing: a TRP-depletion day and a non-depletion (placebo) day. TRP-depletion will be achieved following a standard protocol (Young et al., 1985). Subjects will eat

a low protein diet (20g) for 24 hours prior to the TRP-depletion day, fast overnight prior to both days of testing and refrain from caffeine or alcohol consumption for 48 hours prior to either test day. They will receive one of two amino-acid drinks in a double-blind, randomized order at 8:00 on the morning of test day, with test days separated by no less than three and no more than seven days. One hundred gram amino acid drinks will be consumed which will differ only in whether they lack or contain 2.3g of tryptophan (TRP-free drink and control drink, respectively, see Table 1). The powdered amino acids (provided by Sigma Aldrich) will be mixed with 250ml of water and 60ml "Quick" chocolate syrup (by Nestle) immediately before administration. Subjects will be instructed to drink the mixture as quickly as possible. Subjects will remain in the laboratory under observation by the experimenter in the time between ingestion and the rebreathing task, 4.5 hours later.

Table 1: Composition of the placebo amino acid mixture

Amino Acid	Composition (g)
L-Alanine	5.5
L-Arginine	4.9
L-Cysteine	2.7
Glycine	3.2
L-Histidine	3.2
L-Isoleucine	8.0
L-Leucine	13.5
L-Lysine	11
L-Methionine	3
L-Phenylalanine	5.7
L-Proline	12.2
L-Serine	6.9
L-Threonine	6.9
L-Tyrosine	6.9
L-Valine	8.9
L-Tryptophan	2.3

In the tryptophan depleted mixture, L-Tryptophan will be left out.

Plasma Tryptophan Measurement

Blood will be drawn on each test day to obtain plasma tryptophan measures ensuring sufficient depletion just prior to testing. Subjects will have blood drawn twice: once during an initial assessment visit to determine baseline amino acid drink levels. A sample will be drawn during test day, 2.5 hours after ingestion of the beverage, prior to the rebreathing procedure. A qualified nurse will draw small blood samples (10 mL) each time.

The blood samples will be spun at 3000 rpm for 10 minutes at a temperature of 4 ° C in a cold centrifuge to separate serum from the pellet. Subsequently, the serum will be stored at -80 ° C and analysed for levels of tryptophan using high pressure liquid chromatography (HPLC).

Procedure

The subject will first undergo a 5-minute hyperventilation period, which will lower the end-tidal partial pressure of CO_2 between 20-24 mmHg. The subject will then be switched to the rebreathing bag and will be asked to take 3 deep breaths to ensure that the end-tidal partial pressure of carbon dioxide and oxygen in the bag, lungs and arterial blood quickly equilibrate with the mixed venous partial pressure, which serves as an estimate of the partial pressure in large tissue. Adequate equilibration will be evidence by a proper plateau in the end-tidal partial pressure of carbon dioxide.

The end-tidal partial pressure of carbon dioxide will rise linearly with time and the test will be terminated at 60 mmHg. At this time the subject will be switched back to room air and will remain seated and recover for several minutes.

Experimental Schedule

First Visit

Healthy Volunteers will be screened and assessed for eligibility by medical and psychiatric history, SCID-NP (structured psychiatric interview for non-patients) and the Symptom Checklist 90 (SCL-90). Laboratory tests will include blood hematology and chemistry, urinalysis, urine drug screen, and EKG. Blood will also be tested for baseline tryptophan levels. Eligible subjects will also be informed of the symptoms that they might experience as a result of the breathing procedure or the TRP-depleted/placebo drink. Eligible subjects will be instructed to avoid caffeine and alcohol 48 hours before each visit and maintain a low tryptophan diet for 24 hours prior to the day of testing. Upon completion of the first visit, subjects will be provided with the date of their second and third visits and a copy of the consent form.

Panic Disorder subjects will be screened and assessed for eligibility by medical and psychiatric history, SCID (structured psychiatric interview for DSM-IV psychiatric patients) the Symptom Checklist 90

(SCL-90), Anxiety Sensitivity Inventory (ASI), Sheehan Disabilty Scale, and a Panic and Anxiety record. Laboratory tests will include blood hematology and chemistry, urinalysis, urine drug screen, and EKG. Eligible subjects will also be informed of the symptoms that they might experience as a result of the breathing procedure or the TRP-depleted/placebo drink. Eligible subjects will be instructed to avoid caffeine and alcohol 48 hours before each visit and maintain a low tryptophan diet for 24 hours prior to the day of testing. Upon completion of the first visit, Panic Disorder subjects will be provided with a copy of the consent form and a Panic Diary, to record their anxiety and panic symptoms in the week following the first visit including the date of their second and third visit.

Second Visit

Upon arrival subjects will confirm that they have avoided caffeine and alcohol 48 hours before each visit and have followed the low protein diet (20g) regimen in the last 24 hours. Then, at 8:00 the subjects will be asked to drink the TRP-free or placebo mixture as quickly as possible. For the next 2.5 hours the subjects will be kept under observation with rebreathing tests done 4.5-6 hours after drink consumption. The 4.5-6 hour time delay was selected to allow lowest possible tryptophan levels, as tryptophan levels begin to rise again after 7 hours (Delgado et al., 1990; Miller et al., 1992; Young et al., 1985).

To introduce the subject to the rebreathing apparatus, and the prior hyperventilation technique, they will undergo a series of three rebreathing tests. Just prior to the first test, the subjects will have blood drawn the second time for later analysis of plasma amino acid levels in the TRP-depleted (TRP-free drink session) or non-depleted (placebo session) state. The first test will begin 4.5 hours after ingestion of the drink mixture at 13:00, and undertaken to teach the subject how to follow the rebreathing protocol. During this period the subject is shown the apparatus, taught how the study will run and shown how to indicate the onset and offset of symptoms (using an upward hand swing for onset and a downward hand swing for the offset of symptoms). Following this practice rebreathing test there will be a half-hour break.

The order (hyperoxic vs. hypoxic) of the following tests will be assigned randomly for each subject.

A second test, the "Hyperoxic Rebreathing test" will be undertaken to obtain the central chemoreflex

response. The end-tidal partial pressure of carbon dioxide will rise linearly with time under hyperoxic conditions and the test will be terminated at 60 mmHg P_{CO_2}. At this time the subject will be switched back to room air and will remain seated and recover for several minutes.

The third test, the "Hypoxic Rebreathing test," will be conducted undertaken to obtain combined central and peripheral chemoreflex activity. The same conditions will be employed as in the previous run this time under hypoxic conditions with test termination at 60 mmHg P_{CO_2}.

Following completion of each phase, the attending psychiatrist, present throughout the duration of the experiment, will ask the subjects to retrospectively assess the severity of their symptoms using the DSM-IV derived PSS (Panic Symptom Scale).

Third Visit

This visit will take place at least 3 days, but no more than 7 after the second visit and will be identical to it in every respect other than the contents of the drink mixture, which will be administered in a randomized, double-blind fashion of TRP-free drink or placebo (TRP-included). The Panic Diary and Anxiety record will also be returned at this time.

V. Data Collection

Respiratory data collected will include all of the following versus time (s):

(i) Heart rate (beats/min)

(ii) Oxygen saturation (%)

(iii) End-tidal partial pressure of CO_2 (mmHg) and O_2 (mmHg)

(iv) Ventilation (L/min) - Tidal Volume (ml) and Frequency (breaths/min.)

(v) Inspiratory and Expiratory times

The Symptom Checklist 90 (SCL-90), Anxiety Sensitivity Inventory (ASI), Sheehan Disability Scale, and a Panic and Anxiety record will be assessed post hoc to examine for potential correlation with the respiratory data.

A DSM-IV based panic inventory (Panic Symptom Scale, PSS) will be used for recording of

symptoms. The PSS will be undertaken immediately after the subject has completed each test phase. Symptoms spontaneously reported by the subject and not appearing on the inventory will be recorded; they will be recorded as panic symptoms if the subject has the particular symptom following each phase. The symptoms will be rated on a five-point scale, from 0 (not present) to 5 (extremely severe).

VI. Data Analysis

Breath-by-breath values of ventilation and the end-tidal partial pressures of oxygen and carbon dioxide accumulated from the rebreathing tests are analysed using a specially designed spreadsheet (Microsoft, Excel). First, breaths from the initial 3-breath equilibration as well as sighs, swallows and breaths incorrectly detected by the software are excluded from further analysis. Next, the breath-by-breath end-tidal partial pressures of carbon dioxide are plotted against time and fitted with a least-squares regression line. The equation for this line provides a predicted value of end-tidal partial pressure of carbon dioxide vs. time, thereby minimising inter-breath variability due to measurement. Subsequently, tidal volume (ml BTPS), respiratory rate (breaths/min) and ventilation (L/min BTPS) are plotted against the predicted end-tidal partial pressure of carbon dioxide (mmHg).

Each of these plots is fitted with a model made up of the sum of two or three segments separated by one or two breakpoints respectively (Duffin et al., 2000). Model fitting is based on minimising the sum of least squares for non-linear regressions using commercial software (Sigma Plot 5.0, SPSS). The first segment is an exponential decline to a final value, the latter taken as a measure of the basal ventilation, basal tidal volume and basal breathing frequency characteristics. The exponential decline is chosen to fit any waning of short-term potentiation of ventilation produced by hyperventilation that might have occurred, usually observed in only a few tests. In those tests without such a trend the decay constant of the exponential decline reverted to a value less than 1, so that the basal values are equivalent to the mean values below the first breakpoint.

The second and possible third segments are straight lines from the first to the second breakpoint, and above the second breakpoint respectively. The first breakpoint is taken as a measure of the chemoreflex threshold for the ventilation, tidal volume and breathing frequency responses to carbon dioxide. The second breakpoint is taken as the point at which the ventilatory response pattern

changes, in terms of tidal volume and frequency. The slopes of the first straight-line portions are taken as the chemoreflex sensitivity for the ventilation, tidal volume, and breathing frequency responses.

For each subject we will calculate the parameters: basal ventilation, chemoreflex threshold and sensitivity. The above will be calculated for: (a) hyperoxic condition with TRP-free drink; (b) hypoxic condition with TRP-free drink; (c) hyperoxic condition with placebo; (d) hypoxic condition with placebo. Assessments of the central chemoreceptor sensitivity will be undertaken using the hyperoxic condition. Assessment of the peripheral chemoreceptor will be undertaken by subtracting the results of central chemoreflex output to ventilation obtained under the hyperoxic condition, from the combined central and peripheral chemoreflex output to ventilation from the hypoxic condition.

Plasma samples will be analyzed for levels of tryptophan. A difference of 70% in tryptophan levels between the two blood samples (baseline vs. tryptophan depleted state) will be taken as sufficient tryptophan depletion. Subjects whose samples do not meet this difference will be excluded from further data analysis and an additional subject's sample meeting this criterion will be obtained. Blood will be collected in a single 10mL tube that contains EDTA. The samples will be centrifuged at 3000 rpm for 10 minutes under cool (4°C) conditions. One-milliliter aliquots of plasma will be stored at minus 80°C in 1.5 micorfuge tubes. The samples will be batched and shipped in dry ice to a biochemistry laboratory at the University of Edmonton. Quantitative analyses of free tryptophan levels will be determined using the HPLC method.

The team undertaking the analysis of the data will be blinded to the diagnosis.

VII. Sample Size

Based on previous studies that have been published (Goetz et al., 1993; Papp et al., 1993a; Verburg et al., 1995), and our own recent study (Katzman et al., 2000, in preparation), a group of 11 patients and 11 healthy volunteers will be recruited with use of advertisement.

VIII. Safety Issues

Study procedures will be directly supervised by one of the responsible investigators who will monitor subjects until the resolution of all possible symptoms induced by the rebreathing test.

Young, normal healthy adults, differing only in terms of the presence or absence of Panic Disorder can complete the proposed protocol. To ensure the safety of subject:

(i) the end-tidal levels of oxygen and carbon dioxide will be monitored at all times.

(ii) carbon dioxide will not be allowed to rise above 60 mmHg (normal level is 40 mmHg) and oxygen level will not be allowed to fall below 50 mmHg (normal level is 100 mmHg).

(iii) the oxygen saturation will also be continuously monitored with a pulse oximeter and will not be allowed to fall below 99% (normal level is 98%) during hyperoxia and not below 60% during hypoxia.

Exceeding any of these limits will terminate the test. Rebreathing tests have been performed in our laboratory on over 200 subjects, none of whom experienced any adverse effects of the test.

IX. Consent and Confidentiality

Consent will be obtained from the subjects prior to study entry (see Appendix). All data from this study will be analyzed in an anonymous and confidential manner and the subject's name or identifying information will not appear in any publication or presentation. All subjects will be able to withdraw from the study at any time for whatever reason with no further obligation.

NOTE: Apart from the tryptophan depletion intervention, this protocol has been previously approved on July 19/1999 (protocol reference # 4840.)

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Statement of Informed Consent

INVESTIGATORS:

Dr. Martin Katzman and Dr. James Duffin

CLINICAL STUDY TITLE:

EFFECTS OF TRYPTOPHAN DEPLETION ON CENTRAL AND PERIPHERAL CHEMOREFLEXES IN PANIC DISORDER PATIENTS AND HEALTHY VOLUNTEERS

DESCRIPTION AND PURPOSE:

You are being asked to take part in a research study that will investigate one of the potential theories of Panic Disorder. The study will involve consumption of a specially prepared drink mixture and the use of a breathing test in Panic Disorder patients and healthy volunteers.

On the first visit, you will be assessed for suitability through the use of medical and psychiatric histories, a physical examination and various paper and pencil tests. Psychiatric history will be collected via two interviews: the first will gather your psychiatric history using a semi-structured interview (SCID;) the second will be a regular consultation with a psychiatrist to determine any psychiatric condition that you may have. Blood tests, an electrocardiogram (a painless procedure to measure the activity of the heart,) will also be done prior to entry into the study. If eligible for the study, you will be asked to maintain a low protein diet for 24 hours prior to the next two visits, and to avoid caffeine and alcohol 48 hours before these visits. You will also be asked to maintain a fasting diet beginning midnight the night before each test day. You will also be asked to keep a Panic Attack record for the week prior to the second visit, indicating when, where, during what activity you had a panic attack, and at what time it occurred. An anxiety record will also be assigned to similarly record bouts of anxiety that occurred in the week prior to the second visit.

On the second visit, upon arrival at the laboratory at 8:00 a.m. you will be asked to drink a carefully prepared drink. After a period of 2.5 hours a blood sample will be taken from your left arm. Two hours after that, the rebreathing procedure will commence. At that time, a measuring device will be placed on your finger in order to measure heart rate and oxygen concentration in your blood. A clip will be placed on your nose (you will wear it throughout the experiment) to ensure you only breathe through your mouth via a mouthpiece connected to a valve. This valve will allow a switch from room air to the rebreathing bag and back. A tube attached to the valve will sample the concentration of carbon dioxide and oxygen in the air breathed through the mouth.

Once you are prepared, you will undergo a series of three breathing trials. The first trial will begin at 1:30 p.m., 4.5 hours after consumption of the drink mixture and is designed to teach you how the study is undertaken. During this period you will be shown the apparatus, taught how the study will run and shown how to indicate the onset and offset of symptoms (using an upward hand swing for onset and a downward finger swing for the offset of symptoms). The (first and) second trial will take place in a relatively high oxygen condition. The third trial will take place in a safe but relatively lower oxygen condition. Following each run there will be a half-hour break. At the end of the third phase of the study, you will remain in the lab for up to one hour as a precaution to ensure that you are well to leave.

During each phase you will be asked to first hyperventilate (exhale deeply) through a mouthpiece while breathing room air for 5 minutes. Then the valve will be turned for you to allow you to breathe only through the rebreathing bag and you will be asked to take 3 deep breaths. This will allow the experimenter to have a good estimate of the concentration of carbon dioxide and oxygen in your lungs, blood and tissues. You will then be asked to breathe as your regularly do. You will continue breathing from the bag until an adequate measure of the activity of respiratory system has been assessed. At this time, you will be switched back to room air, will remain seated and recover for several minutes. At any time in the study where you notice the onset of symptoms, you will be asked to notify the experimenter by lifting your hand to signify the onset and lower your hand to signify offset of symptoms. Following completion of each phase, the attending psychiatrist, who will be

present throughout the experiment, will ask you to retrospectively assess the severity of symptoms during the run.

The exact same procedure to the above will follow on the third and last visit, which will take place between 3 to 7 days after the second visit.

The drink you will be asked to consume on the second and third visit is a mixture of protein components (amino acids) of slightly different composition (specifically: Alanine, 5.5 grams; Arginine, 4.9 grams; Cysteine, 2.7 grams; Glycine, 3.2 grams; Histidine, 3.2 grams; Isoleucine, 8.0 grams; Leucine, 13.5 grams, Lysine monohydrochloride, 8.9 grams; Methionine, 3 grams; Phenylalanine, 5.7 grams; Proline 12.2 grams; Serine and Tyrosine, 6.9 grams; Threonine, 6.5 grams; Valine, 8.9 grams, Tryptophan, 2.3 grams.) These components will be mixed with 250mL of water and 60mL of "Quick" chocolate syrup to give a full glass drink similar in consistency and content to "boost" energy shakes.

RISKS ASSOCIATED WITH THE STUDY:

Study procedures will be directly supervised by one of the responsible investigators. To ensure your safety, the levels of oxygen and carbon dioxide will be monitored at all times, and if these levels fall above or below the safe limits, the study will immediately be terminated. In addition, it is important to note that rebreathing tests have been performed in our laboratory on over 200 subjects, none of whom experienced any significant adverse effects of any kind. This has also been shown in other labs using the same technique.

Occasionally however, subjects have reported a brief duration of shortness of breath, dizziness, unsteady feeling, palpitations, trembling, sweating, flushes or chills, abdominal distress, feeling unreal, tingling, chest discomfort, nervousness and fear. These symptoms if present usually last less than 2-5 minutes. As a result of consuming the drink mixture some subjects occasionally also experience drowsiness, nausea and abdominal fullness. These sensations are also usually resolved within 2 hours of drink consumption.

On the first visit, you will provide 20mL (2 tablespoons) of blood. The second and third visit

to the laboratory will also require that you provide 10 mL (1 tablespoon) of blood. You must also be aware that you may experience discomfort when having blood drawn; this may be a source of mild pain, and some mild swelling and bruising. Rarely inflammation of the vein may occur at the site of the blood collection. Although it is uncommon, this may also cause you to feel faint, to bleed slightly, or to develop an infection at the site of the blood collection. Someone who is qualified to draw blood will carry out the collection of your blood.

The interviews that you will receive during the course of the study (first visit) involve no specific risks or discomforts beyond those of a standard clinical interview situation, such as feeling upset at a review of your psychiatric status, boredom, or fatigue.

CONFIDENTIALITY:

Your medical records that are related to this study will be maintained in confidentiality. Your name or any information that could identify you will not appear in any reports or publications of the results of this study. However, by law it is required to release information that will result in the saving of lives and in such a case the physician seeing you will be required to do so.

RIGHT TO ASK QUESTIONS AND/OR WITHDRAW FROM THE STUDY:

You have the right to ask any questions concerning the potential hazards of this study at any time. During the study you will be informed of any new information which may affect your safety.

If you have any questions about this study please contact Dr. Martin Katzman: phone number (416) 535-8501 x 4408; pager # 339-1764.

You may also reach Dr. David Goldbloom at 535-8501 x 6915, who is an external contact person.

Your participation in this study is entirely voluntary. You have the right to withdraw from the study at any time. If you decide to be withdrawn from the study this will not affect your

future medical care in any way. The research team may withdraw you from the study if it feels that it is in your best interest.

You will receive a signed copy of this form.

COMPENSATION:

You will be paid for taking part in the study. Upon your completion of the interviews, medical exam and laboratory test, you will be compensated for your time at the end of the first visit, receiving $30.00. Upon arrival for the second visit, you will receive $40.00. Upon fully completing the study you will receive $30.00 on the third visit, for a total of $100.00.

If you have any questions or if you think you have experienced a research-related illness or injury, contact Dr. Martin Katzman at (416) 535-8501 x 4408. Alternatively he may be paged at (416) 339-1764.

CONSENT:

Study Title: **EFFECTS OF TRYPTOPHAN DEPLETION ON CENTRAL AND PERIPHERAL CHEMORECEPTORS IN PANIC DISORDER PATIENTS AND HEALTHY VOLUNTEERS**

I, _____, have read and understand all the preceding information describing this study and all my questions have been answered to my satisfaction. I voluntarily consent to participate in this study.

_____

Signature of Patient

Date: ___/_____/___
DD/MMM/YY

_____

Signature of Witness

Date: ___/_____/___
DD/MMM/YY

_____

Signature of Investigator

Date: ___/_____/___
DD/MMM/YY

Would you like to volunteer in a study of Panic Attacks?

Scientists are seeking volunteers to participate in a study, which will examine a potential cause of panic disorder. The study involves the use of a breathing test and consumption of a drink mixture containing protein components (amino acids) mixed together to give a "boost" type energy shake. The study will take place at the Centre for Addiction and Mental Health-Clarke Division and the Medical Sciences Building at the University of Toronto. All information collected will remain confidential. You will be financially compensated for your participation.

To qualify you must be:

- Physically healthy adult male
- Have no diagnosis of psychiatric illness
- Not have been recently diagnosed with other psychological or medical conditions (ex. cardiovascular disease, COPD, asthma, etc.)
- Not taking any medication
- A non-smoker

For more information, please call:

Lucas at 416-535-8501 x 6436

RESEARCH ETHICS OFFICE

Confidential

CENTRE FOR ADDICTION AND MENTAL HEALTH
RESEARCH ETHICS BOARD
PROTOCOL FORM

(Revised September 28, 2000)

PLEASE NOTE:
This form should be provided in 15 copies with 15 copies of the entire protocol and 15 copies of the principal investigator's curriculum vitae. If the study involves a new drug, please submit 1 copy of the Investigator's Brochure. (This form must be completed in FULL – reference to page numbers in the protocol will not suffice.)
Two-sided copies are appreciated and all forms must be TYPED.

In the case of student research, the protocol form must be signed by the student, the student's supervisor at CAMH and the CAMH divisional head.

Principal Investigator:

Name: Dr. Martin Katzman, B.Sc., M.D., FRCPC

Address: 250 College Street, Toronto, Ont. M5T 1R8

Telephone Number: (416) 535-8501 x 4408 Fax : (416)-979-6853

Email: martin_katzman@camh.net

Hospital/Division: CAMH, Anxiety Disorders Clinic

Purpose of Application: (i.e., undergraduate or postgraduate degree, clinical trial in-house, external granting agency, Personal Award, contract, etc.). If the purpose of this application is to meet the criteria for an external agency please provide the full name of the agency.

Student M.Sc. research: Mr. Lukasz Struzik (Dept. of Physiology)

Grant deadline (if applicable):

Are you planning to conduct the research with or without external funding?
No external funding.

Title: Effects of Tryptophan Depletion on the Central and Peripheral Chemoreflexes in Panic Disorder Patients and Healthy Volunteers

Purpose of Study: (e.g., generalizable knowledge, programme evaluation).

We aim to elucidate the pathophysiology underlying Panic Disorder.

Hypothesis/Objectives of the Study:

We are proposing a study to investigate the possible site of interaction between the serotonin system and the neural respiratory control centres in panic disorder patients and healthy volunteers. To date, no studies have looked at the effect of low brain tryptophan (i.e. serotonin) on the thresholds and sensitivities of the central and peripheral chemoreflexes, and thus no anatomical localization of respiratory-serotonin system interaction has been proposed.

Background and Rationale for the Study:

Many studies in panic etiology have shown that abnormal respiratory responses are observed in panic disorder patients in terms of tidal volume and frequency of response to carbon dioxide as well as other panicogens. Theories have been posited implicating carbon dioxide hypersensitivity in panic etiology, but the physiological site of the supposed pathology has remained elusive. Concurrently, it is now well known that selective serotonin reuptake inhibitors (SSRI's,) agents that putatively elevate brain serotonin levels, are successful in relieving panic. The mechanism of these drugs and their site of efficacy in panic has yet to be elucidated.
Enhanced serotonin neurotransmission has shown to normalize the tidal volume and frequency response in panic patients, while reduced neurotransmission exacerbates the ventilatory response to panicogens such as carbon dioxide. Considering the involvement of the serotonin system in the control of breathing, it is not unreasonable to look for a site of interaction between the two systems at the level of the neural control centres. If pathology of serotonin neurotransmission exists at the level of the chemoreceptors in panic, it should manifest itself as changes in chemoreflex threshold and/or sensitivity of response to carbon dioxide.

Procedures - What procedures will be used in the study? (Describe the procedures and indicate what instruments - if any - will be used. Include with this submission any instruments which are not standard.)

We will use:

(1) The Modified Read rebreathing procedure

-This has been used in previous protocols that were accepted by the current IRB (Protocol reference # 4840)

(2) Tryptophan depletion procedure

-This procedure functions to safely and temporarily lower brain serotonin levels (see Kent et al., 1996; Miller et al., 1996; Klaassen et al., 1998; Miller et al., 2000.)

(3) The study will be undertaken in two populations: panic disorder patients and healthy volunteers.

Subjects will we screened with the following tools: Semi-Structured Clinical Interview, as per DSM-IV criteria (SCID), Anxiety Sensitivity Inventory (ASI), Sheehan Disability Inventory, Symptom Checklist-90 (SCL-90.) In addition, panic and anxiety symptoms will be assessed using an Anxiety Diary and a Panic Attack record. Symptoms experienced as a result of the rebreathing procedure will be assessed using a DSM-IV derived Panic Symptoms Scale.

Participants (Give size of the eligible population and the numbers of participants you wish to have complete the study.)

Fifteen male panic disorder patients and healthy volunteers between 18-50 years of age will undergo the experimental protocol. Subjects will be excluded from the protocol if they are smokers, are currently taking medication, or were born at high altitudes.

The Centre encourages the inclusion in research of women and of individuals over age 65 years.

Female subjects are excluded to avoid effects of the menstrual cycle on the serotonin system, as well as the chemoreflex response to carbon dioxide.

How will the group be selected? (inclusion/exclusion criteria)*

Panic disorder patients: No significant illness upon history, physical examination and laboratory findings; no history of compromised respiratory function; no DSM-IV criteria other then Panic Disorder with or without Agoraphobia; no current use of medications; consent to study.
Healthy volunteers: Same as panic disorder patients, but the controls must not meet any DSM-IV diagnosis.

1) Are there any special issues in the use of this population? (i.e., incompetent patients, subjects who may feel pressured to consent, i.e., those with limited skills in English, etc.)

No.

2) How and by whom are prospective subjects to be approached?

Subjects will be recruited by use of an advertisement. Mr. Lukasz Struzik, who has been trained in the use of all of the assessment tools involved, will do screening of the population using a SCID interview. Dr. Martin Katzman, a specialist in anxiety disorders, will confirm and inform the patients of the diagnosis.

Has the person approaching prospective subjects any prior relationship with them which might make them feel pressured into participating? (For example, is this person their physician or employer or teacher?) Attach a copy of any advertisements for subjects to this submission.

No.

3) If a patient is rejected for the study or withdraws from it, what alternatives are available to him/her?

Subjects not meeting study criteria will, upon consent, receive immediate treatment. They are also not excluded from any future studies or other concurrent studies.

Did you use sample size calculations to justify your sample size? If this has not been done, please describe how you arrived at the sample size.

Estimated sample size was calculated using Sigma Stat 2.02 statistics program. Previous experience in the research area agrees with the estimate of 15 subjects for study.

How was the proposed control group selected (if applicable)?

The control group will consist of healthy male subjects, age and sex matched to the panic disorder patients.

Describe how the data will be analyzed.

Breath-by-breath values of ventilation and end-tidal partial pressures of oxygen and carbon dioxide will be analyzed for patients and control subjects during a low tryptophan session and a placebo (i.e. normal tryptophan blood levels) session. First, a plot of ventilation versus end-tidal carbon dioxide will be obtained for a hypoxic and hyperoxic modified rebreathing test. Under these conditions ventilatory breakpoints, or chemoreflex thresholds will be compared for differences under low tryptophan and normal tryptophan levels. The constant ventilation segment above threshold, fitted to a mean, will be indicative of the slope, or chemoreflex sensitivity which will be analyzed in a similar fashion.

Briefly describe the direct implications of research.

Should respiratory chemoreflex threshold or sensitivity differences be found between panic disorder patients and controls with the no-tryptophan intervention, the implications will be rather profound.

1. The exact site of interaction between the serotonin system and respiratory control would be found (i.e. chemoreflex threshold/sensitivity changes mediated by altered serotonergic neurotransmission.) This will be obtained by examining the control subjects alone.

2. The site of defective serotonin neurotransmission at the respiratory control centres in panic would also be located, elucidating a more exact mechanism mediating carbon dioxide hypersensitivity in this population, greatly contributing to the understanding of panic etiology.

Risk/Benefit Ratio - ***It is the expectation of the Tri-Council that proposed research will be designed to benefit participants where possible. Studies which involve significant risk without a balance of significant benefit may be inappropriate.***

I.

- list risks

The proposed rebreathing procedure has been performed in this laboratory many times and has included the involvement of all current investigators. There are no risks associated with the procedure.

Tryptophan depletion under rebreathing has been previously done with no risk. At most, slight nausea is experienced upon consumption of the tryptophan-free/placebo drink, but any symptoms present disappear within two hours of drink consumption.

- list benefits

The benefit to the patient is to the sense of helping elucidate potential mechanisms of action for an illness which they have and which is poorly understood. The sense of having contributed is something described by more than 50% of subjects in previous studies undertaken by this research team.

The benefits of this study are long term and far reaching. The project will contribute to the understanding of the etiology of panic, in the hope of improving treatment in the long-run. Also, future development of a quick breathing test could help with expedient and accurate diagnosis of panic disorder.

II.

- How are the risks and benefits balanced?

There are no risks to the participants aside from possible slight nausea. Benefits are both to patient and to the understanding of the mechanisms underlying panic pathology.

Consent* - *The Office of Research Services has available general guidelines on drafting consent forms and also samples of appropriate forms. These may be accessed on the Website -

http://www.rir.utoronto.ca/

Click on: U of T Community Site, Ethics, Human Subjects, Forms and Sample Letters, Consent Form

General Considerations:

- consent forms should be written in lay language at an approximate grade 6-8 reading level;
- consents should be written in the first person;
- **the form should be placed on the institutional letterhead and broken into point form;**
- terms like "randomization", "double-blind" and "placebo" should be explained in lay language

Points to Include:

- initially a statement should be included that the individual is being asked to participate in research and why it is they are being asked;
- a comprehensible statement of the research purpose, identity of the researcher, the expected duration and nature of participation and a description of the research procedures; (only procedures which are being done specifically for the research need to be described);
- early in the form a statement should be included concerning alternatives to participation - i.e. what would be considered standard treatment for the condition being investigated;
- a comprehensible description of reasonably foreseeable harms and benefits that may arise from research participation, as well as the likely consequences of non-action should be given;
- an assurance that the prospective subjects are free not to participate and have the right to withdraw at any time without affecting the quality of their care;
- a statement regarding the possibility of commercialization of research findings and the presence of any apparent or actual or potential conflict of interest on the part of researchers, their institutions or sponsors;
- an indication as to who will have access to information collected on the identity of subject's and descriptions of how confidentiality will be protected.
- a statement about any payment to subjects and whether or not it will be prorated.
- the name and telephone number of the principal investigator;
- the name and telephone number of an additional contact person (who is not involved in the study) to whom participants can direct questions;
- the approximate number of participants in the trial and whether the study is multicentred.

In addition, please consider the following: If you anticipate that you may wish to re-contact study participants in the future for further research, please include this information in your initial consent form. Similarly, if you think you may wish to use data from the study for future research

please provide subjects with as much information as possible about future data use; i.e., the type of research and any protections of subject confidentiality and the like. Any videotaping must either be addressed in the consent form or in a separate consent form. For studies involving children, parental consent is required for those under age 16.
Please attach a copy of the proposed consent form and any information sheets and/or letters to the subjects to this form.

Placebo:

- Does the proposed study involve a placebo control?

Yes.

- If yes, how will subjects be assigned?

Subjects perform the rebreathing procedure either under a tryptophan depleted condition or placebo condition. The conditions will be assigned in a randomized, double-blind fashion.

- Is there an alternative standard treatment available?

The study is not a treatment study and therefore, there is no ethical consideration in terms of randomization to the placebo group

- If so, is this clearly conveyed to the subject?

Yes.

Please justify the use of placebo when a known effective treatment exists.

Not applicable.

Deception

- Does the study involve deception? No.

- If yes, why is this thought to be necessary?

- If yes, will subjects be fully debriefed? How will this be done?

Yes, any questions pertaining to the study will be answered in detail at the conclusion of the last visit.

Compensation

- Will participants be compensated for their involvement in the study? Yes

- If yes, on what basis? It is expected that payment will be pro-rated in the case of a subject's withdrawal from the study.

Subjects will be compensated with $30.00 at the end of the first visit when they will undergo a SCID interview, and a physical and laboratory test. Upon arrival for the second visit, subjects will receive $40.00 for the time spent undertaking the rebreathing test and for time involved in keeping a panic attack and anxiety diary in the week prior to this visit. Thirty dollars will follow upon arrival for the third and final visit when the last set of rebreathing tests will be undertaken.

- Is compensation used in such a way or is the amount such that it could be construed as an undue inducement to participate? Please justify the use of compensation.

Compensation will cover time and travel expenses incurred as a result of involvement in the study.

Confidentiality - How will confidentiality be maintained? Are existing records being used (for example, patient charts) and if so, how will confidentiality be protected? Do the researchers have permission to use existing records?

All members with access to patient files have signed the CAMH Confidentiality Act.

Monitoring - Does this study require any special procedures for monitoring by the Committee? If so, what do you propose as an appropriate method of monitoring?

No.

Should patients be asked for their continuing consent on a regular basis?

No, this is not a treatment study. The subjects will not be blinded to the procedure of the study and therefore will not be presented with a schedule for the study for which they were not prepared. While they are not receiving treatment, they will blinded to the order of the rebreathing challenges and the content of the amino acid drink.

Possible Conflict of Interest – None.

Finder's Fees and Other Recruitment Incentives - The University of Toronto has a policy against any payment of finder's fees or other recruitment incentives to researchers.

- What direct or indirect benefits are you receiving as a result of this research? (For example, payment per research subject...)

None.

- Do the researchers have any affiliation with, or financial involvement in, any organization or entity with a direct or indirect interest in the subject matter or materials of this research?

No.

Financial Benefits

- Who is funding this research?

An application for funding will most likely be submitted to the Centre for Addiction and Mental Health. The estimated size of the grant needed to cover this study will be less than $5000.00. This includes $3000.00 for subject compensation, $2000.00 for costs involved in analyzing blood samples by High Pressure Gas Chromatography as well as costs associated with supplies and maintenance of the rebreathing apparatus.

- Is this a grant? No.

- Is this a contract? No.

Please submit 2 copies of your contract and study budget with this proposal.

- What direct or indirect financial benefits will you receive from this research? For example 1) do you get payment per research subject?

Mr. Lukasz Struzik (M.Sc. candidate) is supported by the Ontario Thorasic Society for graduate level work related to this study.

- Do you get personal income from this research? No.

- To whom is any payment made?

The thirty subjects involved in this study will each receive $100.00 compensation for their time spent during their involvement in the study. This will incur a total of $3000.00.

- If this research is a contract or grant has it been signed by an appropriate administrative authority?

Are there any additional ethical issues you would like the Committee to consider?
None, all pertinent issues are outlined in detail in the study protocol.

References

Kent JM, Coplan JD, Martinez J, Karmally W, Papp LA, Gorman JM. (1996). Ventilatory effects of tryptophan depletion in panic disorder: A preliminary report. Psychiatry Research 64: 83-90

Klaassen T, Klumperbeek J, Deutz NEP, van Praag HM, Griez E. (1998). Effects of tryptophan depletion on anxiety and on panic provoked by carbon dioxide challenge. Psychiatry Research 77: 167-174

Miller H, Deakin JEW, Anderson I (1996). Acute tryptophan depletion increases panic anxiety in panic disorder patients. Journal of Psychopharmacology 9: 72

Miller HEJ, Deakin JEW, Anderson IM (2000). Effect of acute tryptophan depletion on CO2-induced anxiety in patients with panic disorder and normal volunteers. British Journal of Psychiatry 176: 182-188

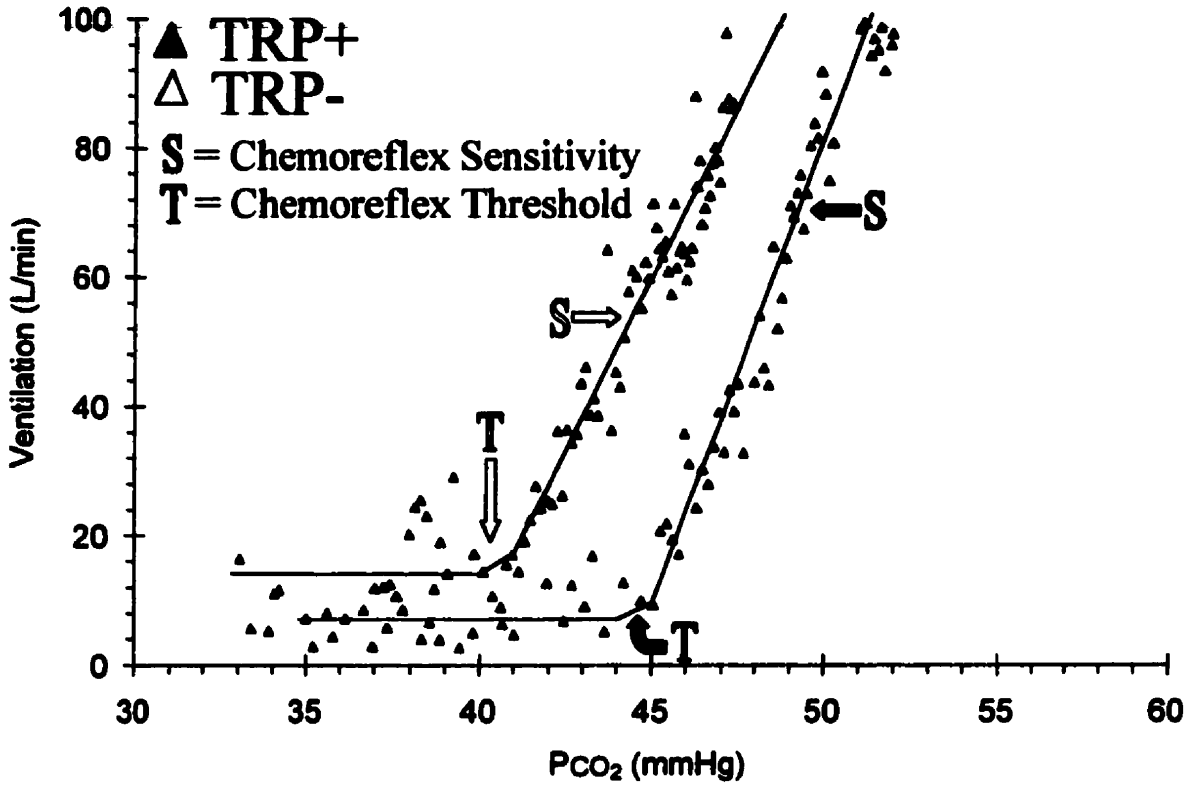

Figure A1: Ventilatory responses during a hyperoxic rebreathing test for subject #1.

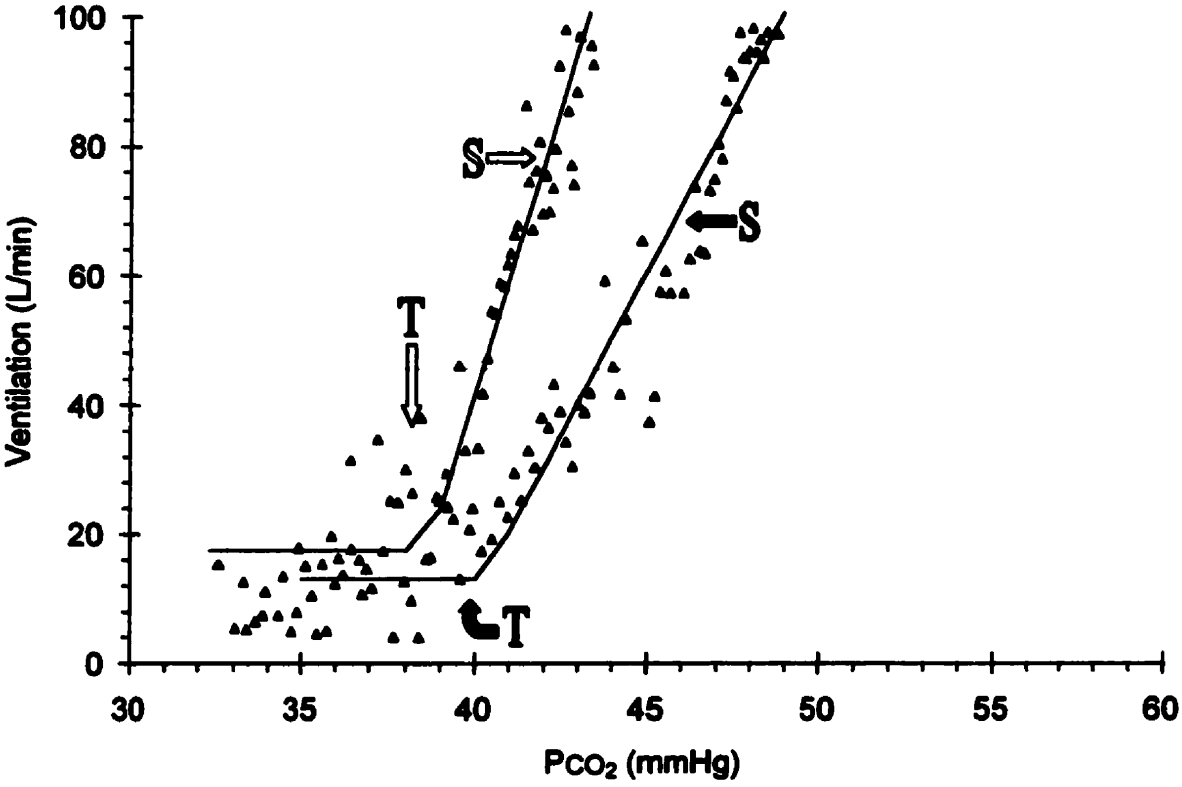

Figure A2: Ventilatory responses during a hypoxic rebreathing test for subject #1.

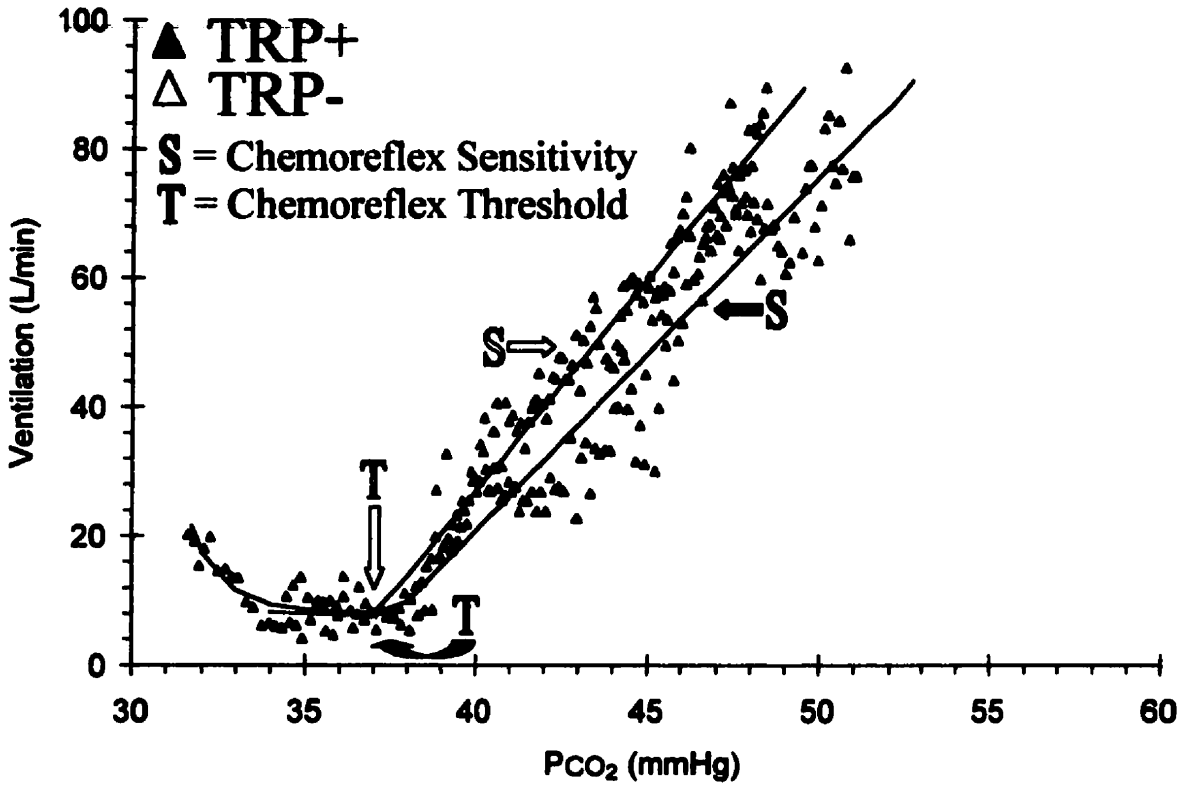

Figure A3: Ventilatory responses during a hyperoxic rebreathing test for subject #2.

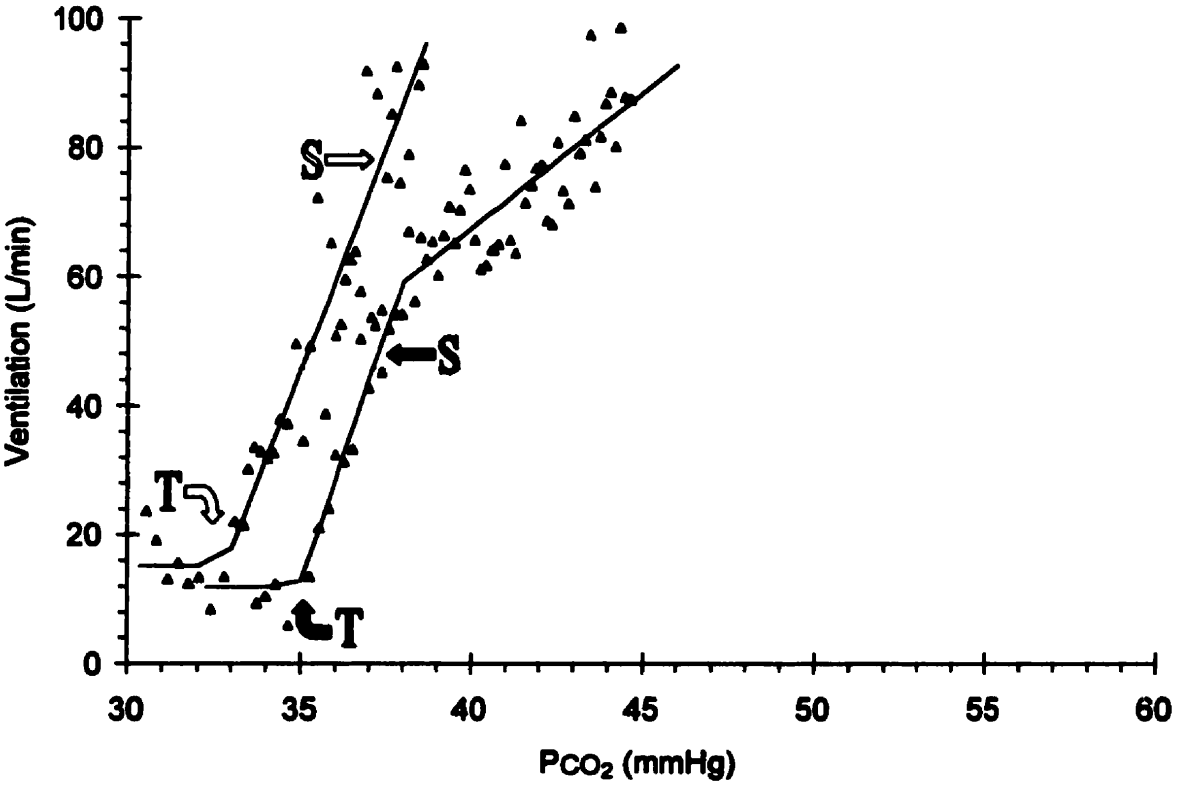

Figure A4: Ventilatory responses during a hypoxic rebreathing test for subject #2.

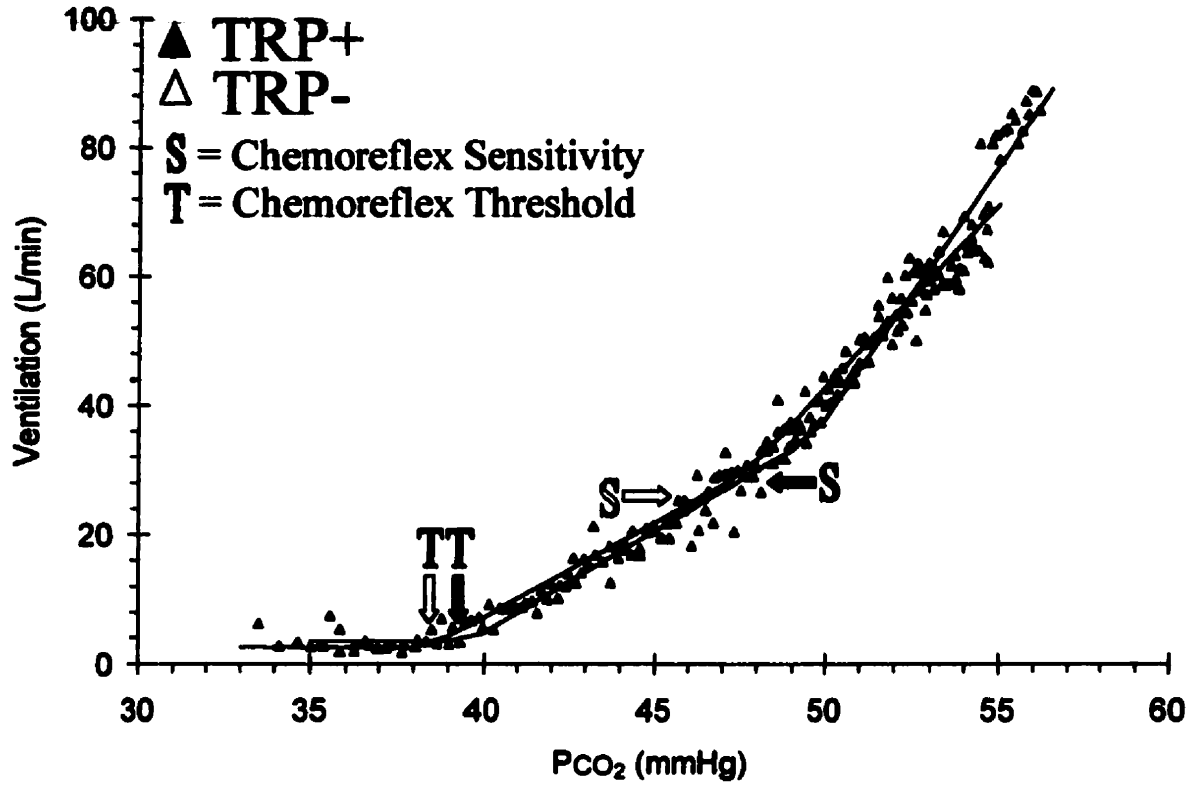

Figure A5: Ventilatory responses during a hyperoxic rebreathing test for subject #3.

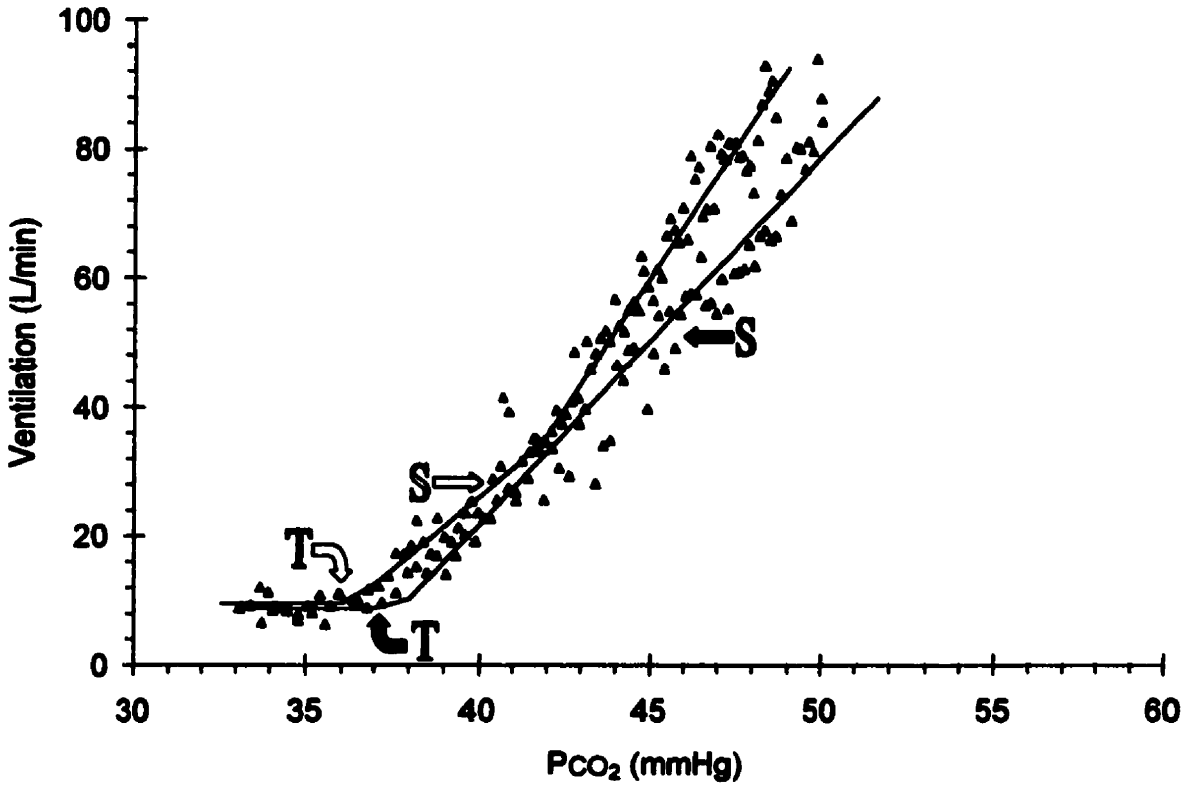

Figure A6: Ventilatory responses during a hypoxic rebreathing test for subject #3.

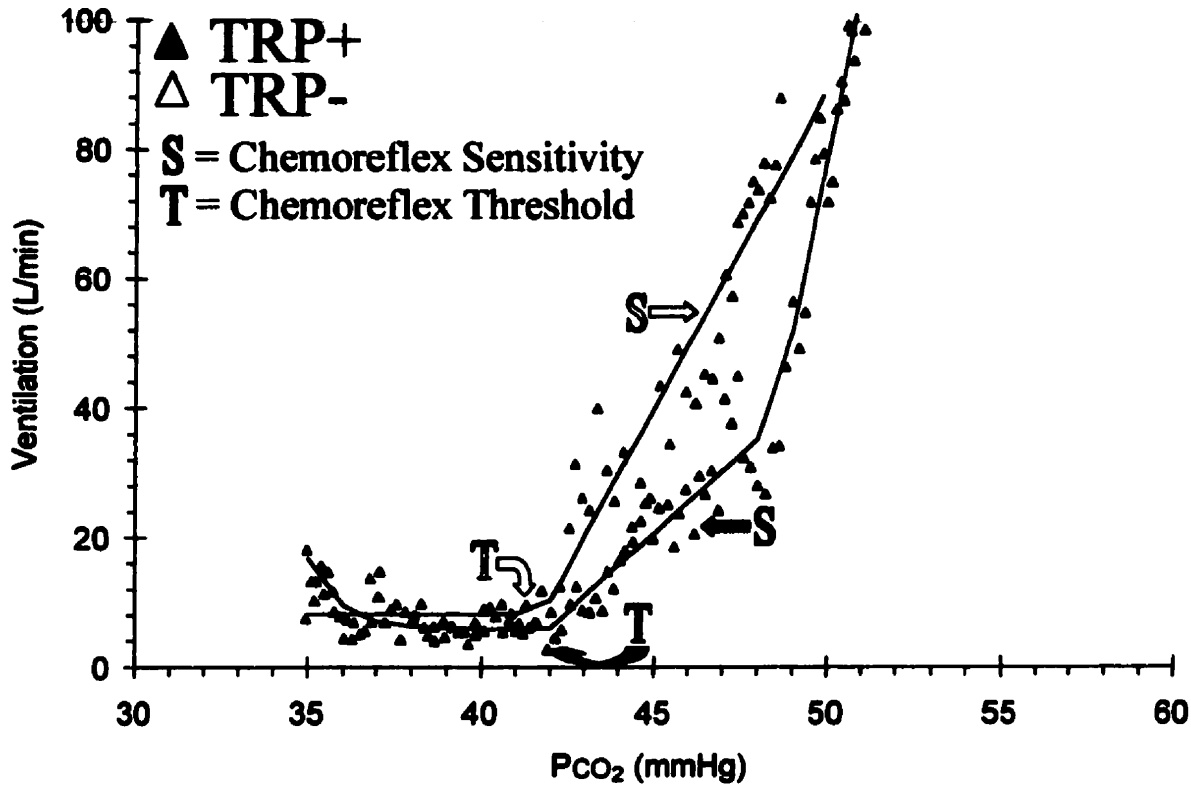

Figure A7: Ventilatory responses during a hyperoxic rebreathing test for subject #4.

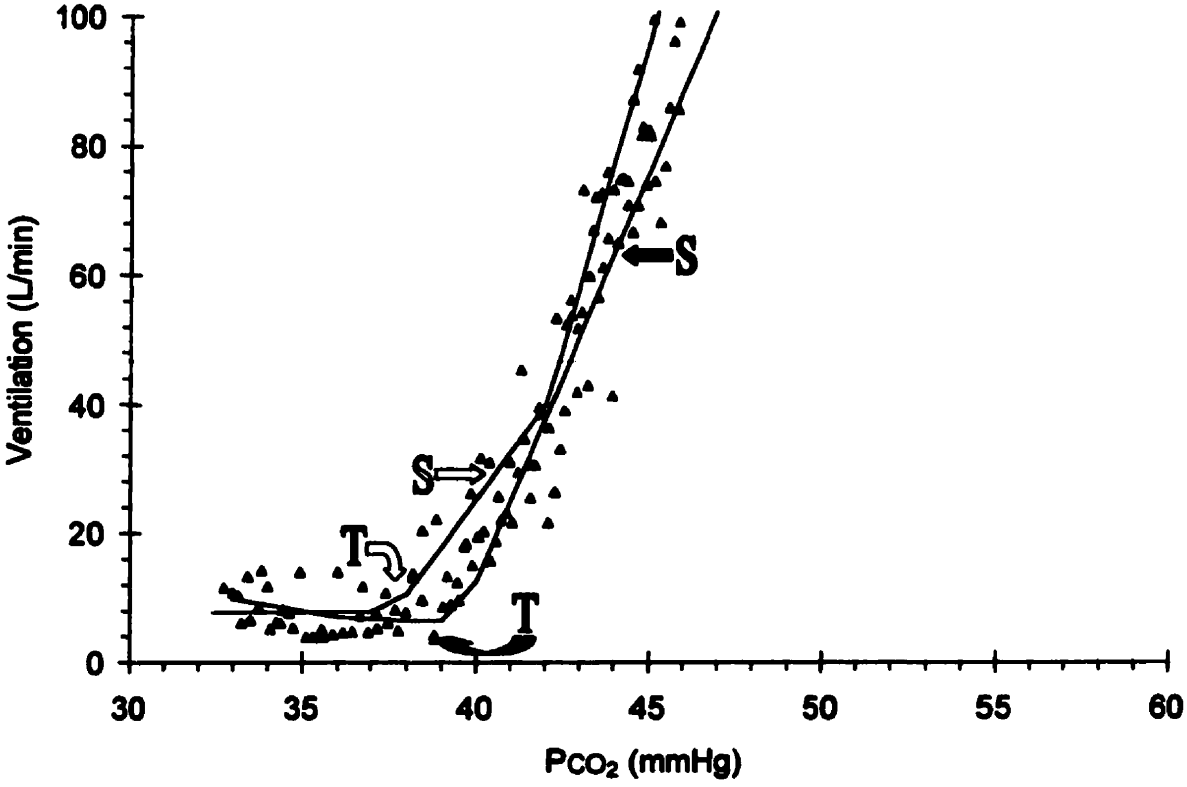

Figure A8: Ventilatory responses during a hypoxic rebreathing test for subject #4.

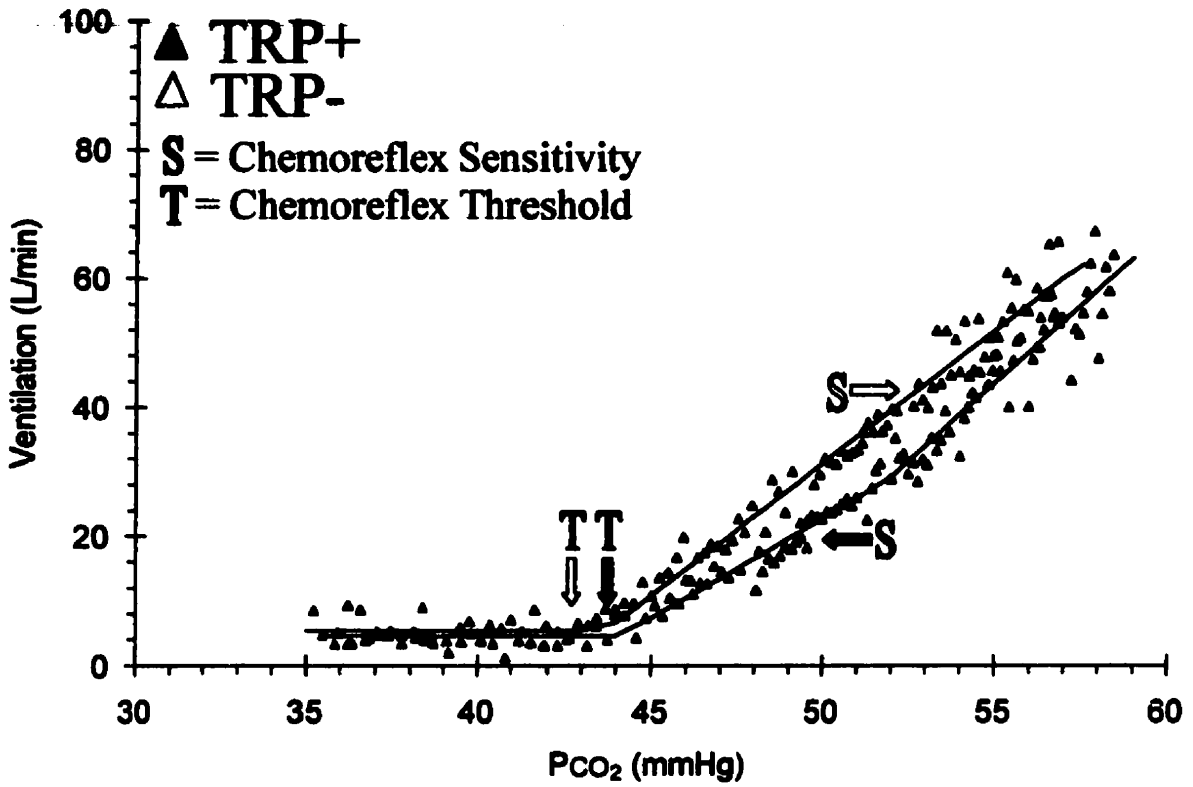

Figure A9: Ventilatory responses during a hyperoxic rebreathing test for subject #5.

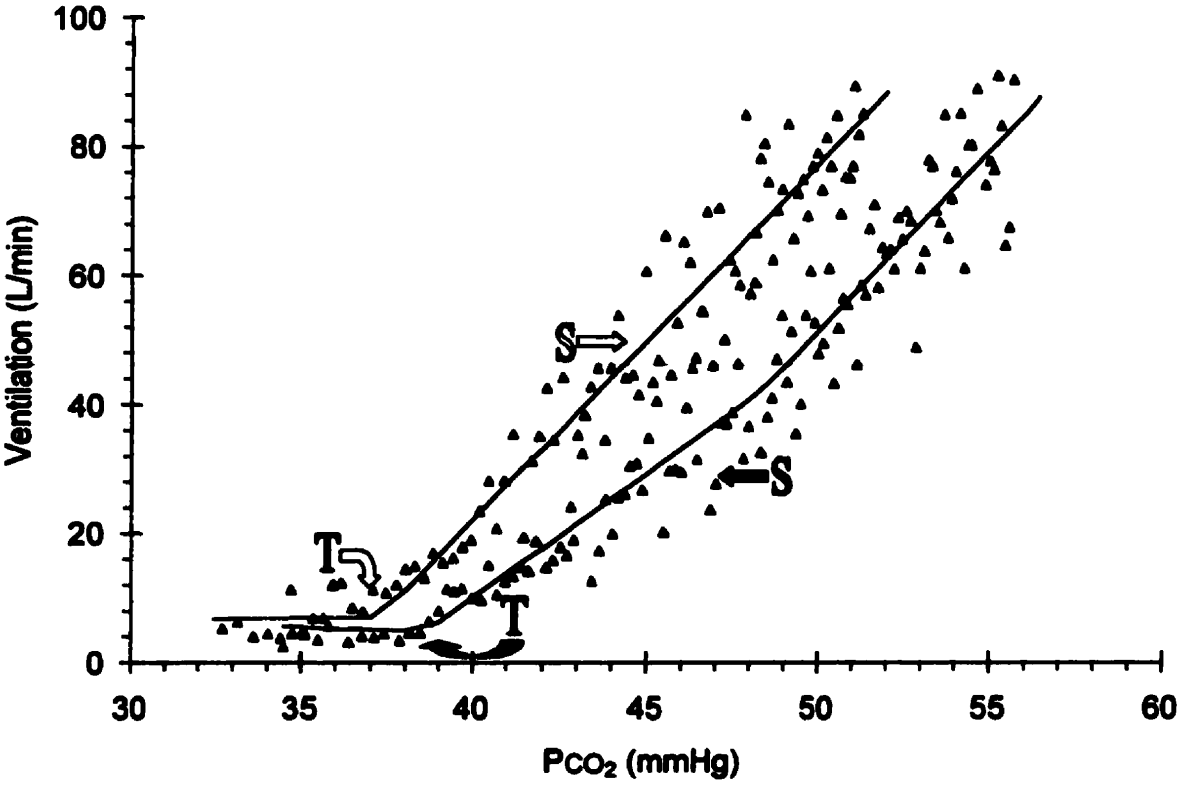

Figure A10: Ventilatory responses during a hypoxic rebreathing test for subject #5.

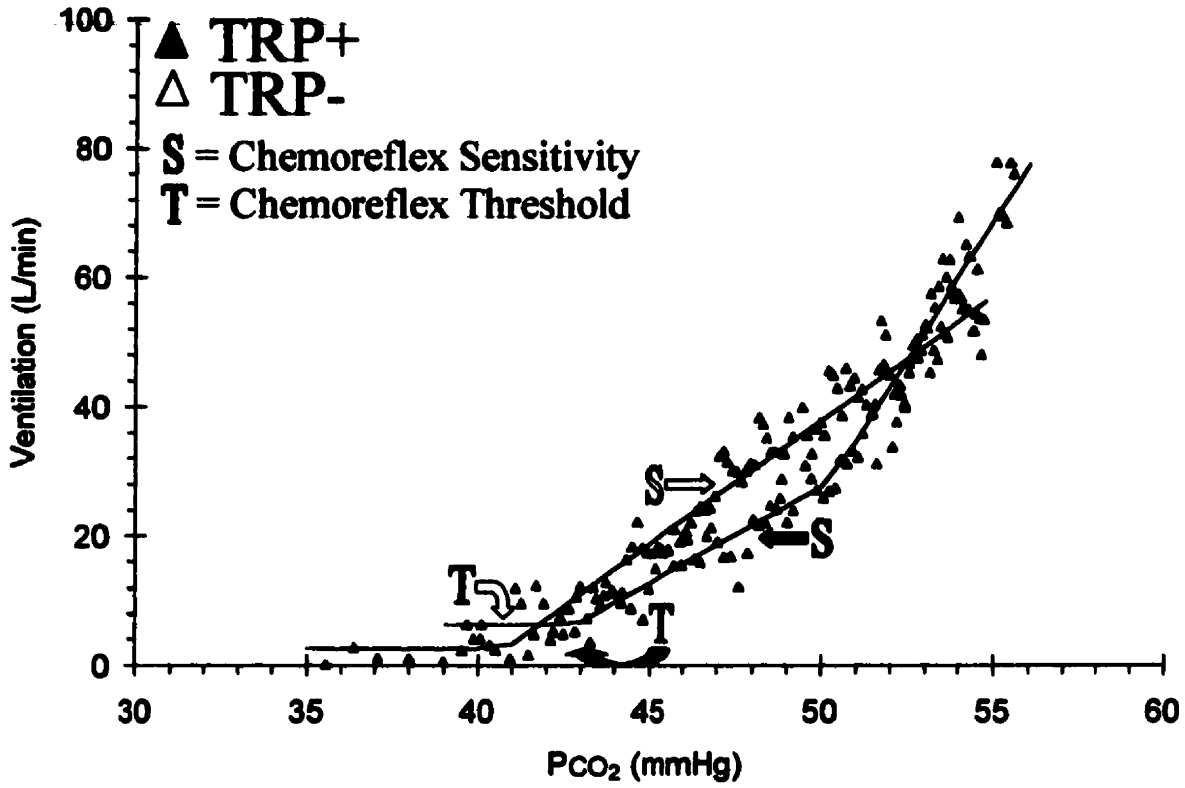

Figure A11: Ventilatory responses during a hyperoxic rebreathing test for subject #6.

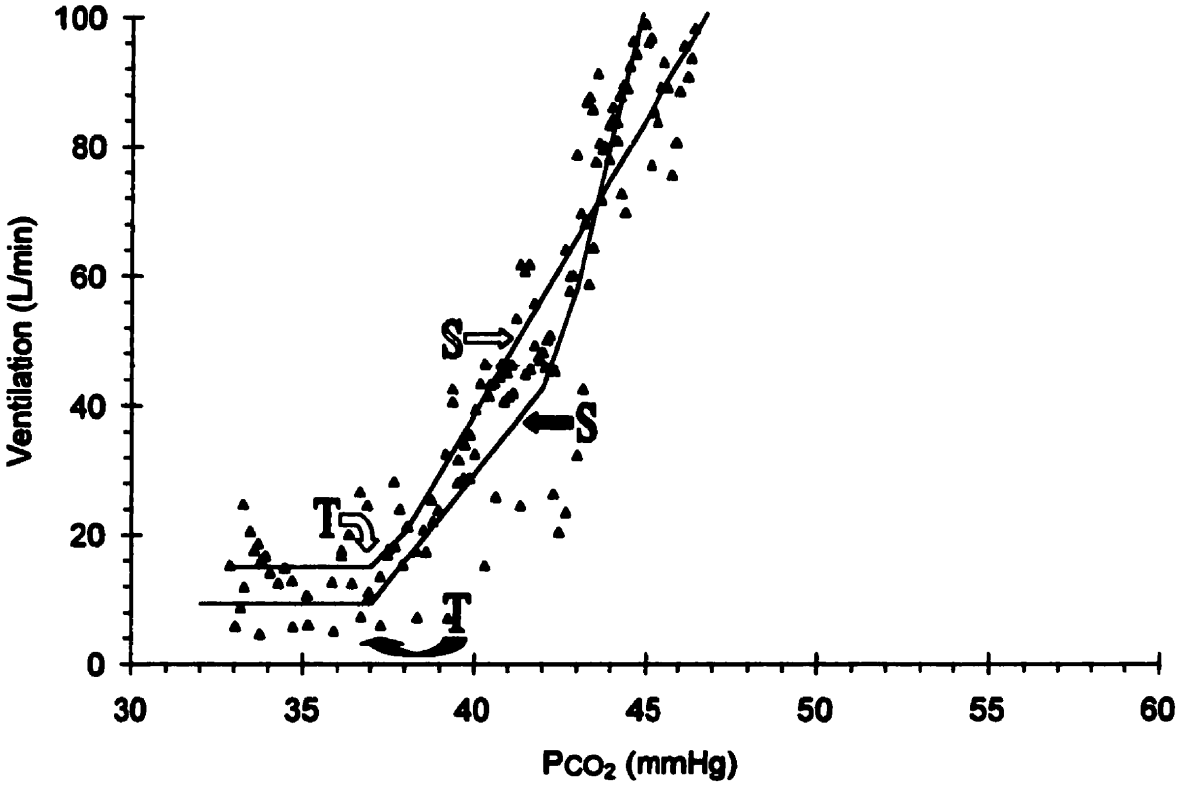

Figure A12: Ventilatory responses during a hypoxic rebreathing test for subject #6.

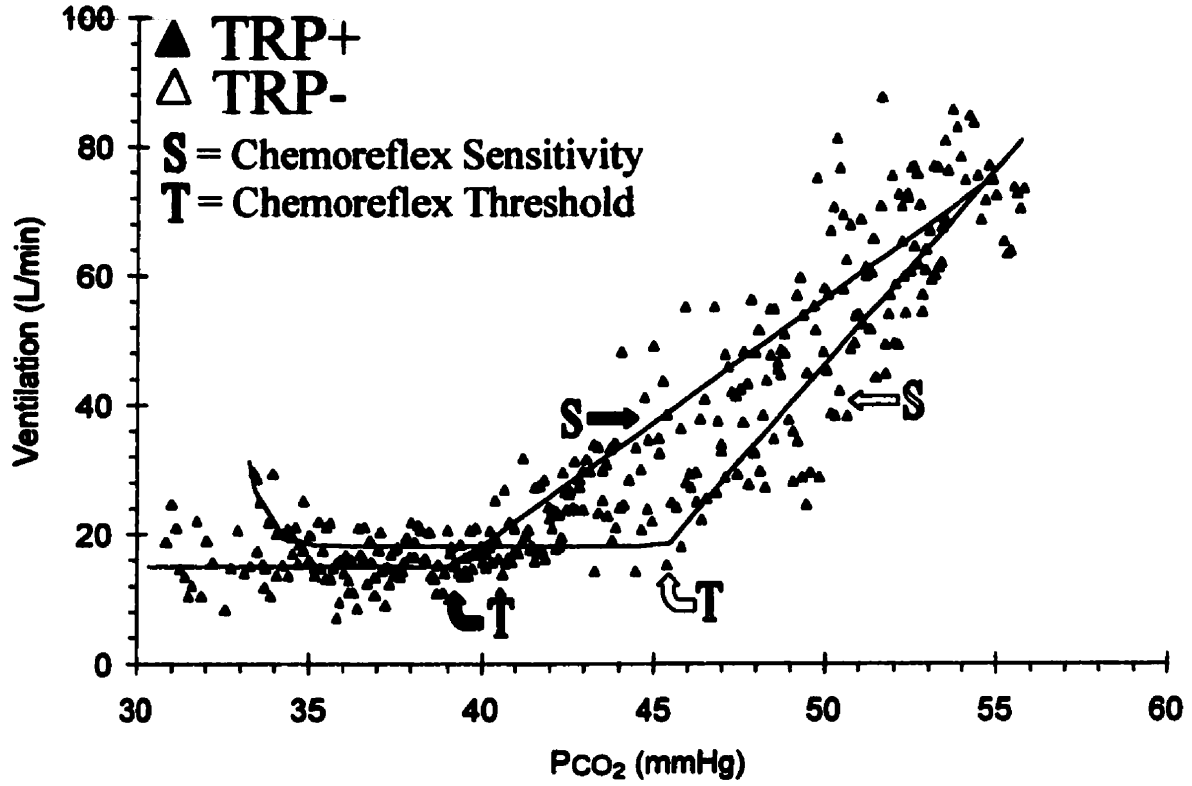

Figure A13: Ventilatory responses during a hyperoxic rebreathing test for subject #7.

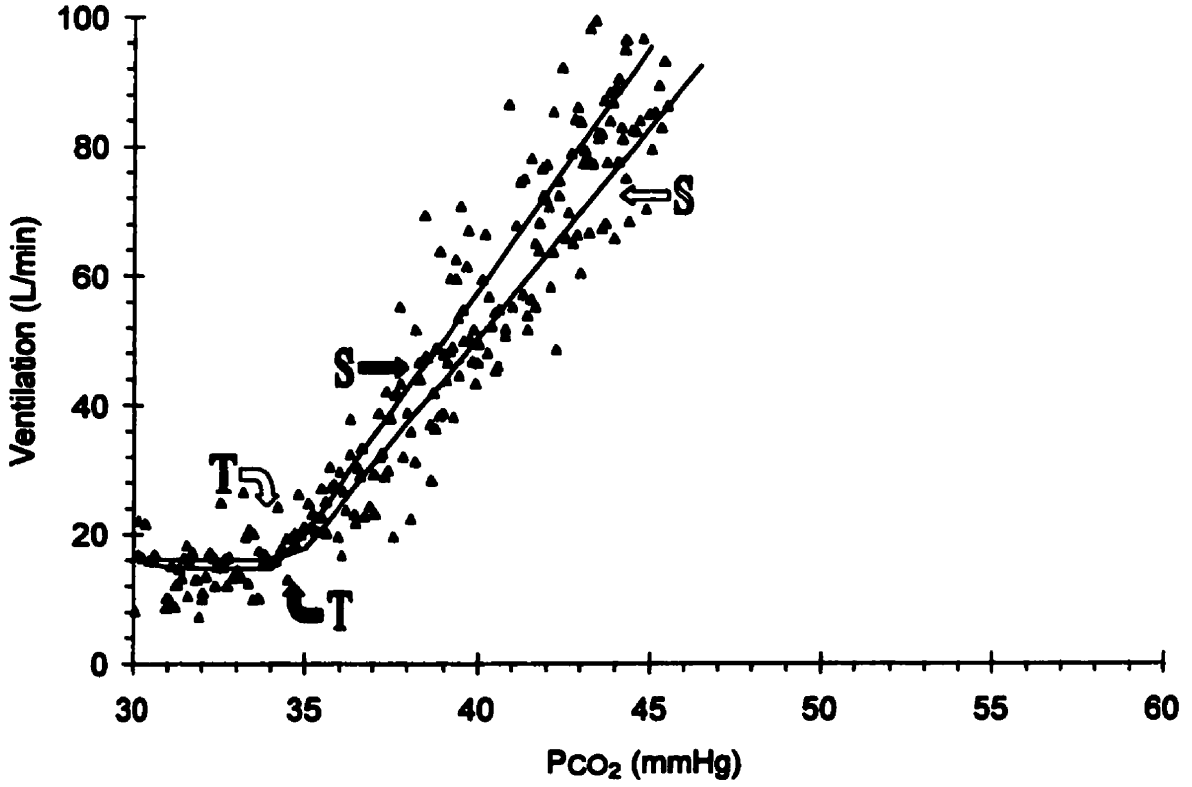

Figure A14: Ventilatory responses during a hypoxic rebreathing test for subject #7.

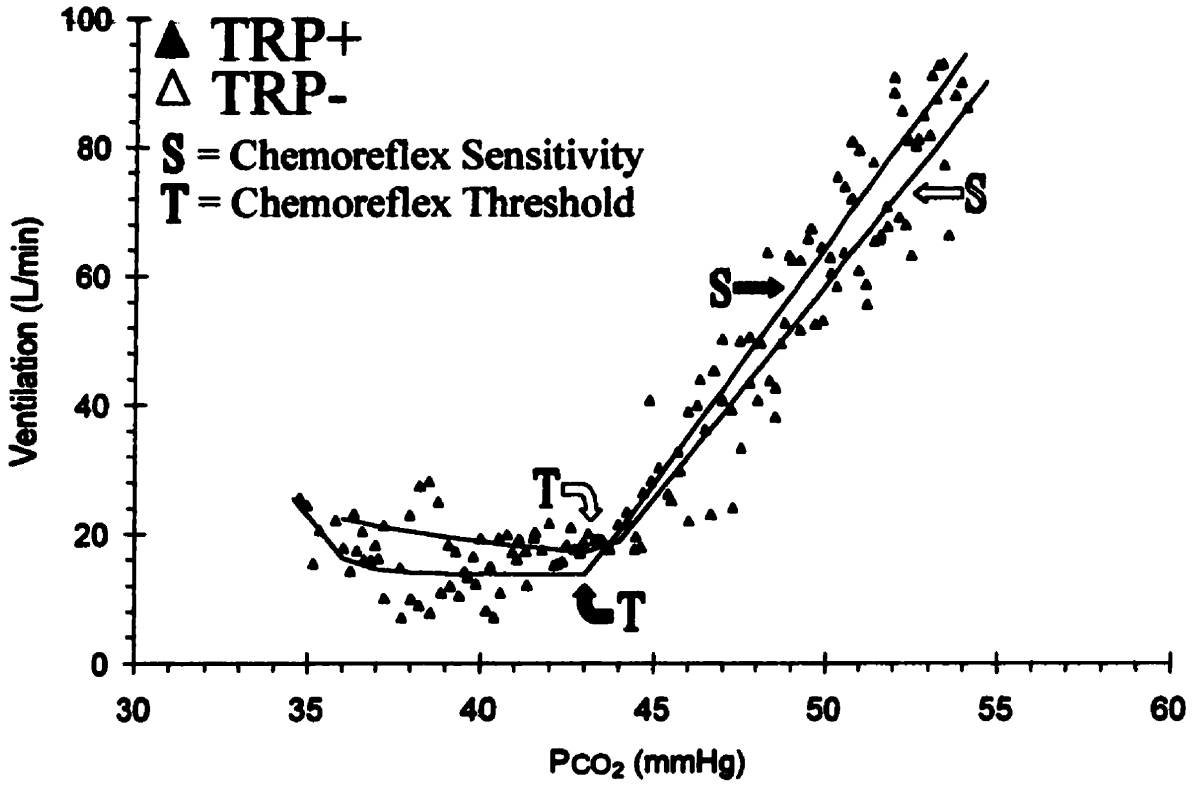

Figure A15: Ventilatory responses during a hyperoxic rebreathing test for subject #8.

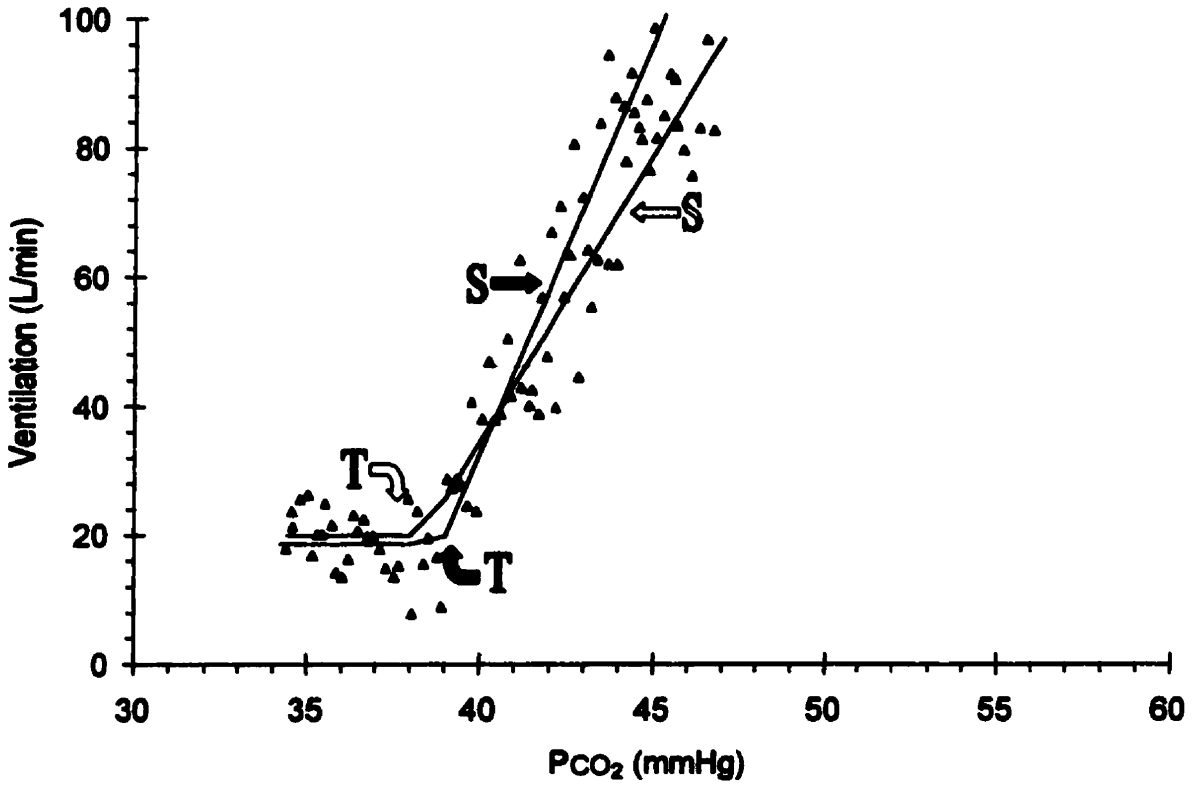

Figure A16: Ventilatory responses during a hypoxic rebreathing test for subject #8.

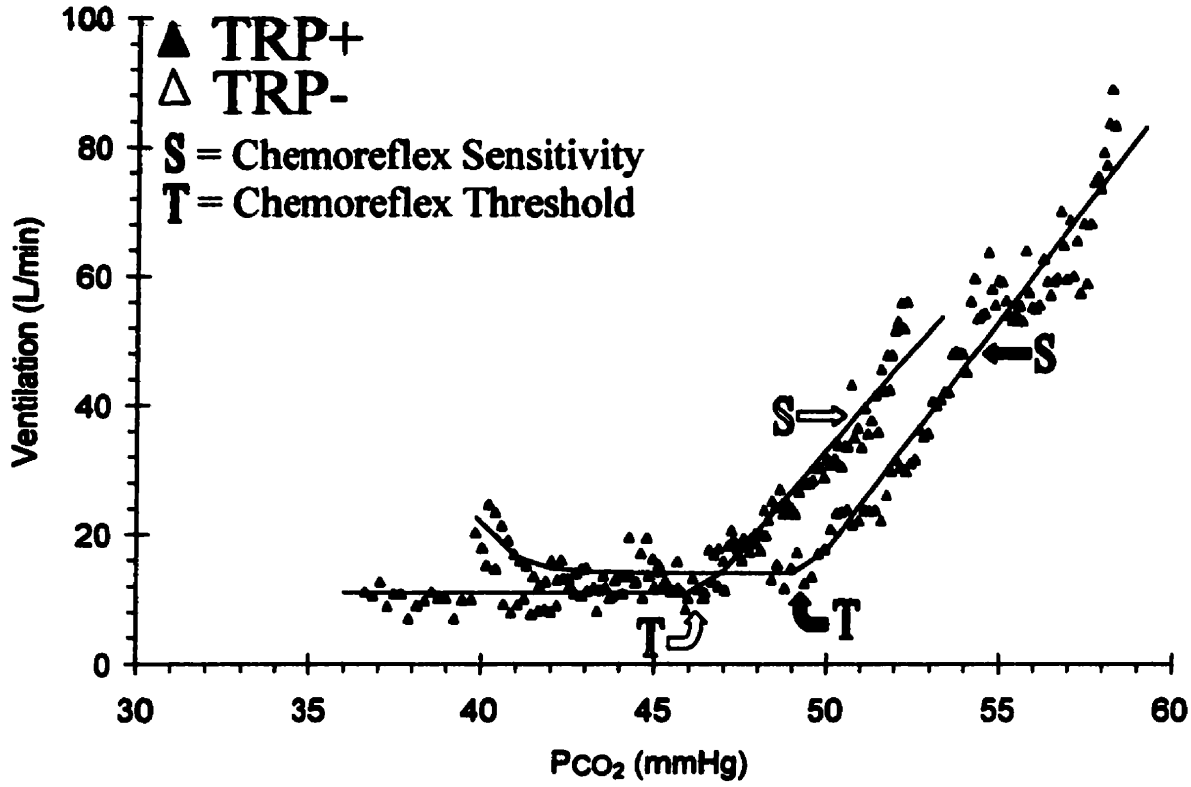

Figure A17: Ventilatory responses during a hyperoxic rebreathing test for subject #9.

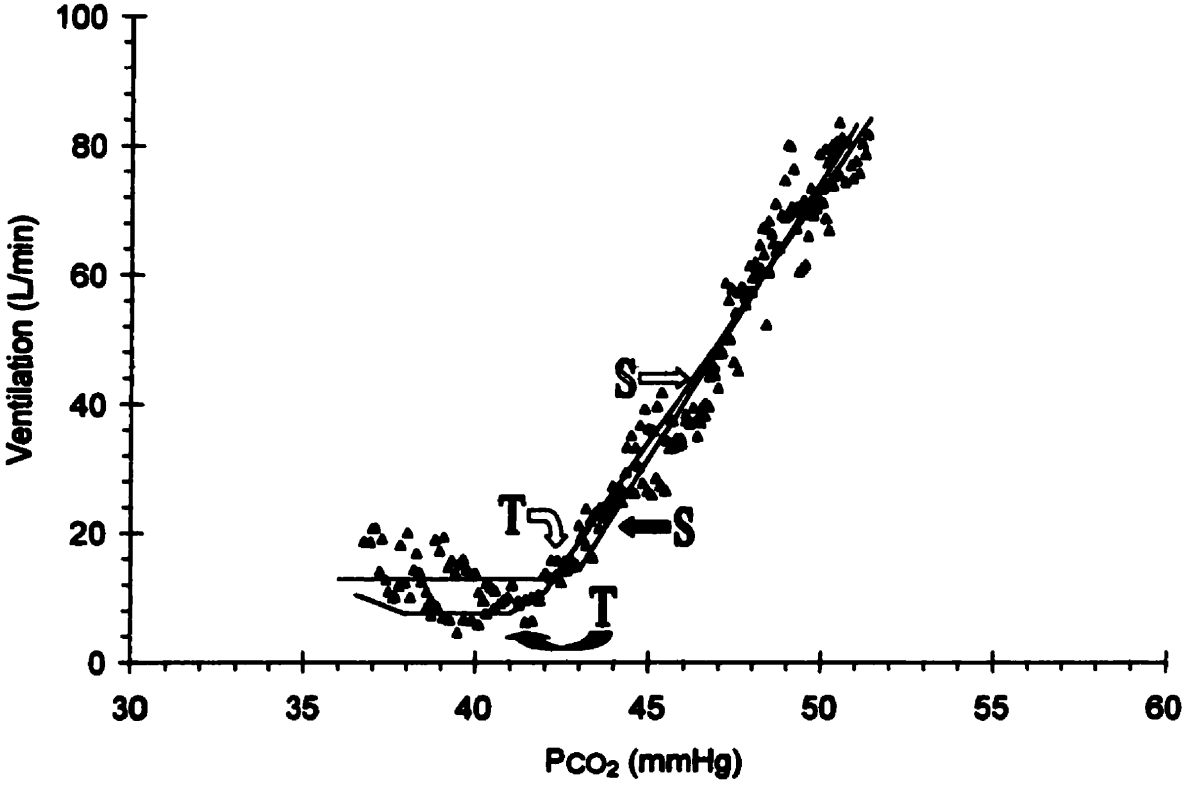

Figure A18: Ventilatory responses during a hypoxic rebreathing test for subject #9.

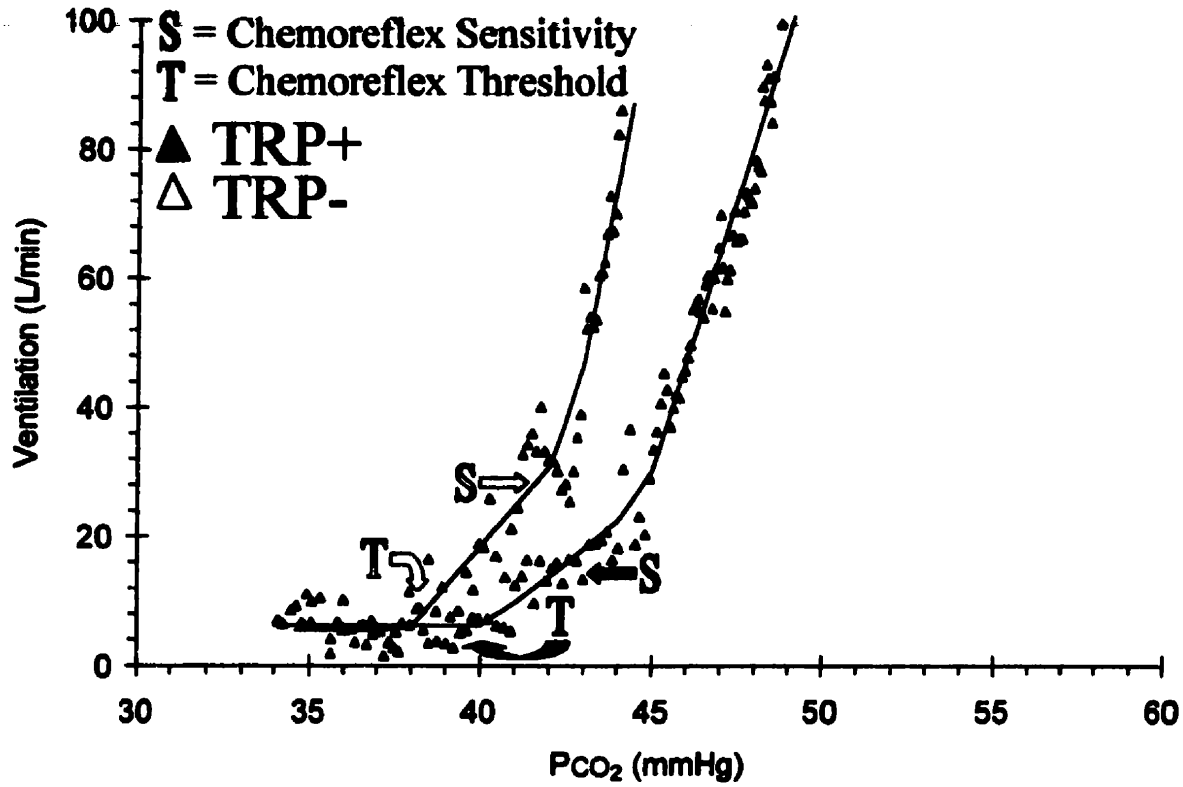

Figure A19: Ventilatory responses during a hyperoxic rebreathing test for subject #10.

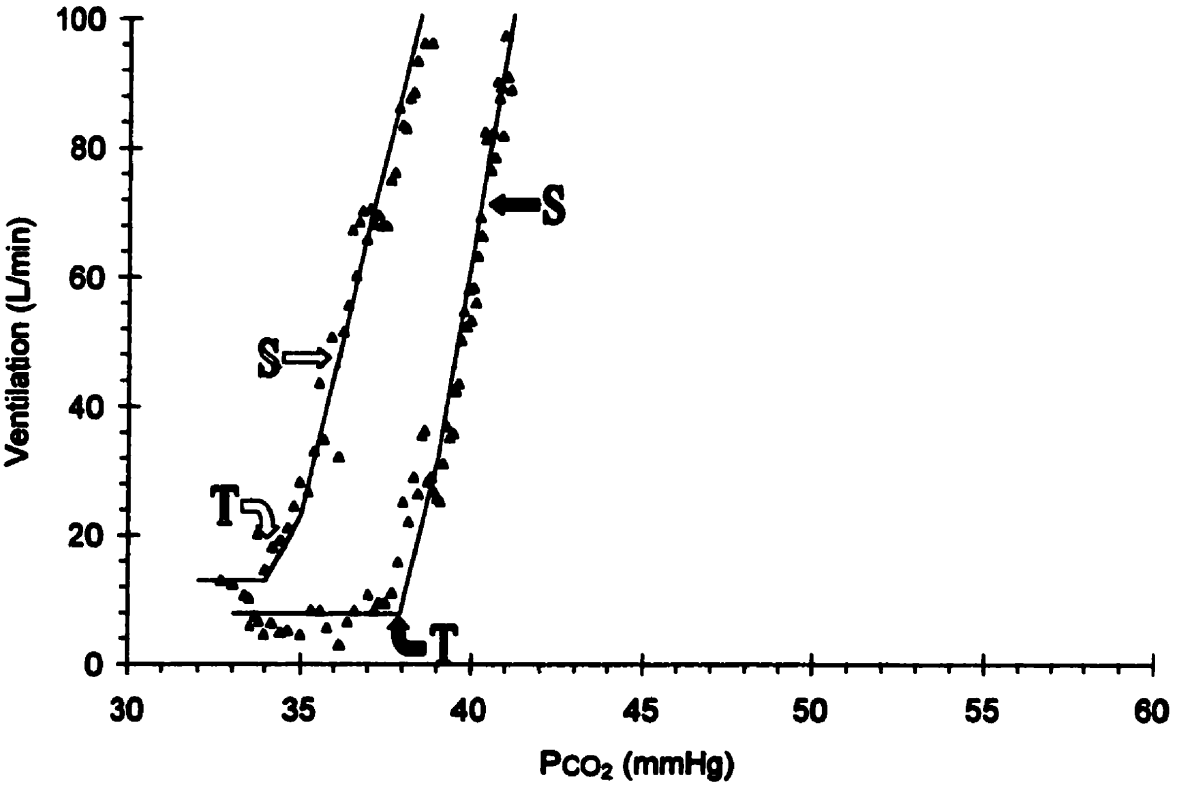

Figure A20: Ventilatory responses during a hypoxic rebreathing test for subject #10.

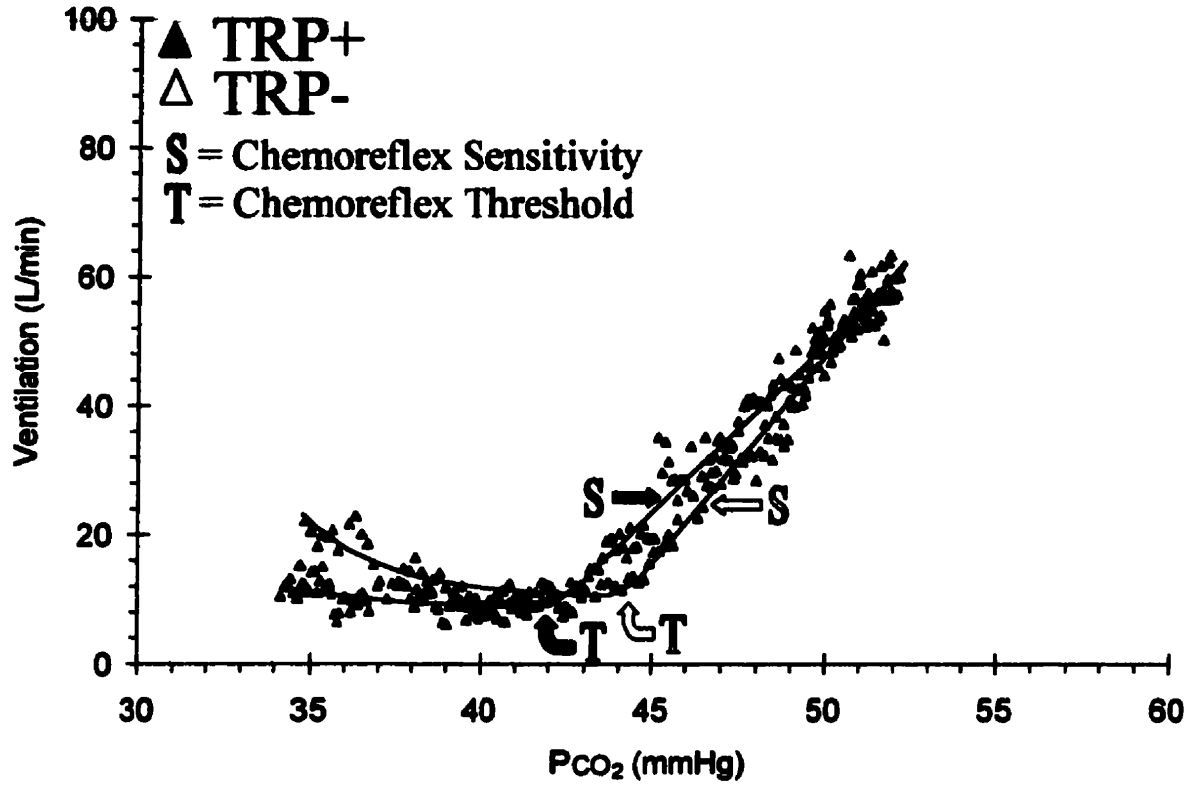

Figure A21: Ventilatory responses during a hyperoxic rebreathing test for subject #11.

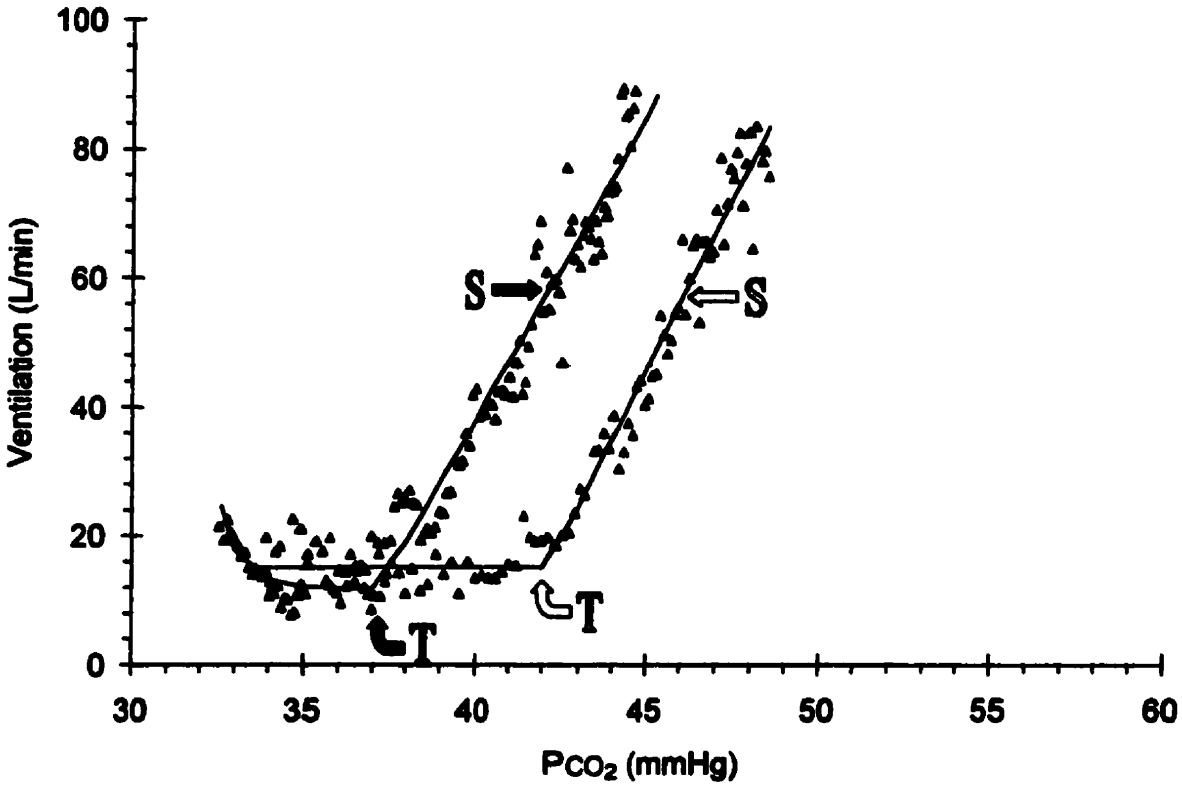

Figure A22: Ventilatory responses during a hypoxic rebreathing test for subject #11.