University of Alberta

MECHANISMS OF FLUMAZENIL-INDUCD PANIC

by

Lenka Zedkova 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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A.S. Joyce (Committee Chair)

N.J. Coupland (Supervisor)

G.B. Baker (Co-Supervisor)

K.M. Hegadoren (Committee Member)

T. Davis (Committee Member)

D. Koszycki (External Examiner)

Date: 16 June, 2003

ABSTRACT

Reduction of GABA neurotransmission in animals causes anxiety, cardio-respiratory and neuroendocrine activation (reactions resembling human panic attacks). In patients with panic disorder (PD), flumazenil (normally an antagonist at the benzodiazepine binding site of the common $GABA_A$ receptor subtypes) may cause panic directly by acting as a partial inverse agonist, thus reducing GABA effect. Alternatively, flumazenil-induced panic may reflect a negative (cognitive) reaction to somatic symptoms experienced during the challenge. Both possibilities were tested. Thyrotropin-releasing hormone (which is not panicogenic in PD patients) induced equal or greater somatic panic-related symptoms than flumazenil in healthy volunteers (HV), suggesting that catastrophic misinterpretation of somatic symptoms may not be the primary mechanism of flumazenil-induced panic in PD. Consequently, behavioral, cardio-respiratory and cortisol responses to flumazenil were compared between patients with 1) pure PD (P), 2) comorbid PD and depression (P+D) and 3) depression, and HV in a double blind, placebo-controlled crossover design. The reproducibility of flumazenil effects was assessed in the P and HV groups. Flumazenil induced panic attacks specifically in patients with PD (P and P+D), although this effect was not highly reproducible in each P subject. The sum intensity of panic symptoms showed better reproducibility. The lack of marked physiological activation during flumazenil-induced panic suggests that flumazenil does not act globally as an inverse agonist in PD. Responses to flumazenil may be consistent with a more restricted regional expression of $GABA_A$ receptor subtypes at which flumazenil exhibits inverse agonist activity. Panic attacks following flumazenil were frequently associated

with catastrophic cognitions, but it is unclear whether these were the causes or concomitants of panic. None of the assessed cognitive variables significantly predicted increases in panic symptomatology in PD patients. Plasma flumazenil concentrations did not differ between PD panickers and non-panickers, ruling out a pharmacokinetic mechanism as the major determinant of responses to flumazenil. The results provide indirect evidence that flumazenil induces panic by a more direct route rather than via cognitive mechanisms, although cognitive factors may modulate the intensity of responses. Possible mechanisms of flumazenil-induced panic and their relation to spontaneous panic are discussed.

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ABBREVIATIONS

aBLA	anterior basolateral amygdala
ACQ	Agoraphobic Cognition Questionnaire
ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
ASI	Anxiety Sensitivity Index
AVP	arginine vasopressin
Asp	aspartate
CBF	cerebral blood flow
CBT	cognitive behavioral therapy
CCK	cholecystokinin
CCK-1R	CCK-1 receptor
CCK-2R	CCK-2 receptor
CCK-4	CCK tetrapeptide
CCK-8S	sulfated CCK octapeptide
CO_2	carbon dioxide
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
CV	coefficient of variation
D	patients with major depression currently experiencing a major depressive episode
DBP	diastolic blood pressure
DMH	dorsomedial hypothalamus

DSM IV	Diagnostic and Statistical Manual of Mental Disorders, 4thEdition
DST	dexamethasone suppression test
ECG	electrocardiogram
FL	flumazenil
FP	flumazenil-induced panic attacks
FPPF	flumazenil-placebo-placebo-flumazenil (order)
FQ	Fear Questionnaire
GABA	γ-aminobutyric acid
GI	gastrointestinal
H	histidine
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance (pressure) liquid chromatography
HR	heart rate
HV	healthy volunteer(s)
ICC	intra-class correlation coefficient
IS	internal standard
iv	intravenous
mACQ	modified Agoraphobic Cognitions Questionnaire
mACQ3+	PD patients rating 3 or more on at least 1 mACQ item
mACQ2-	PD patients *not* rating 3 or more on at least 1 mACQ item
mCPP	meta-chlorophenylpiperazine
MDD	major depressive disorder
Met	methionine

MV	minute volume
P	patients with pure panic disorder
P+D	patients with comorbid panic disorder and major depression currently experiencing a major depressive episode
PAG	periaqueductal gray
PD	panic disorder
PET	positron emission tomography
PFFP	placebo-flumazenil-flumazenil-placebo (order)
PG	pentagastrin
Phe	phenylalanine
PL	placebo
PSS	Panic Symptom Scale
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
RR	respiratory rate
SBP	systolic blood pressure
SCID	Structured Clinical Interview for Diagnosis: DSM-IV version
SP	recent severe panic attacks
SPECT	single-photon-emission computed tomography
SSRI	selective serotonin reuptake inhibitors
TRH	thyrotropin-releasing hormone
Trp	tryptophan
TV	tidal volume
VAS	Visual Analog Scale

1. INTRODUCTION

1.1. General introduction

In the *Diagnostic and Statistical Manual of Mental Disorders*, 4th Edition (DSM IV) (American Psychiatric Association 1994), panic disorder (PD) is defined by recurrent spontaneous panic attacks. Panic attacks are short episodes of fear or discomfort accompanied by at least four somatic or cognitive symptoms, such as palpitations, shortness of breath, sweating, dizziness, fear of losing control, or fear of dying. The symptoms have a sudden onset and usually reach a peak within 10 minutes. Panic attacks must also be followed by either persistent concerns about having additional attacks, worries about implications of the attacks, or significant changes in behavior related to the attacks. The behavioral changes often refer to agoraphobia, which is an avoidance of situations or places from which escape might be difficult, or help unavailable in the event of having a panic attack. Many patients develop anticipatory anxiety before entering these situations.

The lifetime prevalence of PD in the general population throughout the world ranges from 1.5 to 3.5%, and the annual prevalence is generally about 1 to 2% (Bland et al 1988; Eaton et al 1994; Lee et al 1990; Wittchen et al 1992). The rates of PD are approximately 2.5 times higher among women than men (Bland et al 1988; Eaton et al 1994; Wittchen et al 1992). In a cross-national study the mean age of onset of PD was found to be usually early to mid 20s (Weissman et al 1997). Apart from gender and age, the major risk factors for the development of the condition seem to be adverse early-life events, especially parental separation or loss (Battaglia et al 1995; Faravelli et al 1985), or physical or sexual abuse (Stein et al 1996; Friedman et al 2002; Dinwiddie et al 2000;

Safren et al 2002). Later in life, stressful life events, especially if perceived as uncontrollable, often precede the first panic attack (Roy-Byrne et al 1986a). Evidence for genetic factors in the etiology of PD has been reviewed in a recent meta-analysis of family and twin studies (Hettema et al 2001). This study calculated the summary odds ratio for PD as 5 in the first-degree relatives of probands across several family studies (Hettema et al 2001); the relative morbidity risk may be further increased in relatives of PD probands whose panic attacks are accompanied by smothering symptoms (Horwath et al 1997). The estimated heritability for PD derived from large twin studies was 43 % (Hettema et al 2001). Although several studies failed to identify specific genes involved in the disorder, links have been found between PD and the adenosine A_{2A} receptor gene (Deckert 1998), the cholecystokinin-2 receptor gene (Kennedy et al 1999), the monoamine oxidase A gene (Deckert et al 1999), and the catechol-*O*-methyltransferase gene (Hamilton et al 2002). Furthermore, a targeted genome scan guided by homologous mouse chromosomal loci involved in anxiety-like behavior provided evidence for linkage of PD to a locus on chromosome 12 (Smoller et al 2001). These promising findings now need to be replicated.

PD remains a chronic condition, with "most patients improved, but few cured" (Roy-Byrne and Cowley 1994). Some patients continue to experience panic attacks on a regular basis, others experience outbursts of attacks separated by months or years of remission. The major predictors of poorer outcome include longer duration of illness, the presence of agoraphobia, and comorbid depression or personality disorder (Iketani et al 2002; Pollack et al 1993; Roy-Byrne and Cowley 1994; Roy-Byrne et al 2000). Frequent comorbid conditions also include alcoholism and other anxiety disorders (Kushner et al

1999; Pollack and Marzol 2000). Patients with PD also appear to be at higher risk for cardiovascular diseases and have increased morbidity and mortality (Fleet et al 2000). Some studies have found that PD is associated with an increased risk for suicidal ideation and suicide attempts (Johnson et al 1990; Lepine et al 1993), although this often may be due to comorbid affective or personality disorders, or substance abuse (Friedman et al 1999; Warshaw et al 1995).

The chronicity of the disease usually requires long-term management. Pharmacological treatment includes antidepressants and the anxiolytic benzodiazepines. Selective serotonin reuptake inhibitors (SSRIs) have become the first-line treatment, due to their broad efficacy across anxiety and depressive symptoms and their tolerability and increased safety relative to other classes of antidepressants (Ballenger 1999; Kasper and Resinger 2001). These drugs may exert their antipanic effects by increasing deficient inhibitory serotonergic transmission in several brain regions involved in panic responses (Gorman et al 2000). Benzodiazepines are not usually the first choice in PD, due to the risk of developing physical dependence or withdrawal symptoms, and to their low efficacy in the treatment of comorbid depression. They can be initially co-administered with antidepressants, if a rapid therapeutic onset or a reduction of anxiety sometimes associated with early SSRI treatment is required. These drugs can also alleviate anticipatory anxiety accompanying panic attacks (Goddard et al 2001a; Kasper and Resinger 2001). New potential antipanic agents and non-pharmacological treatments of PD will be described in later sections of this thesis.

Overall, PD (frequently associated with psychiatric comorbidity), can be a disabling condition often leading to chronic unemployment, financial dependency and

significant direct and indirect economic costs (Greenberg et al 1999; Kessler et al 2001). The etiology of PD largely remains unclear, but research has implicated the role of both biological and cognitive factors.

1.2. Biological aspects of PD

Apart from evidence for genetic diathesis (Hettema et al 2001), many other findings suggested a biological basis for PD. Brain anatomical and functional abnormalities were evident in electroencephalographic studies (Bystritsky et al 1999; Landry et al 2002) and neuroimaging studies, such as those employing magnetic resonance imaging, positron emission tomography (PET) or single-photon-emission computed tomography (SPECT) (Gorman et al 2000). Some of the studies will be discussed in later sections. Strong evidence for biological abnormalities in PD has been provided by pharmacological challenge studies.

1.2.1. Pharmacological challenge studies: rationale and limitations

In laboratory studies, numerous pharmacological challenges have provoked panic responses significantly more often in patients with PD than in patients with other disorders or in healthy volunteers. These panicogenic agents include sodium lactate (Liebowitz et al 1984; Pitts and McClure 1967), carbon dioxide (CO_2) (Griez et al 1987; Kent et al 2001; Perna et al 1995), bicarbonate (Gorman et al 1989), the presynaptic α-2 adrenoreceptor antagonist yohimbine (Charney et al 1984; Charney et al 1992), the serotonergic agents *meta*-chlorophenylpiperazine (mCPP) and fenfluramine (Apostolopoulos et al 1993; Klein et al 1991), cholecystokinin (CCK)-2 receptor agonists

(Abelson and Nesse 1994; Abelson et al 1994; Bradwejn and Koszycki 2001), the benzodiazepine antagonist flumazenil (Nutt et al 1990; Woods et al 1991), the adenosine receptor antagonist caffeine (Charney et al 1985), the respiratory stimulant doxapram (Abelson et al 1996a), and the sympathomimetic epinephrine (Veltman et al 1996; Veltman et al 1998). Due to the difficulties related to capturing and studying spontaneous panic attacks, these agents have been used to study the biology of PD in the laboratory. By inducing panic, they may implicate systems they interact with in the disorder. To date several neural systems have been implicated in the etiology of PD (for reviews see Bourin et al 1998; Nutt and Lawson 1992); however, the exact mechanisms and sites of the panicogenic actions of these agents are still being investigated.

Several factors should be considered when interpreting the results of pharmacological challenge studies. First, the exact relationship between pharmacologically induced panic attacks and spontaneous panic attacks is still unknown. Several criteria for an "ideal" panicogenic agent have been established in order to assess its suitability for studying the neurobiology of panic (Guttmacher et al 1983). The agent should:

1. be safe for human use
2. induce emotional and somatic symptoms of a panic attack
3. reproduce symptoms of a spontaneous panic attack
4. display specificity for PD
5. show dose-dependent and reproducible effects
6. be antagonized by antipanic agents
7. not be antagonized by non-antipanic agents

To date, only some agents used in biological research on PD have been systematically tested for these criteria. However, even in the ideal case that an agent meets the criteria, the relationship between the mechanism of its panicogenic action and the biology of spontaneous panic remains unclear. The assumption that at least some of the agents (although often provoking phenomenologically similar panic attacks) target different neuronal systems, together with patients' interindividual differences in sensitivity to these agents, suggests the existence of PD subtypes (Nutt and Lawson 1992; Strohle et al 1998). This may be reflected in patients' interindividual differences in their responses to pharmacological treatments. On the other hand, some of the agents may have a final common panicogenic pathway, due to a complex interaction among systems. In addition, regardless of whether different agents have common or independent pathways, a single biological abnormality is unlikely to underlie panic attacks in fully developed PD. Even if a single system is initially affected, compensatory changes in other systems may take place. Therefore, it is unclear whether an agent may reveal a primary abnormality that causes panic attacks, or a compensatory 'downstream' change that occurs later in the course of the disease.

Second, the assessment of behavioral, physiological and cognitive responses to pharmacological challenges may give an idea about their panicogenic mechanisms, but is unable to describe neural networks activated by these challenges in detail. Therefore, the results from challenge tests should be correlated with information provided by neuroimaging studies.

Finally, although the initial aim of challenge tests was to clarify the biological basis of panic, many studies have emphasized the role of cognitive factors in challenge-

induced panic attacks. These factors are described in the section on cognitive aspects of PD.

Despite its limitations, the challenge strategy has generated a number of hypotheses concerning the etiology of PD and continues to play an important role in biological research on this disease.

Research projects described in this thesis employed challenge tests with the CCK-2 receptor agonist pentagastrin and with flumazenil, a ligand for the benzodiazepine site on the γ-aminobutyric acid$_A$ ($GABA_A$) receptor. The following sections review the role of GABA and CCK systems in the neurobiology of PD.

1.2.2. GABAergic system in PD

1.2.2.1. GABA and $GABA_A$ receptors

GABA is the major inhibitory neurotransmitter in the mammalian brain, present in approximately 20-50% of all central synapses (Iversen and Bloom 1972; Sieghart 1995). In many brain regions, neuronal inhibition is mediated predominantly by local circuit neurons. In addition, some areas contain longer GABAergic projections (e.g., the striatonigral and striatopallidal neurons, or the cerebellar efferent Purkinje cells). GABA is released from the nerve terminal upon depolarization and its action at the synapse is terminated primarily by ion-dependent reuptake into both nerve terminals and surrounding glial cells (Olsen and DeLorey 1999).

The action of GABA is mediated primarily by two different types of receptors, $GABA_A$ and $GABA_B$, which differ in their pharmacological properties. $GABA_A$ receptors

are pentameric ligand-gated chloride ion channels. Molecular cloning has identified a large number of structurally related peptide subunits. On the basis of 30-40% amino acid sequence identity, the subunits are now divided into 7 classes - α, β, γ, δ, ε, θ and ρ. In vertebrate brain, $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ and $\rho_{1\text{-}3}$ subunit isoforms, each encoded by separate genes, have been identified so far (Mohler et al 2002). Within each class, the subunit isoforms share about 70% sequence identity. Various combinations of five subunits, which are assembled to form the central ion channel, give rise to a large diversity of $GABA_A$ receptor subtypes. The major receptor subtype is believed to consist of $\alpha 1\beta 2\gamma 2$ subunits (Pirker et al 2000). The $\rho 1$ and $\rho 2$ subunits form homo-oligomeric channels displaying large GABA-induced currents. Due to their distinct pharmacology (e.g., insensitivity to the benzodiazepine site ligands or barbiturates, see Cutting et al 1991; Kusama et al 1993), these receptors are sometimes classified as $GABA_C$ receptors (Johnston 1996), although they represent a specialized subset of $GABA_A$ receptors (Barnard et al 1998).

GABA, upon binding to the receptor, opens the ion channel. Chloride ions, following their concentration gradient, enter the neuronal cell and this influx leads to membrane hyperpolarization associated with a reduced neuronal excitability (Bormann 1988). $GABA_A$ receptors are the targets for a variety of other centrally acting compounds, such as benzodiazepines, barbiturates, steroids, the convulsants picrotoxin and pentylenetetrazole, anesthetics, polyvalent cations and ethanol. These drugs interact with their binding sites at the receptor complex and some act as positive or negative allosteric modulators of the receptor response to GABA stimulation. Interaction of the binding sites seems to be complex, since the binding of one compound, by inducing a conformational

change in the receptor, can modify the binding properties of other sites (for a review see Sieghart 1995).

$GABA_A$ receptors show an ubiquitous distribution within the CNS, which may explain the implication of abnormal GABAergic activity in a number of psychiatric and neurological disorders, such as anxiety and mood disorders, epilepsy, schizophrenia, Huntington's disease or alcoholism (Kent et al 2002; Sanacora et al 2000; Bohme and Luddens 2001; Carlsson et al 2001; Chesselet and Delfs 1996; Davis and Wu 2001).

1.2.2.2. Benzodiazepine site

Many ligands with a benzodiazepine or nonbenzodiazepine structure have a high affinity for the benzodiazepine site. Agonists, like the benzodiazepines, enhance GABAergic transmission by increasing both the frequency of chloride channel openings and the affinity of the receptor for GABA and its agonists (positive intrinsic efficacy). These drugs have anxiolytic, anticonvulsant, relaxant, sedative/hypnotic, amnestic and ataxic effects. Inverse agonists, represented by some β-carbolines, reduce GABA effects by a mechanism opposite to that of agonists, and display anxiogenic, proconvulsant and stimulant properties (negative intrinsic efficacy). Antagonists, like flumazenil (Ro 15-1788), have virtually no intrinsic efficacy but competitively block the actions of both agonists and inverse agonists. Partial agonists and partial inverse agonists exhibit intermediate actions on the spectrum from full agonism to full inverse agonism (for reviews see Hevers and Luddens 1998 and Sieghart 1995).

This describes the situation at the most common $GABA_A$ receptor subtypes. However, the characteristics of the receptors vary with the presence of different subunits.

For example, receptors containing the α4 or the α6 subunit were found to be insensitive to the benzodiazepine agonists (Knoflach et al 1996; Wafford et al 1996). This is caused by a specific arginine residue in the α4 or α6 subunits, replaced by histidine in the corresponding position of the α1, α2, α3 and α5 subunits (Wieland et al 1992). A replacement of histidine (H) at a conserved position in the α subunit of diazepam sensitive receptors (α1-H101, α2-H101, α3-H126 and α5-H105) with arginine renders these receptors diazepam-insensitive; introduction of these point mutations into mouse lines resulted in the identification of the α subunit isoforms that may selectively mediate pharmacological effects of benzodiazepines (see section 1.2.2.7) (Rudolph et al 1999; Low et al 2000; McKernan et al 2000). Apart from the above α subunit isoforms, the presence of the γ2 subunit is also required for the sensitivity of $GABA_A$ receptors to classical benzodiazepines (Mohler et al 2002).

Subunit composition can further determine the affinity of the receptor for GABA, channel kinetics, and the efficacy of various allosteric site ligands (Knoflach et al 1996; Wafford et al 1996; Smith et al 2001; Bianchi et al 2002; Brown et al 2002). Findings relevant to this thesis will be described in the following sections.

1.2.2.3. GABA, anxiety and panic

The link between GABA, anxiety and panic has been supported by many studies. In both humans and animals, drugs that increase GABA function (benzodiazepine full or partial agonists, inhibitors of GABA breakdown or reuptake, and some neuroactive steroids) are anxiolytic (Rupprecht et al 2001; Sandford et al 2001; Sieghart 1995; Zwanzger et al 2001a), whereas those that reduce GABA function (benzodiazepine

inverse agonists, picrotoxin or pentylenetetrazol) are anxiogenic (Dorow et al 1983; Sieghart 1995).

Benzodiazepines reduce anxiety in animal models of anxiety or panic (Griebel et al 1996; Russo et al 1993). In animals, electrical stimulation of the dorsolateral periaqueductal gray (PAG) (a midbrain region surrounding the aqueduct) and the dorsomedial hypothalamus (DMH), which is interconnected with the PAG, elicited flight and escape behavior and autonomic activation (tachycardia, increased blood pressure and hyperventilation; see Graeff 1993 and Jenck et al 1995). These aversive reactions, which to some extent resemble human panic attacks, were attenuated when GABA activity in these areas was increased by microinjection of GABA, its agonists, or other drugs that increase GABA activity (Audi and Graeff 1984; Brandao et al 1982; Milani and Graeff 1987). In contrast, panic–like responses were observed when GABA activity in the PAG, DMH or the anterior basolateral amygdala (aBLA) was impaired by GABA antagonists, or inhibitors of GABA synthesis (Brandao et al 1986; Milani and Graeff 1987; Sanders and Shekhar 1995). GABA dysfunction in the DMH and aBLA also enhanced anxiogenic and cardiovascular effects of sodium lactate, a panicogen (Sajdyk and Shekhar 2000; Shekhar et al 1996). Responses to electrical or pharmacological stimulation of the above areas were modified by different panicogenic (yohimbine, caffeine) or antipanic (imipramine, benzodiazepines) agents, suggesting an interaction between several neural systems at the level of PAG and DMH (Jenck et al 1995; Shekhar 1994).

In humans, stereotactic stimulation of the dorsal PAG in awake neurosurgical patients induced intense emotional responses (severe fear and distress) and autonomic changes similar to those observed in animals under this stimulation (Nashold et al 1969).

Although the direct effects of GABAergic transmission on this stimulation were not tested in humans, other studies provided evidence for altered GABA function in PD. Systemically administered drugs that facilitate GABA effects through different mechanisms reduced both spontaneous (Goddard et al 2001a; Kasper and Resinger 2001; Sandford et al 2001; Zwanzger et al 2001a; Zwanzger et al 2001c) and challenge-induced (Liebowitz et al 1995; Sanderson et al 1994; Zwanzger et al 2001b) panic attacks. Furthermore, when PD patients discontinue use of long-term benzodiazepines, they may develop severe (rebound) anxiety (Rickels and Schweizer 1993). Plasma (Goddard et al 1996; Roy-Byrne et al 1992) and cerebrospinal fluid (CSF) levels of GABA may be within the normal range in PD patients; however, low baseline CSF GABA correlated with a poor treatment response to alprazolam and imipramine (Rimon et al 1995). In addition, PD patients, particularly those with a family history of anxiety or mood disorders, may exhibit reduced total occipital cortex GABA levels (Goddard et al 2001b; Kent et al 2002).

Thus, there is preclinical and clinical evidence for altered GABA function in PD. The findings of animal studies cited above suggest that the loss or reduction of GABA inhibitory tone in brain regions involved in the generation of panic-like responses may underlie panic attacks. The following section describes changes in $GABA_A$ receptors that may lead to impaired GABAergic transmission in PD.

1.2.2.4. $GABA_A$ receptor dysregulation in PD

Several lines of evidence implicate abnormal $GABA_A$ receptor function in PD. First, PD patients, compared with healthy controls, showed reduced sensitivity to

benzodiazepine agonists. High-potency benzodiazepines, such as clonazepam or alprazolam, or low-potency benzodiazepines in higher doses, are usually required to suppress panic attacks in PD (Dunner et al 1986; Kasper and Resinger 2001). PD patients were less sensitive than controls to the slowing effect of diazepam on saccadic eye movements (a valid test of benzodiazepine sensitivity) (Roy-Byrne et al 1996; Roy-Byrne et al 1990). PD patients also exhibited smaller reductions in arterial plasma catecholamine levels and heart rate in response to diazepam (Roy-Byrne et al 1989).

Second, regional reductions in benzodiazepine site binding in PD were observed in several SPECT studies in frontal (Kaschka et al 1995; Kuikka et al 1995; Schlegel et al 1994), temporal (Kaschka et al 1995; Schlegel et al 1994) and occipital (Schlegel et al 1994) cortical areas, and in left hippocampus and precuneus (Bremner et al 2000). Notably, in the latter study, a relative decrease in benzodiazepine site binding was observed in prefrontal cortex in patients who had a panic attack during the time of the scan, suggesting a link between receptors in this region and the state anxiety level. One PET study found a global cortical reduction in benzodiazepine site binding, with the greatest magnitude in right orbitofrontal and insular cortices (Malizia et al 1998). Except for two studies (Bremner et al 2000; Malizia et al 1998), these investigations were limited by the use of PD patients on medication or with comorbid psychiatric illness, or by the use of diseased patients (with epilepsy or dysthymia) as control groups. Despite these methodological limitations, reduced benzodiazepine site binding seems to be a reliable finding in patients with PD. This is consistent with reports of reduced benzodiazepine binding primarily in prefrontal cortex and hippocampus in rats exposed to inescapable stress (Drugan et al 1989; Medina et al 1983; Weizman and Gavish 1989),

or in genetically modified rats displaying anxious behavior (Crestani et al 1999; Robertson et al 1978). Prefrontal cortex and hippocampus are part of a neural network that is theoretically implicated in PD, and may play a role in cognitive aspects of panic (i.e., misinterpretation of sensory information) and phobic avoidance (i.e., fear of the context of previous panic attacks), respectively (Gorman et al 2000; Kent et al 2002).

Third, flumazenil has repeatedly provoked panic attacks in PD patients, but not controls (Nutt et al 1990; Woods et al 1991), although this panicogenic effect was not replicated on two occasions (Strohle et al 1999; Strohle et al 1998). These findings will be described in detail in the second paper of this thesis (chapter 3). Flumazenil, a water-soluble imidazobenzodiazepine, competitively antagonizes the effects of benzodiazepines at the most abundant $GABA_A$ receptor subtypes (Hevers and Luddens 1998; Sieghart 1995), and is used in clinical practice for reversal or termination of benzodiazepine-induced CNS depression (sedation or general anaesthesia) and for the management of benzodiazepine overdose (McCloy 1995; Whitwam and Amrein 1995). In healthy volunteers, flumazenil has shown no, or minimal intrinsic activity (Darragh et al 1983; Higgitt et al 1986; Schopf et al 1984).

Interpretation of the above independent observations implicating $GABA_A$ receptors in PD is rather difficult, because they may not reflect a single abnormality. There are several hypotheses about the mechanism of the panicogenic action of flumazenil, some of which may accommodate the other findings suggesting $GABA_A$ receptor dysfunction in PD.

1.2.2.5. Panicogenic action of flumazenil

Abnormal responses to flumazenil in PD have helped to distinguish some previous hypotheses about the changes in the GABA system in this disorder (Malizia et al 1995). It has been suggested that PD patients have an excess of an endogenous benzodiazepine inverse agonist, produced continuously or during panic attacks. The existence of endogenous benzodiazepine site ligands has been investigated to clarify whether these sites are physiological receptors, or only drug acceptors. Diazepam binding inhibitor, its metabolites, and a monoamine oxidase inhibitor, tribulin, were extracted from the brains of animals and humans and were found to have inverse agonist properties (Corda et al 1984; Elsworth et al 1986). However, their significance in anxiety disorders remains uncertain. In addition, if there were increased availability of an inverse agonist in PD, flumazenil would block its effect and reduce anxiety or prevent panic attacks. Another hypothesis postulated that PD patients lack an endogenous agonist normally present in the CNS of healthy individuals and released continuously, or in response to stress. This possibility was supported by the detection of benzodiazepine-like immunoreactivity post-mortem in the brains of individuals who died before the first synthesis of benzodiazepines (Sangameswaran et al 1986). Benzodiazepines could also be stored in the brain after eating plants that produce them (Nutt and Malizia 2001). In this case, however, flumazenil would provoke anxiety in healthy controls by blocking the agonist tone. In addition, in healthy controls, pretreatment with flumazenil did not increase anxiety induced by CCK tetrapeptide (CCK-4), a validated panicogenic agent (Bradwejn et al 1994a), which does not support the idea of stress-induced endogenous benzodiazepine agonist secretion.

After refuting the above hypotheses, Nutt and coworkers (1990) suggested two explanations for the panicogenic action of flumazenil. Flumazenil could block a putative endogenous agonist produced in PD patients in an attempt to reduce anxiety caused by primary abnormalities in another system or systems. Although this compensatory mechanism would not be sufficient to prevent spontaneous panic attacks, its blockade could trigger them (Nutt et al 1990). The presence of an endogenous ligand that competes for receptor occupancy could explain the reduced benzodiazepine receptor binding in PD (Bremner et al 2000; Kaschka et al 1995; Kuikka et al 1995; Malizia et al 1998; Schlegel et al 1994).

The other possible explanation is a shift of the benzodiazepine site spectrum of action (i.e., its set point) towards inverse agonism in PD (Figure 1.1) (Nutt et al 1990). According to this "set point" hypothesis, full agonists would function as partial agonists, partial inverse agonists as full inverse agonists and antagonists as partial inverse agonists. This would explain both panic responses to flumazenil and the reduced sensitivity to benzodiazepines in PD patients (Roy-Byrne et al 1996; Roy-Byrne et al 1990). A similar shift in the efficacy of benzodiazepine site ligands was observed in mice that had developed tolerance to benzodiazepine agonists (Little et al 1988).

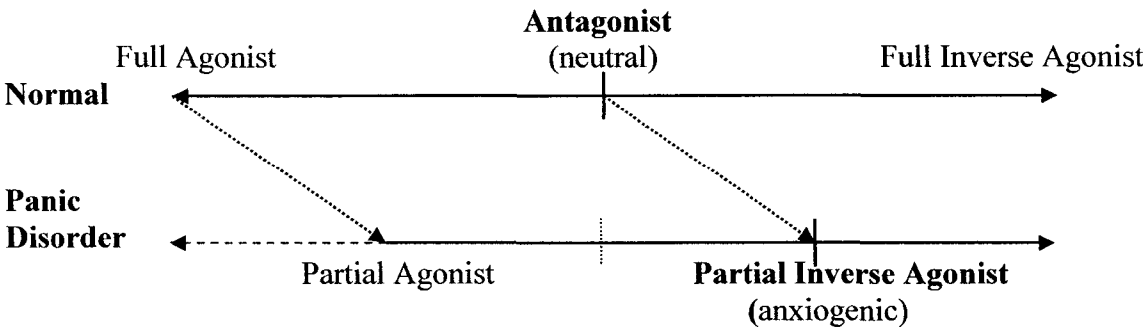

Figure 1.1. Proposed shift in the activity of benzodiazepine site ligands towards inverse agonism.

1.2.2.6. Molecular basis and etiology of $GABA_A$ receptor dysregulation

The above set point hypothesis has become rather influential and has been tested in several studies (Maddock 1998; Strohle et al 1999; Strohle et al 1998; Woods et al 1991). However, the molecular basis of the proposed shift in the benzodiazepine site pharmacology remains unclear. In benzodiazepine-tolerant mice (Little et al 1988) this phenomenon may be caused by altered receptor subunit composition, since chronic exposure to benzodiazepines results in changes in $GABA_A$ receptor subunit expression. Although the effects of benzodiazepines on subunit isoform mRNA levels may vary with dose regimens, measurement techniques and brain regions (Holt 1996), reduced expression of the α_1 or γ_2 subunits in rat cortex has been the most consistent finding (Heninger et al 1990; Holt et al 1996; Kang and Miller 1991; Primus and Gallager 1992). It has been suggested that during long-term benzodiazepine exposure the transcription of genes encoding these subunits, clustered on chromosome 5, is inhibited, while the expression of receptors containing subunits encoded by genes clustered on chromosome 4 (namely the $\alpha4$ and $\beta1$ subunits) and 15 (the $\alpha5$ and $\gamma3$ subunits) is increased (Holt et al 1996). If chronic benzodiazepine treatment in humans leads to analogous molecular changes that would manifest as the receptor shift, and if rebound anxiety after benzodiazepine discontinuation (Rickels et al., 1993) is related to this shift, then similar changes may occur in PD.

At this stage these conclusions are highly speculative. However, a significant inverse agonist activity of flumazenil has recently been reported at a recombinant $\alpha4\beta1\delta$ $GABA_A$ receptor subtype (Dunn et al 2003). Although the gene encoding the δ subunit on the human chromosome 1 is not clustered with genes for the $\alpha4$ and $\beta1$ subunits on

chromosome 4, there is now evidence that the expression of the δ and α4 subunits, which are often present in the same receptor, is coordinated (Korpi et al 2002; Peng et al 2002). Thus, panic reactions to flumazenil in PD patients might occur due to increased expression of this receptor subtype.

Both genetic and environmental factors may contribute to the development of $GABA_A$ receptor changes in PD. A new approach to investigate the effects of specific subunits on brain function is the generation of mice that fail to express certain subunits, or in which the subunit is rendered insensitive to neurotransmitters or pharmacological agents by point mutations that affect their binding. For example, mice heterozygous for the γ2-subunit (showing ~50% reduction in γ2 levels) exhibited anxiety-like behavior in several models of anxiety (Crestani et al 1999), presumably due to a decreased receptor clustering resulting in altered synaptic GABAergic transmission. These mice also showed decreased flumazenil binding, particularly in cerebral cortex and hippocampus, which are areas of reduced benzodiazepine binding in patients with PD (Bremner et al 2000; Malizia et al 1998). These findings indicate that such mutants may represent a genetically defined model of human anxiety (Crestani et al 1999) and allow for speculation that an inherited defect of the γ2 subunit encoding gene represents a risk factor for developing PD.

Receptor subunit expression may also be affected by environmental stimuli, including stress-related events (Caldji et al 2000; Kang et al 1991; Montpied et al 1993), which often precede PD (Roy-Byrne et al 1986a; Safren et al 2002), chronic alcohol consumption (Devaud et al 1997), or by fluctuations in the concentrations of endogenous neuroactive steroids, some of which are positive $GABA_A$ receptor modulators (Smith et

al 1998; Concas et al 1999; Gulinello et al 2002). In fact, a disturbed homeostasis of these steroids could alter GABAergic transmission and be a risk factor for the development of anxiety disorders regardless of changes in the receptor composition. Irrespective of the specific structural changes in $GABA_A$ receptors in PD, altered receptors could be differentially modulated by physiological fluctuations of the endogenous steroids during different phases of the menstrual cycle, pregnancy or postpartum period (Consas et al 1999), which might contribute to the variable severity of the disease during these conditions (Cowley and Roy-Byrne 1989).

1.2.2.7. Implications for treatment

Understanding the nature of $GABA_A$ receptor abnormalities in PD may result in the development of antipanic drugs that will selectively target the subsets of dysfunctional receptors. Meanwhile, studies have tried to find GABAergic anxiolytics that are devoid of the undesirable side effects of benzodiazepines. Compounds enhancing GABA function by acting as partial agonists at the benzodiazepine site (Sandford et al 2001), or by blocking GABA transaminase (GABA-catabolizing enzyme) (Zwanzger et al 2001a) or GABA reuptake (Zwanzger et al 2001c) recently showed promising antipanic effects without dependency or withdrawal problems in initial studies. However, the future use of vigabatrin, an irreversible GABA-transaminase inhibitor, in PD is uncertain, since it has been associated with increased occurrence of depression and psychosis (Levinson and Devinsky 1999) and persistent visual field defects (Nousiainen et al 2001) in patients with epilepsy. Gene-manipulation studies revealed that actions of benzodiazepines are receptor subtype-specific. *In vivo* "knock-in" point mutations of

different isoforms of the α subunit, making the mutated receptor benzodiazepine-insensitive, demonstrated that the sedative, amnestic and some anticonvulsant effects of benzodiazepines are mediated by α1-containing receptors in mice, whereas the anxiolytic effects are mediated by α2-containing receptors. These findings offer new strategies in drug development. Benzodiazepine site ligands with subtype selectivity are now being investigated in preclinical and clinical studies (for a review see Mohler et al 2002).

1.2.3. CCK system in PD

1.2.3.1. CCK and CCK receptors

CCK is a peptide originally found in the gastrointestinal tract, where it induces gallbladder contraction and stimulates pancreatic secretion. It was discovered subsequently that CCK is the most abundant neuropeptide in the mammalian brain (Rehfeld et al 1985; Vanderhaeghen et al 1975). High concentrations of CCK were found in the cortex, hippocampus, amygdala, brainstem and striatum (Emson et al 1982). CCK exists in several molecular forms, all of which share the terminal Trp-Met-Asp-Phe-NH_2 amino acid sequence. The most abundant form in the brain is the sulfated octapeptide (CCK-8S), followed by the desulfated CCK-8 and the smaller fragments CCK-5 (pentagastrin) and CCK-4 (Rehfeld and Nielsen 1995). Two main types of CCK receptors have been identified. CCK-1 receptors (CCK-1Rs; formerly CCK-A receptors) are localized predominantly in the gastrointestinal tract, and to a lesser extent in restricted brain regions. These receptors have a high affinity for CCK-8S and a lower affinity for CCK-8, CCK-5 and CCK-4. CCK-2 receptors (CCK-2Rs; formerly CCK-B) are widely distributed in the CNS, mainly in the cortex and limbic structures, although they have

been found also in the stomach, lymphocytes and monocytes. CCK-2Rs have a high affinity for all the above CCK forms (for reviews see Bradwejn and Koszycki 2001; van Megen et al 1996).

1.2.3.2. CCK, anxiety and panic

The role of CCK in anxiety was first indicated by electrophysiological experiments, which showed that benzodiazepine agonists could attenuate CCK-8S-induced excitation of rat hippocampal neurons (Bradwejn and de Montigny 1984). Basic researchers later found that CCK-4 or pentagastrin induced anxiety-like behavior in rodents (Harro and Vasar 1991; Harro et al 1993; Singh et al 1991) and monkeys (Ervin et al 1991).

In a series of human studies, CCK-4 showed replicable, dose-dependent panicogenic effects, which were significantly stronger in PD patients than in healthy controls, or in patients with other anxiety disorders (Bradwejn et al 1990; Bradwejn et al 1992a; Bradwejn et al 1992b). The CCK-4 induced response was described by PD patients as identical or similar to their naturally occurring panic attacks. Systematic evaluation revealed that CCK-4 meets all the criteria of a panicogenic agent and that CCK-4 challenge may be used as a model of panic attacks (Bradwejn and Koszycki 1994). Similar findings were later reported with pentagastrin (Abelson and Nesse 1994; Abelson et al 1994; van Megen et al 1994).

1.2.3.3. Panicogenic action of CCK-2R agonists

The mechanism of anxiogenic action of CCK-2R agonists remains unclear. Observations that, in animals, CCK-2R antagonists showed anxiolytic effects and abolished CCK-4- or pentagastrin-induced anxiety-related behavior (van Megen et al 1996) suggested that the anxiogenic effect of CCK fragments is mediated via CCK-2Rs.

The site of action of the agents is also not clear, partly because it is not certain how well the peptides penetrate the blood-brain barrier. Actions at CCK-2Rs in brainstem regions, such as the nucleus tractus solitarius, medulla and parabrachial nucleus, have been suggested (Bradwejn and Koszycki 2001). These structures are not fully protected by the blood-brain barrier, and are involved in the control of respiratory and cardiovascular systems, both of which are activated by CCK (Abelson and Nesse 1994; Bradwejn et al 1992a; Bradwejn et al 1998). These structures have a connection with the locus coeruleus, which is activated during states of fear and anxiety and has been implicated in panic attacks (Charney et al 1990). Emotional responses to CCK-2R agonists could result from the direct or indirect activation of brain stem structures and, consequently, higher CNS regions that are connected with the brainstem (Bradwejn and Koszycki 2001).

More specific information about the neuroanatomical correlates of CCK-4-induced anxiety came from PET studies. One study evaluated brain activity at two time points after CCK-4 administration to healthy volunteers. The early scan corresponding with the onset of panic symptoms showed cerebral blood flow (CBF) activation of the brainstem-hypothalamic region, which was followed by claustrum-insular activation at the peak expression of panic symptoms (Javanmard et al 1999). This finding supports the

initial activation of the brainstem structures. This hypothalamic activation may be related to hormonal responses to CCK-2R agonists (see section 1.2.4.3; Abelson et al 1994; Koszycki et al 1998; Shlik et al 1997). The results are also consistent with CCK-4-induced CBF activation in the claustrum-insular region reported earlier (Benkelfat et al 1995). However, since the insular cortex is an interoceptive area activated during a variety of emotions (Craig 2002), its activation during CCK-4 challenge may be a consequence of peripheral sensations associated with panic, rather than a specific cause of CCK-4-induced anxiety.

Peripheral actions of CCK-2R agonists at stomach gastrin receptors, or CCK-2Rs on the vagus nerve, with secondary activation of central sites may also contribute to anxiety (McCann et al 1994).

If PD is associated with a dysregulation of the CCK system, its nature has yet to be determined. It is possible that panic responses to CCK-4 and pentagastrin are caused by increased sensitivity or numbers of CCK-2Rs. Increased intracellular signaling in CCK neurons may also take place; CCK-4 induced intracellular calcium mobilization in T cells was increased in untreated PD patients relative to treated PD, depressed or schizophrenic patients, or healthy controls (Akiyoshi et al 1997; Akiyoshi et al 1996). Further, PD patients exhibited lower CSF and lymphocyte CCK-8S concentrations compared to healthy volunteers, which may indicate altered CCK turnover (Brambilla et al 1993; Lydiard et al 1992). Some recent studies have shown associations between PD and CCK-2R gene polymorphisms, indicating that variations in gene expression could be

a risk factor for PD (Hattori et al 2001; Kennedy et al 1999). However, not all studies have found associations (Hamilton et al 2001).

In the future, the use of CCK-2R radioligands in PET studies may help to clarify the role of CCK system in the pathophysiology of PD.

1.2.3.4. Implications for treatment

The results of clinical studies assessing the anxiolytic effects of CCK-2 antagonists have been less consistent than those of animal studies (van Megen et al 1996). The CCK-2R antagonist L-365,260 reversed the anxiogenic effect of pentagastrin in healthy volunteers (Lines et al 1995) and attenuated the panicogenic effect of CCK-4 in PD patients (Bradwejn et al 1994b). Another antagonist, CI-988, modestly reduced CCK4-induced panic attacks in healthy volunteers (Bradwejn et al 1995), but was no more effective than placebo in blocking CCK4-induced symptoms in PD patients (van Megen et al 1997). Both drugs appeared to be ineffective in treatment of PD (Kramer et al 1995; Pande et al 1999), but the inconsistent effects of these drugs may be related to their low bioavailability (e.g., low gastrointestinal absorption or a poor ability to cross the blood-brain barrier). The development of CCK-2R antagonists with superior pharmacokinetic properties will clarify their therapeutic potential as antipanic drugs.

The assessment of neuroendocrine functioning is another research strategy used in biological research on PD. Findings on the hypothalamic-pituitary-adrenal (HPA) axis activity in PD are relevant to the major project in this thesis and are briefly reviewed in the following section, with the focus on its activity during panic attacks.

1.2.4. HPA axis in PD

1.2.4.1. HPA axis - physiology

Activation of the HPA axis is a part of a general adaptive response to stress-related stimuli, when an organism attempts to counteract the imbalance caused by stressors and restore homeostasis. Corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), produced in the parvocellular component of the paraventricular nucleus (PVN) of the hypothalamus, promote the release of adrenocorticotropin hormone (ACTH) in the anterior pituitary, which, in turn, leads to the release of glucocorticoids (cortisol in humans) from the adrenal cortex. The secreted hormones exert a negative feedback, by acting on receptors in the hippocampus, hypothalamus or in the pituitary glandular cells (Chrousos and Gold 1992).

The precise manner in which glucocorticoids help adaptation to stress is not fully understood. It is certain, however, that if the stress-related stimulus is excessive or prolonged (in chronic stress) or if the stress response is not terminated at the end of the stress exposure, the previously protective mechanism then becomes maladaptive. Prolonged hypersecretion of CRH may contribute to the pathogenesis of disorders characterized by anxiety and/or depression (Chrousos and Gold 1992). Dysregulation of the HPA axis has been recognized in patients with stress-related conditions, such as major depression or posttraumatic stress disorder (PTSD) (for reviews see Holsboer 1999; Yehuda 2002). Dysregulation of the HPA axis activity may play a role in the pathophysiology of PD, but the supporting evidence is less consistent.

1.2.4.2. HPA activity under baseline conditions and during standardized tests

When detected, HPA abnormalities in PD tend to be similar to those seen in depression (Holsboer 1999). Some, but not all, studies have reported elevated basal cortisol levels in plasma, saliva or urine in PD patients (for a review see Wedekind et al 2000). In studies using standardized tests, some PD patients showed a non-suppression of cortisol levels by a low dose of the synthetic steroid dexamethasone (dexamethasone suppression test; DST) and blunted ACTH responses to CRH (CRH challenge test), (Coryell et al 1991; Holsboer et al 1987; Roy-Byrne et al 1986b), but some did not (Brambilla et al 1992; Curtis et al 1997; Curtis et al 1982; Rapaport et al 1989). During a combined dexamethasone – CRH challenge, PD patients had higher ACTH and cortisol levels than healthy controls, but lower levels than depressed patients (Schreiber et al 1996).

These inconsistent results may be attributed to variations in study protocols that could affect stress hormone levels, such as the time of blood sampling for stress hormone analysis (i.e., during different phases of the circadian rhythm), fractions of cortisol measured (bound or unbound to corticoid-binding globulin), emotional states of patients during experimental conditions, and sizes and clinical characteristics of samples. The latter variable refers to possible current or past depression or subclinical depressive symptoms in PD patients in some studies (Holsboer et al 1987; Roy-Byrne et al 1986b) and to the severity of PD and/or agoraphobia, which have been found to affect the degree of HPA dysfunction (Abelson and Curtis 1996; Coryell et al 1991; Wedekind et al 2000; Westberg et al 1991). Finally, in some patients, the baseline HPA hyperactivity may be related to anticipatory (generalized) anxiety and situational attacks that may develop later

in the course of PD, rather than to PD *per se* (Klein 1993).

1.2.4.3. HPA activity during panic attacks

Inconsistencies in HPA responsiveness have been observed during challenge-induced panic attacks. Sodium lactate infusion (Hollander et al 1989; Liebowitz et al 1985), 5% CO_2 inhalation (Sinha et al 1999; Woods et al 1988) and doxapram (Abelson et al 1996b) did not increase plasma cortisol. In contrast, CCK-2R agonists (Abelson et al 1994; Koszycki et al 1998; Shlik et al 1997), yohimbine (Charney et al 1987; Charney et al 1992), mCPP (Klein et al 1991), caffeine (Charney et al 1985) and the benzodiazepine partial inverse agonist FG7142 (Dorow et al 1983) elevated cortisol levels.

Hormonal changes that accompany naturally occurring panic attacks, especially spontaneous attacks, are poorly documented. Plasma cortisol was increased in PD patients during attacks upon exposure to fearful situations (Woods et al 1987), but these findings could have been affected by novelty, nonspecific experimental stress or venipuncture stress. Spontaneous panic attacks were not accompanied by a significant increase in plasma cortisol, but patients stayed in bed throughout this 36-hour study, which could have reduced their anxiety (Cameron et al 1987). More recently, a subtle but significant increase in salivary cortisol was reported during spontaneous panic attacks (Bandelow et al 2000). Salivary cortisol correlates highly with the biologically active free (unbound to corticoid-binding globulin) plasma cortisol, and is a better indicator of adrenal cortical secretion than plasma cortisol (Vining et al 1983). In addition, the collection of samples is non-invasive and can be done by patients in naturalistic settings.

The lack of HPA activation in response to two "classic panicogens", sodium

lactate and carbon dioxide, and during some natural panic attacks prompted a suggestion that spontaneous panic has a pathophysiology distinct from natural fear and is not associated with activation of the HPA axis. In Klein's view (Klein 1993), only the pharmacological agents that cause marked dyspnea (which he sees as the key component of panic, see section 3.1 for his suffocation alarm hypothesis) without HPA activation, are the "true panicogens". Agents that do activate the HPA system, with (caffeine, CCK-2R agonists) or without (yohimbine, m-CPP, fenfluramine, flumazenil) marked respiratory distress (Sinha et al 1999) elicit states resembling natural (situational) fear, stress or pain, rather than spontaneous panic (Klein 1993).

The lack of HPA activation during lactate-induced panic attacks may be caused by the release of atrial natriuretic hormone, which antagonizes CRH-induced ACTH release (Kellner et al 1995), but it is unclear whether CRH is also released during CO_2-induced or spontaneous panic. However, studies that collected samples only within 20 minutes after a challenge administration (Coplan et al 2002; Sinha et al 1999) may not have detected a delayed cortisol response, especially if measured in saliva (Aardal-Eriksson et al 1998). In addition, inhalation of 35% CO_2 was recently reported to significantly increase cortisol levels in healthy volunteers, suggesting that a higher CO_2 concentration may be necessary for neuroendocrine activation (Argyropoulos et al 2002). On the other hand, increases in cortisol in PD patients following panicogens often do not correlate with behavioral changes, and may occur in healthy controls as well (Abelson et al 1994; Charney et al 1985; Charney et al 1992). This indicates that the hormonal and panicogenic effects of a challenge may be independent.

1.2.4.4. The nature of HPA dysfunction in PD

The cause of HPA axis abnormalities found in some PD patients is not well understood. There is evidence that the abnormalities in depression are linked to CRH overdrive, downregulation of glucocorticoid receptors and reduced negative feedback (Curtis et al 1997; Holsboer 1999), although the primary defect remains to be determined. It is not clear whether this model is applicable to PD. Although two studies that found a blunted ACTH response to CRH related these findings to desensitization of pituitary CRH receptors secondary to episodic increases of CRH during panic (Roy-Byrne et al 1986; Holsboer et al 1987), there is no direct evidence for CRH overdrive in PD. For example, unlike in depressed patients, in PD patients CSF levels of CRH are not elevated (Fossey et al 1996; Jolkkonen et al 1993). Some researchers suggested that the attenuated ACTH response could rather be caused by an enhanced glucocorticoid negative feedback, as described in PTSD (Kellner and Yehuda 1999). This suggestion was based on the lack of consistent HPA activation during panic (e.g., Liebowitz et al 1985; Sinha et al 1999, Cameron et al 1987), and a trend towards hypersuppression, rather than non-suppression, of cortisol in DST in two studies with PD patients (Poland et al 1985; Carson et al 1988). This idea remains highly speculative, since numerous studies have reported the opposite results (see above). Thus, it has to be clarified whether the HPA dysfunction in PD resembles that in other disorders, or has a distinct nature.

Future research, using uniform study protocols, should focus on HPA reactivity during spontaneous panic and on the role of atrial natriuretic hormone in the feedback regulation of the axis.

1.3. Cognitive Aspects of PD

Other research approaches to the etiology of PD emphasize the importance of cognitive factors. Most cognitive models of PD are based on the patients' tendency to process somatic sensations in a threatening way and associate them with disastrous outcomes (Clark 1993; Rapee 1995; McNally 2002).

1.3.1. Clark's cognitive theory

A cognitive theory formulated by Clark (1986) attracted considerable attention. According to Clark, panic is a result of patients' catastrophic misinterpretation of somatic sensations (Clark 1986). Palpitations, for example, are perceived as a heart attack, dizziness as a brain tumor, feelings of derealization as insanity, etc. These somatic sensations can be triggered externally by a feared situation, or physical activity, or internally by images or changes in internal environment. These sensations are perceived as a threat, which leads to apprehension and further somatic sensations. Catastrophic misinterpretation of these sensations results in a further increase in apprehension, and this vicious circle escalates into a panic attack (Clark 1986).

Several studies confirmed that PD patients have catastrophic thoughts during panic attacks (Ottaviani and Beck 1987; Westling and Ost 1993; Rachman et al 1987) and that these thoughts distinguish them from patients with other anxiety disorders or healthy controls (Hibbert 1984; Taylor et al 1992; Clark et al 1997).

The theory further holds that catastrophic misinterpretation also underlies panic provoked by challenges (Clark 1986; Clark 1993). Catastrophic cognitions were reported by PD patients during challenge-induced panic (Rapee et al 1986; Rapee et al 1992), even

when the differences in objective physiological measures were not significant between patients and normal controls (Rapee et al 1992; Asmundson et al 1994). If the theory is accurate, pre-challenge instructional manipulations designed to reduce patients' tendency to misinterpret should reduce panic responses to the challenges. Prior to a single inhalation of 50% CO_2/50% oxygen, some PD subjects were informed about all the possible sensations that the gas could cause, while the control PD group was given minimal information about the procedure. As the cognitive theory predicted, the informed group experienced less catastrophic cognitions and significantly fewer panic attacks than the control group. However, the results were difficult to interpret, since the informed group might have breathed less gas (Rapee et al 1986). In a study using a similar design but a controlled amount of inhaled CO_2, the instructional manipulation did not modify the rate of panic attacks (Papp et al 1995). Nevertheless, a detailed description of somatic symptoms to be expected during a challenge test, with an emphasis on their harmlessness, reduced panic responses to sodium lactate (in Clark 1993), doxapram (Abelson et al 1996b) and epinephrine (Veltman et al 1998). Conversely, when possible catastrophic misinterpretation was activated by a false heart rate feedback, PD patients experienced increased anxiety and physiological arousal relative to controls (Ehlers et al 1988).

Cognitive researchers also identified behavioral patterns that may explain the persistence of catastrophic beliefs in patients who have repeatedly "survived" many panic attacks (Clark 1999). Patients often engage in safety-seeking behaviors, which they believe will prevent the feared catastrophe. For example, feeling a rapid heart beat, they sit or lie down and believe that this maneuver is the only reason why they did not die. The avoidance of activities associated with physical sensations, like exercise, is another

strategy that prevents them from recognizing that the symptoms are benign. PD patients who dropped their safety behavior upon exposure to a feared situation experienced significantly less anxiety and negative beliefs during a subsequent exposure to the same situation than patients who did not (Salkovskis et al 1999).

Another factor that may play a role in the maintenance of PD is patients' enhanced awareness and monitoring of their internal environment. PD patients were reported to be more accurate than controls at detecting their heart rate and changes in airway resistance than controls (Ehlers and Breuer 1992; Ehlers and Breuer 1996), but other studies failed to confirm this superior accuracy (Asmundson et al 1993; Rapee 1994).

1.3.1.1. Critiques of the cognitive theory

Clark's cognitive theory gave rise to at least five main criticisms. First, numerous studies that reported the occurrence of catastrophic thoughts in PD patients used interviews which inquired about panic experience retrospectively (Ottaviani and Beck 1987; Hibbert 1984). In other studies, patients' answers were based on a hypothetical situation, such as "You feel discomfort in your chest area. Why?" (McNally and Foa 1987). It is possible that under these conditions PD patients may exaggerate the intensity of their symptoms or their negative interpretations (Hayward et al 2000).

Second, catastrophic misinterpretation may not be necessary for triggering panic. Not all natural or challenge-induced panic attacks were associated with frightening thoughts (Papp et al 1995; Rachman et al 1987; Aronson et al 1989).

Third, studies often did not report whether the occurrence of catastrophic thoughts preceded, accompanied or followed panic experience. In other words, they did not indicate whether the thoughts were a cause (consistent with the cognitive theory), a symptom (e.g., fear of losing control or fear of dying) or a consequence of a panic attack. Studies that did examine the order of events reported inconsistent findings (Ley 1985; Wolpe and Rowan 1988).

Fourth, the theory predicts that conditions or drugs that produce somatic sensations should trigger panic. However, PD patients suffering from pheochromocytoma, a tumor of the adrenal medulla, which due to paroxysmal production of catecholamines causes symptoms of elevated autonomic arousal, did not experience panic in response to this episodic catecholamine secretion (Starkman et al 1985). Further, thyrotropin-releasing hormone produced some panic-related somatic symptoms in healthy volunteers, yet did not trigger panic attacks in PD patients (Stein and Uhde 1990; Stein and Uhde 1991).

Fifth, if panic is caused by misinterpretations, it is difficult to explain nocturnal attacks, especially given that they occur during non-rapid eye movement periods, when patients are less likely to be dreaming. Clark argued that the misinterpretations need not be conscious (Clark 1988). This, however, threatens the falsifiability of the theory, as it is difficult to find evidence against this statement.

Although these arguments suggest that Clark's theory may not be applicable to all panic attacks, it remains extremely influential.

1.3.2. Anxiety sensitivity

Another cognitive factor that may play a role in anxiety disorders (PD in particular) is anxiety sensitivity, which refers to the fear of anxiety-related symptoms, based on beliefs that these symptoms have harmful somatic, psychological, or social consequences. For example, an individual with high anxiety sensitivity believes that palpitations could lead to a heart attack, whereas an individual with low anxiety sensitivity perceives them as merely unpleasant (McNally 2002).

The anxiety sensitivity concept is distinct from other cognitive explanations of panic. Individuals with high anxiety sensitivity do not necessarily catastrophically misinterpret physical sensations, although they may be prone to such misinterpretations; they may know very well that the symptoms are caused by anxiety, but still believe in their dangerous consequences. Further, anxiety sensitivity is not a conditioned response to physical sensations, but a pre-existing belief about their harmfulness; the experience of a panic attack is therefore not necessary for the development of high anxiety sensitivity (McNally 1994). There has been a discussion about whether anxiety sensitivity is simply a measure of trait anxiety (Lilienfeld et al 1989), which is a tendency to respond fearfully to stressors in general. McNally argues that "a person with high trait anxiety need not have an additional fear of anxiety symptoms themselves" (McNally 1994, p.116).

The most widely used measure of anxiety sensitivity is the Anxiety Sensitivity Index (ASI), which is a valid and reliable 16-item self-report inventory assessing beliefs about the harmfulness of anxiety symptoms and fears of these symptoms. The ASI items are related to physical concerns, mental incapacitation concerns and social concerns (McNally 2002).

1.3.2.1. Anxiety sensitivity in PD

To test the association between anxiety sensitivity level and the incidence of panic attacks, in several studies the ASI was administered to college students, whose anxiety levels were subsequently classified as high, medium or low. The rates of spontaneous and situational panic attacks were significantly higher among students with high ASI scores, compared with students with medium or low scores (Donnell and McNally 1990; Cox et al 1991; Asmundson and Norton 1993). In these studies 40-70% of students with high anxiety sensitivity never experienced a panic attack, confirming that the fear of anxiety can be acquired elsewhere.

PD patients were found to have significantly higher anxiety sensitivity than normal controls (Reiss et al 1986; Rapee et al 1992) or patients with other anxiety disorders, except for PTSD, where a trend towards significance was found (Taylor et al 1992). In the latter study, PD patients did not differ from other anxiety disorder groups in their trait anxiety, which confirms that elevated anxiety sensitivity is not simply a concomitant of non-specific stress (Taylor et al 1992).

These reports on non-clinical and clinical samples prompted an hypothesis that high anxiety sensitivity may be a risk factor for the development of PD. High ASI scores predicted the development of panic attacks in several longitudinal studies with non-clinical subjects (Ehlers 1995; Harrington et al 1996; Plehn and Peterson 2002; Schmidt et al 1999).

The origins of high anxiety sensitivity are unclear. A twin study estimated the effects of genetic and environmental factors on anxiety sensitivity by gender and found that the ASI scores are likely to be heritable only in women (Jang et al 1999), but this

was not replicated in an independent sample (Stein MB, personal communication). Heightened anxiety sensitivity may also result from childhood experience of parental concerns about anxiety-related symptoms, or parental reinforcement of child's fear of these symptoms (Stewart et al 2001). However, twin studies have not provided convincing evidence that shared environmental factors (e.g., family environment) influence the level of anxiety sensitivity (Jang et al 1999; Stein et al 1999).

It has been suggested that anxiety sensitivity may underlie sensitivity to panicogenic challenges. In PD patients, baseline measures of anxiety sensitivity appeared to predict panic responses in some tests (Rapee et al 1992; Rassovsky et al 2000; Shipherd et al 2001), but not others (Koszycki and Bradwejn 2001; Koszycki et al 1996; Veltman et al 1998). Since a positive correlation between anxiety sensitivity and challenge-induced panic responses does not necessarily indicate any causal relationship, but merely the association between high anxiety sensitivity and PD, challenge studies have been done with healthy volunteers with high scores on the ASI. As with PD patients, these subjects (including those who never experienced a panic attack) showed exaggerated anxiety responses in some, but not all, challenge tests (Holloway and McNally 1987; McNally and Eke 1996; Rapee and Medoro 1994; Aluoja et al 1997; Koszycki et al 1993). The role of anxiety sensitivity in challenge studies will be discussed in more detail in chapter 4.

1.3.3. Other cognitive factors in PD

Studies have used experimental manipulations of several cognitive factors to determine their influence on responses to pharmacological challenges and on the

occurrence of panic outside the laboratory. The manipulated variables included patients' expectancy of a panic attack (Margraf et al 1989), their perceived safety (Rapee et al 1991; Carter et al 1995), or their perceived control (Sanderson et al 1989; Abelson et al 1996b; Veltman et al 1998). These findings will also be discussed in chapter 4.

1.3.4. Implications for treatment

Cognitive models have provided a theoretical rationale for psychological treatments of PD, of which a cognitive behavioral therapy (CBT) appears to be the most effective (Barlow and Lehman 1996; Goldberg 1998). Clinical improvement during CBT has been associated with reduction of both ASI scores and the strength of catastrophic beliefs (Telch et al 1993; Bakker et al 2002). Although different programs emphasize different techniques (Clark 1989; Barlow and Cerny 1988), CBT essentially consists of two phases. The first, cognitive phase, includes patients' identification of their negative beliefs about bodily sensations, after which they are helped to substitute these beliefs with more benign, rational interpretations (cognitive restructuring). During the second, behavioral phase, the patients test the validity of these alternative interpretations in different experiments, which include exposure to somatic sensations induced, for example, by voluntary hyperventilation. During the procedure the patients are encouraged not to engage in avoidance behavior. These techniques demonstrate the real causes of patients' symptoms and enable disconfirmation of their negative thoughts. The behavioral phase may also include breathing retraining to control hyperventilation during panic attacks, and relaxation training (Goldberg 1998).

After the start of pharmacological treatment for initial symptom control, the addition of CBT may help the patients to stay on a lower dose of medication, or increase the rate of its successful discontinuation (Otto et al 1993; Bruce et al 1995). CBT is also an option for treatment-refractory patients (Pollack et al 1994), or for those refusing pharmacotherapy.

Numerous theories have been generated about the etiology and pathogenesis of PD. It has become apparent that no single abnormality underlies panic, and initial one-dimensional biological or cognitive theories are now being replaced by more complex models. Future research should also address the possible existence of subtypes of PD with distinct pathophysiology.

The main objective of the studies presented in this thesis was to investigate further the role of GABAergic system in PD by exploring mechanisms of panic induced by flumazenil, a ligand for the benzodiazepine site on the $GABA_A$ receptor. Two clinical studies employed challenge procedures (chapters 2-4). In the third study, plasma levels of flumazenil were determined in PD participants undergoing challenge tests in order to assess the relevance of the pharmacokinetics of the drug in its actions (chapter 5).

Studies presented in chapters 2 and 5 have been published in *Anxiety and Depression* and the *Journal of Pharmacological and Toxicological Methods,* respectively (Zedkova et al 2003; Zedkova et al 2001). References within the text and the reference lists in these chapters are presented in the style required by these journals.

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2. PANIC-RELATED RESPONSES TO PENTAGASTRIN, FLUMAZENIL AND THYROTROPIN RELEASING HORMONE IN HEALTHY VOLUNTEERS

2.1. Introduction

It is difficult to design a study that would provide conclusive information on the role of biological and cognitive factors in the mediation of panic. Manipulation of cognitive variables (see section 1.3.3) (Margraf et al., 1989; Rapee et al., 1986; Rapee et al., 1991; Papp et al., 1995; Sanderson et al., 1989; Abelson et al., 1996), can add an element of deception, which raises ethical concerns if important information is withheld from the subject, and evidence that it modulates panic responses does not necessarily exclude biological mediation. Neutral placebos may be inadequate comparators for panicogenic challenges because they do not control for patients' misinterpretations of physical symptoms produced by an agent. This might be achieved by using an active comparator that provokes somatic symptoms of panic without normally inducing panic attacks.

Thyrotropin releasing hormone (TRH) appears potentially to meet these criteria. TRH is a tripeptide neuromodulator found in multiple brain regions and in some peripheral organs (Merchenthaler et al., 1985; Morley, 1979). In studies of its cardiovascular and subjective effects, healthy subjects report nausea, apprehension, palpitations, paraesthesia and hot and cold flushes, which are symptoms occurring during panic attacks (Coupland et al., 1995). However, TRH administered to PD patients did not provoke panic attacks (Tancer et al., 1990; Stein and Uhde, 1990, 1991). The cardiovascular actions of TRH have been suggested to be peripheral, on resistance vessels or in the heart, because they were not attenuated by ganglion blockade (Bouloux

et al., 1996). TRH is suitable for comparison with panicogenic agents with a similar intravenous (iv) route of administration, such as the CCK-2R agonist pentagastrin, or the benzodiazepine antagonist flumazenil.

If the elevated rates of panic symptoms in response to pentagastrin (see section 1.2.3.3) and flumazenil (section 1.2.2.5) in PD represent *purely* cognitively-mediated reactions to somatic symptoms (Clark, 1986; 1993), doses of TRH, pentagastrin and flumazenil that provoke a similar number and intensity of panic-related *somatic* symptoms in healthy volunteers should induce similar panic responses to each other in PD patients (when administered under the same conditions). If pentagastrin and flumazenil have *specific* panicogenic effects, they should provoke a higher rate of panic attacks in PD than a matched dose of TRH. The main aim of this study was to compare these agents directly in healthy volunteers, in order to find suitable doses for studies in PD. Behavioral, cognitive and physiological responses were assessed.

2.2. Methods

2.2.1. Subjects

Twenty eight volunteers, 12 males and 16 females, with a mean age of 26.6±7.1 years were recruited through notice boards. They were medically healthy, as confirmed by medical history, physical examination, electrocardiogram (ECG) and routine laboratory blood tests (e.g. complete blood count, calcium, phosphate, and indicators of renal, hepatic and thyroid function). All subjects were medication free and had no current or prior history of Axis I psychiatric disorders, as evaluated by the Structured Clinical Interview for Diagnosis: DSM-IV version (SCID) (First et al., 1997). Subjects scored

within the normal range on the total score (11.2 ± 7.8) and the blood/injury (4.7 ± 4.3), social phobia (4.1 ± 2.9) and agoraphobia (1.9 ± 2.3) subscales of the Fear Questionnaire (Marks and Mathews, 1979) and on the Beck Depression Inventory (2.7 ± 3.2) (Beck et al 1961). The study was approved by the university research ethics committee. All subjects gave written informed consent to participate. They were informed of a list of symptoms that the study drugs could produce, and were told that the drugs were "likely to produce some symptoms" of panic, but that they were not harmful. This information did not divide the list of symptoms into those that are characteristic of each individual drug.

2.2.2. Design and drugs

The study design is presented in Figure 2.1. A blinded between-subjects comparison was made between two doses of TRH (600μg, n=17 or 1200μg, n=10), in order to confirm the expectation that effects would be already maximal at 600μg. Previous studies had shown that TRH 500μg produces a maximal effect on thyroid stimulating hormone (Garbutt et al., 1994) and that a dose of 1000μg does not produce significantly greater cardiovascular effects than 50μg (Bouloux et al., 1996). Since a dose-response was not predicted, a between-subjects approach was used for the TRH dose comparison, with the expectation that the TRH data could be pooled for comparison with the other agents. As expected, there were no dose-dependent effects of TRH on any of the measures (see section 2.3.2.2). Results for the two doses were therefore pooled.

A double-blind, balanced-order, randomized cross-over design was used to compare the agents. Subjects were challenged with one test drug on each test session. Flumazenil (Anexate: Roche Products Ltd.), pentagastrin (Peptavlon: Wyeth-Ayerst Ltd.)

and TRH (Relefact TRH: Roche Products Ltd.) were obtained from the hospital and a local pharmacy supplier. In the first 4 subjects pentagastrin 0.3μg/kg (n=4) was compared with TRH 600μg (3 completed). In a previous pilot study pentagastrin 0.3μg/kg induced similar cardiovascular and subjective responses as TRH 500μg in a healthy volunteer (Coupland et al 1996). However, in this study the intensity of somatic panic symptoms (pentagastrin mean =13.8, TRH mean = 5.3; rated on a Panic Symptom Scale, see section 2.2.4.2) was obviously much greater. In the next 9 subjects a reduced dose of pentagastrin (0.2μg/kg) was compared with TRH (a cross-over design, Study 1). In the following 15 subjects, the lowest dose of pentagastrin (0.1μg/kg) was compared with TRH and with flumazenil 2mg, using a cross-over design (Study 2). This dose of flumazenil has been reported to induce panic attacks in 80% of PD patients (Nutt et al 1990). In order to blind the difference in volume and rate of injection for the peptides and flumazenil, a double-dummy method was used, in which a 5ml rapid bolus of pentagastrin or TRH was followed by a 60 second infusion of saline placebo, or the 60 second infusion of flumazenil was preceded by a rapid 5ml bolus of saline placebo.

2.2.3. Procedures

Subjects reported to the Psychopharmacology Research Unit for each test session. A urine sample was taken before the first test to screen for use of illicit drugs or benzodiazepines. A pregnancy test was performed for female subjects. An intravenous catheter was placed in an antecubital vein. Monitoring devices, i.e. impedance plethysmography (Respitrace) bands, ECG electrodes and an automated sphygmomanometer (see physiological measurements for detail) were attached and

checked. Subjects then rested in a semi-supine position for 30 min, after which baseline measures were made and the continuous recordings started. The test drug was administered at 0 min and responses were measured up to 60 min.

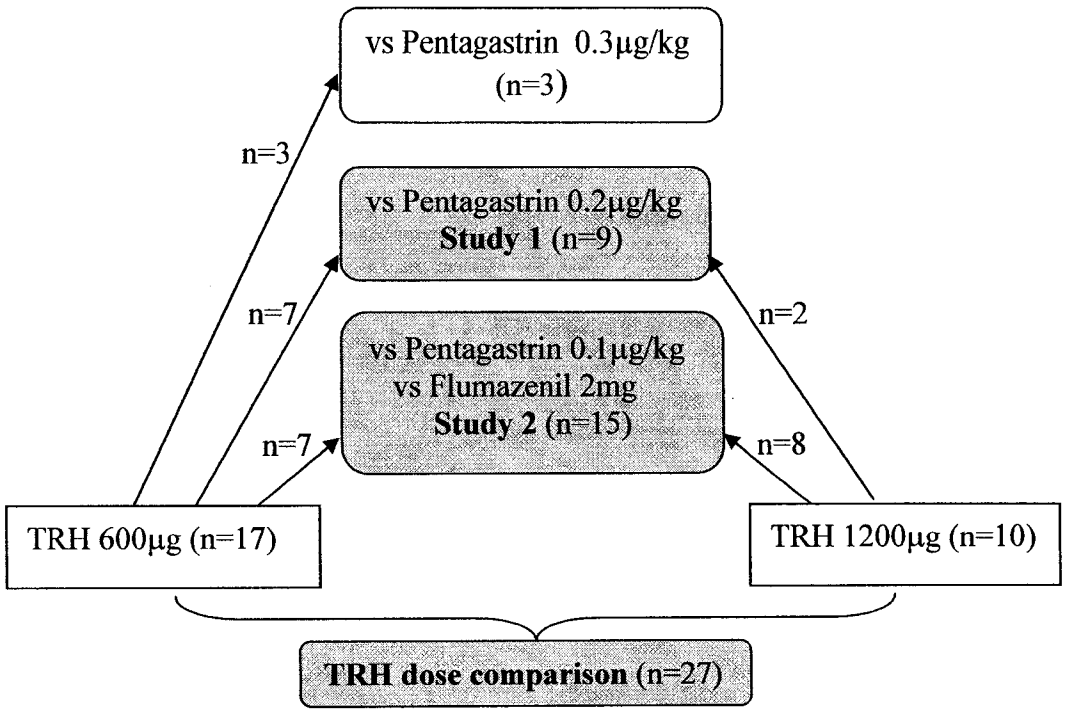

Figure 2.1. Study flow-chart.

2.2.4. Measures

2.2.4.1. Cognitive measures

At the screening session, participants completed the ASI (Reiss et al., 1986). This 16-item questionnaire measures fear of harmful consequences of anxiety symptoms (items are related to physical concerns, mental incapacitation concerns and social concerns); each item is rated on a 0 to 4 point scale. Total ASI scores were assessed in this study.

Before each injection subjects were asked to give subjective ratings on a number of variables identified by Clark (1993) as potential cognitive predictors of panic responses to challenge tests. These were panic expectancy ("How likely are you to panic during this test?"), perceived safety ("How safe do you think you will feel during this test?") and perceived control ("How much control will you have over your reactions to this test?"). Each answer was rated by the subjects on a 100mm Visual Analog Scale (VAS).

Cognitions following the injections were rated using a modified Agoraphobic Cognitions Questionnaire (mACQ) (Chambless et al., 1984). This 14-item questionnaire measured how strongly subjects had believed each of a list of catastrophic cognitions after the injection (e.g., "I am going to pass out", "I will have a heart attack"). Each item was rated from 0="did not believe at all" to 4="totally believed". The items were taken from the ACQ, which evaluates catastrophic ideation associated with "real life" anxiety/panic. A similar modification has been reported previously (Carter et al., 1995).

2.2.4.2. Subjective measures

Panic symptoms were measured using a Panic Symptom Scale (PSS) that has been used previously in multiple CCK-4 challenge studies by Bradwejn and colleagues (Bradwejn et al., 1991, 1992a,b). The PSS is a checklist of panic symptoms. At 10 min after each injection subjects rated the peak response to the injection for each symptom (0=not present to 4=very severe). In this study we calculated the number (i.e. items rated at least 1) and sum intensity of DSM IV somatic symptoms for each test, not including

the anxiety/apprehension and cognition items (fear of dying, losing control or going crazy). This was because the study aimed to find drug doses that would provoke a similar overall intensity of *somatic* symptoms.

Subjective anxiety was rated on a 100mm VAS (0mm=not at all anxious, 100mm=the most anxious I have ever felt). Dyspnea was rated on the Borg Scale of Respiratory Exertion (Borg, 1982), which asks the subject to rate the subjective effort of breathing on a scale from 6-20 (7="very, very light" and 19="very, very hard"). Subjects were asked to rate by pointing to the appropriate score on the scale with a pointer. This scale has previously been shown to capture increases in dyspnea during panic challenges (Papp et al., 1995). Subjects rated each of these items at –5, -1, 1, 2, 3, 4, 5, 7.5, 10, 30, 45 and 60 min. At 5 min after the injection they also rated their peak anxiety and dyspnea.

2.2.4.3. Physiological measures

Respiratory: Measures of respiratory rate (RR), tidal volume (TV) and minute volume (MV) (MV=TV x RR) were derived from continuous recording from 5 min before to 15 min after each injection, using impedance plethysmography (Respitrace: Ambulatory Monitoring, Inc., Ardsley, NY). A spirometer calibration was performed before each test. A syringe was used to inject 5 liters of air into the spirometer (Ohio 842 Spirometer, Airco). The voltage from the spirometer was measured in order to find the relationship between volume and voltage. The subject was then asked to breathe into the spirometer while wearing the Respitrace bands. Data were accumulated from the Respitrace bands and spirometer by the computer. A multiple regression program was used to find the 'best

fit' parameters to relate volume (dependent variable) from the spirometer and voltages (independent variables) from the Respitrace. The measured volume was then compared with the calculated volume to determine the goodness of fit of the parameters obtained from the multiple regression model.

Cardiovascular: Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured by automated sphygmomanometer (Dinamap: Critikon, Canada) at –4 and –2 min and then every 20 sec after the start of the injection.

2.2.5. Data analysis

The cardiovascular and respiratory data up to 5 min were averaged over 1-min intervals. Repeated measures (anxiety, and cardiovascular parameters) and continuous data (respiratory parameters) were analyzed by repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser corrections [epsilon (ε) indicated in text], after logarithmic or square root normalization, where indicated. Nonparametric tests were used where the data could not be normalized. In the TRH dose comparison, time was used as a within- and dose as a between-group factor. In Studies 1 and 2, drug and time were used as within-group factors. Since the repeated measures ANOVA might not detect changes of short duration that might nevertheless act as a somatic cue, peak changes in measures were also compared, using the cardiovascular data collected at 20 sec intervals and the respiratory data averaged over 15 sec intervals (i.e., peak measures within the 5 min post-injection interval minus baseline measures). These were compared using unpaired or

paired t-tests, Mann-Whitney, Wilcoxon, ANOVA or Friedman tests as appropriate. All results were considered significant at $p \leq 0.05$ (two-tailed test).

2.3. Results

2.3.1. Pre-challenge assessment

The mean score of the 27 completers on the ASI was 11.3±5.7. In the TRH-dose comparison and in Studies 1 and 2, there were no significant differences between the ratings on cognitive predictors prior to each drug challenge (Table 2.1).

Table 2.1. Baseline cognitive predictors (VAS)

	Panic expectancy	Perceived safety	Perceived control
TRH comparison			
TRH 600 ug (n=17)	22.5 (12.0-37.5)	82.0 (58.5-95.0)	73.5 (46.8-81.8)
TRH 1200 ug (n=10)	11.5 (5.0-36.0)	93.5 (87.3-97.0)	82.5 (50.8-92.0)
	NS	NS	NS
Study 1 (n=9)			
Pentagastrin 0.2ug/kg	15.0 (9.0-25.5)	85.0 (57.5-94.5)	81.0 (69.5-88.0)
TRH	20.0 (15.0-28.0)	87.0 (69.0-93.5)	72.0 (37.5-79.5)
	NS	NS	NS
Study 2 (n=15)			
Pentagastrin 0.1ug/kg	18.0 (10.0-41.0)	92.0 (88.0-97.0)	83.0 (51.0-90.0)
TRH	15.0 (5.0-34.0)	88.0 (72.0-97.0)	81.0 (55.0-92.0)
Flumazenil	17.0 (10.0-47.0)	89.0 (76.0-94.0)	73.0 (51.0-91.0)
	NS	NS	NS

Values are median (25^{th} – 75^{th} percentiles).

2.3.2. Challenge results

2.3.2.1. TRH-dose comparison: 600μg vs 1200μg

Subjective measures: There was a significant time effect ($F=15.4$, $df=5,130$, $p<0.001$, $\varepsilon=0.5$) and dose effect ($F=4.5$, $df=1,26$, $p=0.044$) for VAS anxiety. The dose effect was

caused partly by significantly higher baseline ratings before the administration of TRH 600μg (t=2.6, df=26, p=0.001). The only significant post-injection dose effect was found at 1 min, with TRH 600μg inducing a greater response (t=2.4, df=26, p=0.026). The effects of the two doses on the peak increase in anxiety were not significantly different. There were no significant differences between the two doses for PSS (sum intensity and number of somatic symptoms), the total score on the mACQ or the peak increase in dyspnea (Table 2.2).

Cardiovascular measures: Both doses of TRH increased all cardiovascular parameters, with peaks at 1 min (HR and DBP) and at 2 min (SBP), and a decline to baseline or below by 3 min after injection. These changes were reflected in a significant time effect for HR ($F=32.8$, $df=3,75$, $p<0.001$), SBP ($F=32.7$, $df=5,125$, $p<0.001$, $\varepsilon=0.7$) and DBP ($F=49.1$, $df=5,125$, $p<0.001$, $\varepsilon=0.6$). There were no significant dose effects or time x dose interactions and no significant differences between the two doses of TRH in the peak increases in HR and blood pressure (Table 2.2).

Respiratory measures: Both doses increased MV and TV, with the peak responses at 1 and 2 min, respectively. The values returned to baseline within 3 min. There was a significant time effect in MV ($F=23.9$, $df=5,115$, $p<0.001$, $\varepsilon=0.6$) and TV ($F=8.3$, $df=5,115$, $p<0.001$, $\varepsilon=0.6$). No time effects were found for RR. No dose effects or time by dose interactions were detected for any of the respiratory measures. As in the cardiovascular responses, the maximum increases in the respiratory responses following the two doses of TRH were not significantly different (Table 2.2).

Table 2.2. Responses to two different dosages of TRH in healthy controls.

	TRH		t-test			Mann-Whitney test	
	600 ug (n=17)	1200 ug (n=10)	t	df	p	U	p
PSS- Somatic symptoms							
Sum intensity	6.4±5.0	5.1±4.3	0.7	26	0.5		
Number	4.2±2.9	3.7±3.1	0.4	26	0.7		
VAS anxiety (mm)	11.5 (5.9-23.4)	7.8 (4.9-26)				82.5	0.7
mACQ	1.0 (0.0-3.0)	1.0 (0.0-1.3)				63.0	0.2
Borg dyspnea	2.0 (1.8-4.0)	2.0 (0.0-4.0)				76.5	0.5
HR (per min)	23.3±11.9	18.9±6.5	1.1	26	0.3		
SBP (mmHg)	19.0 (11.5-31.0)	18.5 (12.5-24.0)				83.5	0.8
DBP (mmHg)	14.0±6.4	12.2±6.4	0.7	26	0.5		
MV (L/min)	4.1±2.9	2.4±2.1	1.6	23	0.1		
RR (per min)	0.0 (0.0-4.0)	4.0 (-2.0-4.0)				70.5	0.9
TV (L)	0.5 (0.2-0.9)	0.4 (0.2-0.5)				53.0	0.3

Values, except for PSS responses, represent peak changes from baseline. Normally distributed values are mean ±SD, nonparametric values are median (25^{th} – 75^{th} percentiles).

2.3.2.2. Study 1: Pentagastrin 0.2μg/kg vs TRH

Subjective measures: A significant time effect was found for VAS anxiety ($F=11.0$, $df=5,40$, $p<0.001$, $\varepsilon=0.3$), with the peak responses following both drugs at 1 min. No significant drug effect or drug-by-time interaction was found for this measure. However, compared with TRH, the subjects responded to pentagastrin with significantly greater peak increases in anxiety. Following pentagastrin, subjects scored significantly higher on the sum intensity and number of somatic PSS symptoms and on the peak increase in dyspnea. The two drugs did not cause significantly different total scores on the mACQ (Table 2.3).

Cardiovascular measuers: Both drugs briefly increased HR and DBP (peak response at 1 min) and SBP (peak at 2 min). There was a significant time effect on HR ($F=24.7$, $df=5,35$, $p<0.001$, $\varepsilon=0.4$), SBP ($F=26.4$, $df=5,35$, $p<0.001$) and DBP ($F=43.4$, $df=5,35$, $p<0.001$). The drug effects were not significant. A significant drug x time interaction was found only for DBP ($F=6.39$, $df=5,35$, $p<0.001$). TRH produced a significantly greater peak increase in DBP than pentagastrin. The differences between the drugs in peak change in HR and SBP were not significant (Table 2.3).

Respiratory measuress: Following both drugs, brief increases lasting 1-2 min were observed in MV, TV and RR. There was a significant time effect on MV ($F=15.4$, $df=5,40$, $p<0.001$, $\varepsilon=0.4$), RR ($F=4.4$, $df=5,40$, $p=0.003$) and TV ($F=7.4$, $df=5,40$, $p<0.001$). There were no significant drug effects or drug x time interactions. No significant differences were observed in peak increases in the respiratory responses. (Table 2.3).

2.3.2.3. Study 2: Flumazenil 2 mg vs pentagastrin 0.1μg/kg vs TRH

Subjective measures: All three drugs increased VAS anxiety, with peaks at 1 min, with flumazenil (FL) producing only a mild response, but the drug effect was not significant. There was a significant time effect ($F=20.6$, $df=5,70$, $p<0.001$, $\varepsilon=0.5$) and drug x time interaction on these ratings ($F=3.2$, $df=10,140$, $p=0.01$, $\varepsilon=0.4$). Table 2.4 shows PSS responses and the peak changes in other subjective measures. *Post-hoc* tests showed that pentagastrin (PG) caused a significantly greater peak increase in anxiety than did both TRH and FL ($Z=-2.9$, $p=0.004$ for PG vs TRH; $Z=-2.7$, $p=0.008$ for PG vs FL). PG

Table 2.3. Responses to 0.2ug/kg pentagastrin and TRH in 9 healthy controls.

			t-test (df=8)		Wilcoxon test	
	Pentagastrin	TRH	t	p	Z	p
PSS - Somatic symptoms						
Sum intensity	11.4±6.7	5.6±4.5	2.6	0.003		
Number	6.3±3.0	3.6±2.7	2.9	0.02		
VAS anxiety	34.5 (30.3-54.5)	11.5 (4.8-22.8)			-2.19	0.03
mACQ	1.0 (0.0-4.5)	1.0 (0.0-2.5)			-0.8	0.4
Borg dyspnea	4.0 (2.0-7.0)	2.0 (1.5-2.0)			-2.04	0.04
HR (per min)	33.6±7.0	28.9±11.6	0.9	0.38		
SBP (mmHg)	16.0±8.5	22.1±12.2	-1.8	0.10		
DBP (mmHg)	7.3±4.1	14.6±3.3	-4.0	0.004		
MV (L/min)	5.4±3.3	4.6±3.1	0.4	0.67		
RR (per min)	0.0 (0.0-4.0)	0.0 (0.0-6.0)			-0.71	0.48
TV (L)	0.6 (0.4-0.9)	0.5 (0.2-0.8)			-1.13	0.26

Values, except for PSS responses, represent peak changes from baseline. Normally distributed values are mean ± SD, nonparametric values are median (25^{th} – 75^{th} percentiles).

caused a significantly greater sum intensity of somatic panic symptoms than FL (t=-2.3, df=14, p=0.039). Both PG and TRH produced a greater number of somatic symptoms than FL, but the difference was not significant. Analysis of individual PSS somatic symptoms showed that the three drugs differed significantly on PSS scores for gastrointestinal (GI) symptoms (PG>TRH>FL; Z=-2.2, p=0.027 for PG vs TRH; Z=-2.0, p=0.044 for PG vs FL; Z=-2.8, p=0.004 for TRH vs FL). PG caused higher ratings than both TRH and FL on palpitations (Z=-2.3, p=0.02 for PG vs TRH; Z=-1.5, p=0.026 for PG vs FL), choking feelings (Z=-2.04, p=0.041 for PG vs TRH; Z=-2.39, p=0.017 for PG vs FL) and chest pain/or discomfort (Z=-2.1, p=0.035 for PG vs TRH; Z=-2.7, p=0.007 for PG vs FL). Both PG and TRH caused higher PSS dyspnea than FL (Z=-2.4, p=0.019 for PG vs FL; Z=-2.1, p=0.034 for TRH vs FL). This was consistent with dyspnea on the

Borg scale, where both PG and TRH provoked significantly larger increases than FL (Z=-3.0, p=0.003 for PG vs FL; Z=-2.0, p=0.045 for TRH vs FL). The total scores on the mACQ following the three drugs were not significantly different.

Cardiovascular measures: PG and TRH briefly increased HR and DBP, with peaks at 1 min (Figure 2.2). There was a significant time effect ($F=18.7$, $df=5,65$, $p<0.001$, $\varepsilon=0.4$) and drug x time interaction for HR, due to a lesser response to FL than to PG and TRH. For DBP there was a significant drug effect ($F=6.0$, $df=2,26$, $p=0.007$), time effect ($F=58.7$, $df=2,26$, $p<0.001$, $\varepsilon=0.4$) and drug x time interaction, due to a decrease in blood pressure after FL. The response in SBP was not analyzed by the repeated measures ANOVA because the data could not be transformed to a normal distribution. *Post-hoc* analysis showed a significantly greater peak increase in HR in response to PG and TRH compared with FL ($t=-3.6$, $df=13$, $p=0.003$ for PG vs FL; $t=-3.3$, $df=13$, $p=0.005$ for TRH vs FL), and significant differences between all three drugs in their effects on SBP (TRH>PG>FL; $Z=-2.2$, $p=0.028$ for PG vs FL; $Z=-3.2$, $p=0.001$ for TRH vs FL; $Z=-2.5$, $p=0.012$ for TRH vs PG) and DBP (TRH>PG=FL; $t=-4.5$, $df=13$, $p=0.001$ for TRH vs FL; $t=-2.4$, $df=13$, $p=0.033$ for TRH vs PG) (Table 2.4).

Respiratory measures: The effects of the drugs on MV and TV are shown in Figure 2.3. PG and TRH briefly increased both MV and TV (peaks at 1-2min). In both measures, PG provoked the highest and FL the lowest response, but the drug effects were not significant. There was a significant time effect for MV ($F=38.1$, $df=5,65$, $p<0.001$, $\varepsilon=0.6$) and TV ($F=22.5$, $df=5,65$, $p<0.001$) and significant drug x time interactions for these

variables. *Post-hoc* testing revealed that PG caused a significantly greater peak increase in TV than FL (Z=-2.3, p=0.022). The differences in other respiratory parameters were not significant (Table 2.4).

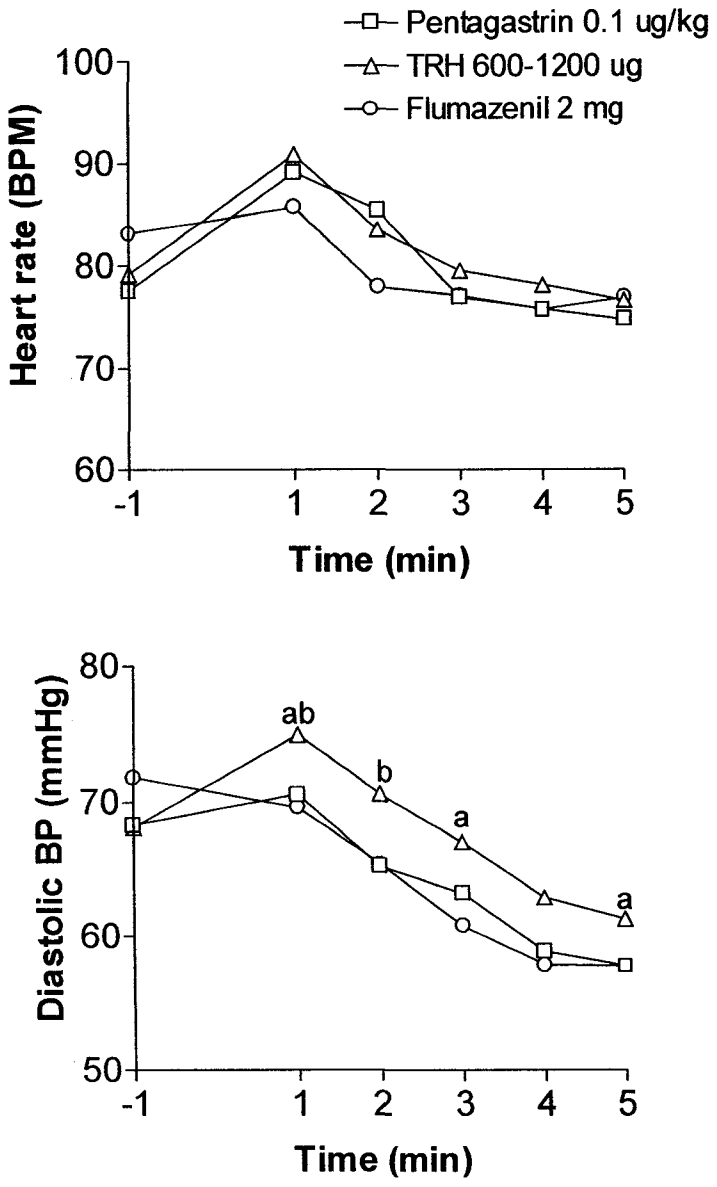

Figure 2.2. Time course of heart rate and diastolic blood pressure responses to flumazenil, pentagastrin and TRH in 15 healthy volunteers. [a]Significantly different ($p<0.05$) from flumazenil, [b]significantly different from pentagastrin.

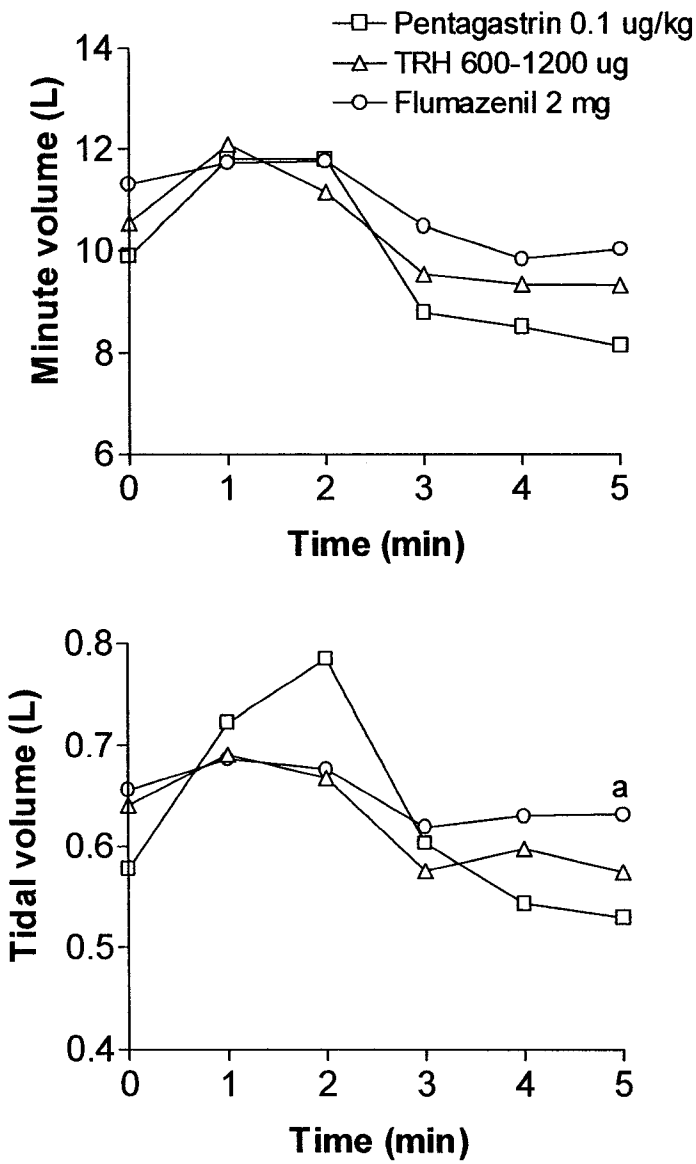

Figure 2.3. Time course of minute and tidal volume responses to flumazenil, pentagastrin and TRH in 15 healthy volunteers. [a]Significantly different ($p<0.05$) from pentagastrin.

Table 2.4. Responses to 2 mg flumazenil, 0.1ug/kg pentagastrin and TRH in 15 healthy controls.

				ANOVA (df=2,26)		Friedman test (df=2)
	Flumazenil	Pentagastrin	TRH	F	p	p
PSS- Somatic symptoms						
Sum intensity	4.5±4.2	9.0±6.9	6.3±4.6	4.2	0.04	
Number	2.0 (0.0-5.0)	5.0 (4.0-8.0)	5.0 (1.0-7.0)			0.062
VAS anxiety	2.0 (-0.5-12.0)	21.0 (7.5-36.5)	9.5 (5.5-24.5)			0.002
mACQ	0.0 (0.0-1.0)	1.0 (0.0-3.0)	1.0 (0.0-2.0)			0.26
Borg	0.0 (0.0-3.0)	3.0 (1.0-5.0)	3.0 (1.0-4.0)			0.003
HR (per min)	9.6±8.4	18.9±8.9	18.1±8.7	7.8	0.002	
SBP (mmHg)	5.0 (0.8-10.3)	12.5 (9.0-19.0)	19.0 (14.3-30.0)			0.001
DBP (mmHg)	3.8±7.4	7.4±5.9	13.7±8.1	9.4	0.001	
MV (L/min)	1.8 (0.4-2.9)	4.4 (3.0-6.0)	2.0 (1.6-6.1)			0.168
RR (per min)	4.0 (0.0-4.0)	4.0 (0.0-4.0)	4.0 (0.0-5.0)			0.976
TV (L)	0.3 (0.1-0.5)	0.5 (0.3-0.9)	0.4 (0.2-0.7)			0.046

Values, expect for PSS responses, represent peak changes from baseline. Normally distributed values are mean ± SD, nonparametric values are median (25^{th} – 75^{th} percentiles).

2.4. Discussion

The study aimed to identify doses of pentagastrin, flumazenil and TRH producing a similar intensity of panic-related somatic symptoms in healthy volunteers. Comparison of the effects of these doses in PD patients could help to clarify the role of cognitive and biological factors in flumazenil- and pentagastrin-induced panic. Three comparison studies were performed. Subjects did not show significant differences on VAS ratings of perceived control, safety or panic expectancy before the start of each different challenge session, which could have been due to a balanced order of administration of the injections. Thus, baseline differences in these cognitive variables were unlikely to explain differences in responses to the challenge agents. Furthermore, the different responses were unlikely to be strongly related to post-injection catastrophic cognitions, since the total mACQ scores were relatively low and similar across the challenges. This indicates a low tendency in healthy subjects to enagage in catastrophic ideation.

The TRH comparison study showed that, in line with other research on the endocrine and cardiovascular effects of TRH (Garbutt et al., 1994; Bouloux et al., 1996), there was no evidence for dose-dependent effects of TRH on any of the measures, confirming that the effects of the 600μg dose were already likely to be maximal. Results for the two doses were therefore pooled.

In Study 1, pentagastrin 0.2μg/kg produced significantly greater subjective ratings (PSS, VAS anxiety, dyspnea) than did TRH, without differences in agoraphobic cognitions. The only significant difference in the physiological measures was a greater increase in DBP due to TRH.

In Study 2, with 15 subjects, the dose of pentagastrin was lowered to 0.1μg/kg and the comparison with flumazenil (2mg) was also introduced in a three-way crossover. Even at this low dose, pentagastrin produced a significantly greater change in VAS anxiety than TRH. Although there were no longer significant differences on the overall PSS measures (i.e., the number and sum intensity of somatic symptoms) or dyspnea, pentagastrin caused higher ratings on the PSS items for palpitations, choking feelings, chest pain/discomfort and GI symptoms than TRH. In terms of objective measures, there were no significant differences in HR and respiratory variables between pentagastrin and TRH, but the latter had greater pressor effects. The higher level of anxiety caused by pentagastrin could not therefore be explained on the basis of its having greater objective cardiorespiratory effects. Its greater anxiogenic effect at this low dose might be a result of other peripheral or indirect central actions.

These effects at low dose clearly do not preclude the possibility that PD patients are more sensitive to the greater cardiorespiratory effects of higher pentagastrin doses typically used in panic-challenge studies. However, since panic rates were not found to differ between patients receiving 0.1, 0.3, or 0.6 μg/kg of pentagastrin (van Megen et al., 1994), other factors may play a role. In addition to the suggested peripheral action on the stomach or vagus nerve receptors, pentagastrin has a lower selectivity for CCK-2Rs than CCK-4 and also binds to CCK-1Rs elsewhere in the GI tract (Hughes et al., 1990). Panic reactions may therefore occur as an increased reactivity to marked somatosensory stimulation, perhaps mediated via cognitive mechanisms, or perhaps by indirect central pathways. In any case, the finding of this study that even a much lower dose of pentagastrin than is typically used produces greater subjective effects than TRH in

healthy volunteers shows that TRH would not be a credible comparator for pentagastrin in PD studies in order assess the relevance of cognitive mechanisms in pentagastrin-induced panic.

Of the three drugs, flumazenil provoked the mildest subjective and objective responses. Although pentagastrin did not cause a significantly greater number of somatic panic symptoms than flumazenil, all other behavioral responses and cardiovascular parameters (HR, SBP) were significantly greater after pentagastrin. The overall PSS and VAS anxiety ratings in response to flumazenil and TRH were not significantly different, although responses on each measure were larger after TRH. The only significant differences between flumazenil and TRH were that the latter produced greater dyspnea and GI symptoms on the PSS and greater objective cardiovascular responses.

If a panic attack is an escalation of anxiety caused by bodily sensations, then it might be predicted that TRH would be a stronger panicogenic agent in PD, particularly given the salience to PD patients of dyspnea, cardiovascular and GI responses, which were significantly greater than those following flumazenil. However, whereas in previous studies TRH did not trigger panic attacks in PD patients (Tancer et al., 1990; Stein and Uhde 1990; Stein and Uhde, 1991), flumazenil has provoked panic responses in three separate samples, including two that used the same dose regimen (Bell et al., 2002; Nutt et al., 1990; Woods and Charney, 1991). Given that these studies were performed under different settings, patients may have had quite different expectations, for example of an endocrine 'test' versus a panic 'challenge'. The use of TRH as a direct comparator,

with assessment of baseline cognitive variables and catastrophic cognitions, could clarify whether flumazenil has specific panicogenic effects in PD that are not explained simply by cognitive or behavioral sensitivity to cardiovascular or respiratory responses.

A potential limitation of the present study is that tests were not timed to menstrual phase, which might affect sensitivity to panicogenic agents (Harrison et al., 1989; Perna et al., 1995). However, neither panic-related responses to flumazenil nor to CCK-4 differ significantly between the follicular and luteal phases of the menstrual cycle in healthy women (Le Mellédo et al., 1999 and personal communication, 2001), so that menstrual phase is unlikely to have had a major influence in the present study. Another limitation is a possible inclusion of healthy subjects with family history of anxiety or mood disorders, which previously have shown increased sensitivity to some panicogenic agents (Perna et al., 1995; Coryell, 1997). While family history may not have markedly affected the generally mild responses to flumazenil in this study, it has yet to be determined if genetic factors play a role in sensitivity to pentagastrin, to which the subjects showed higher subjective and objective responses.

In conclusion, this study suggested that comparison of TRH (600μg) and flumazenil (2 mg) in PD patients may be a useful method to test whether panic induction by flumazenil can be explained as a secondary response to bodily sensations, or is more likely to involve more direct (GABAergic) mechanisms.

2.5. References

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3. SUBJECTIVE AND PHYSIOLOGICAL RESPONSES TO FLUMAZENIL IN PATIENTS WITH PANIC DISORDER, MAJOR DEPRESSION, AND COMORBID PANIC DISORDER AND MAJOR DEPRESSION

3.1. Introduction

The benzodiazepine antagonist flumazenil has been reported to cause panic attacks in patients with PD, but not in healthy controls (Bell et al 2002; Nutt et al 1990; Woods et al 1991). Nutt et al (1990) have proposed that the function of the benzodiazepine site of the $GABA_A$ receptor is shifted towards inverse agonism in PD, such that flumazenil acts as an anxiogenic partial inverse agonist. Other studies, however, did not find flumazenil to be panicogenic (Strohle et al 1999; Strohle et al 1998). Methodological differences (Potokar et al 1999) and the fact that none of the studies controlled for cognitive variables, which may modulate responses to pharmacological challenges (Rapee 1995), might have contributed to the inconsistency.

If there is, at least in some PD patients, a functional shift on the benzodiazepine site towards inverse agonism, several responses to flumazenil might be expected. In animals, a pharmacological reduction in GABAergic inhibitory transmission resulted not only in anxiety-related behavior (Ninan et al 1982; Brandao et al 1986; Schmitt et al 1986), but also in respiratory, cardiovascular (Sanders and Shekhar 1995; Shekhar and DiMicco 1987) and HPA axis (Keim and Shekhar 1996) activation. In these studies, GABAergic transmission was selectively decreased in the PAG, hypothalamus or amygdala, brain regions that have been suggested to form the main neural pathways involved in the generation of panic (Gorman et al 2000; Graeff et al 1993). Similar reactions, i.e. severe anxiety accompanied by cardiorespiratory and HPA activation, were

seen in healthy humans who were administered the partial inverse agonist FG 7142 (Dorow et al 1983).

In healthy volunteers, flumazenil had minimal effects on respiratory function (Forster et al 1993) and decreased or did not affect cardiovascular (Higgitt et al 1986; Nutt et al 1990; Strohle et al 1999) and HPA axis measures (Strohle and Wiedemann 1996; Strohle 1999). Flumazenil-induced physiological activation in PD patients would therefore strongly indicate $GABA_A$ receptor dysfunction.

Respiratory and neuroendocrine responses to different panicogenic agents have varied widely (Sinha et al 1999). According to Klein, spontaneous panic represents firing of an inborn blood CO_2- and brain lactate-sensitive suffocation alarm, which has a pathologically low threshold in PD (Klein 1993). He discriminates spontaneous panic from other forms of stress response (situational fear or chronic anxiety) by the relative prominence of respiratory distress leading to hyperventilation, and the lack of HPA activation. Klein described flumazenil as "fear-provoking" rather than "panic-provoking", and in reference to the receptor shift hypothesis (Nutt et al 1990) predicted that, since inverse agonists mimic situational fear in raising cortisol (Dorow et al 1983), then flumazenil-induced panic should not increase ventilation (Klein 1993; Klein 1996). However, although not objectively measured, heavy breathing was observed in the healthy subjects experiencing anxiety provoked by the partial inverse agonist FG 7142 (Dorow et al 1983). Further, if flumazenil elicits a state close to situational fear, one might expect patients experiencing fear of social situations in social phobia, or fear related to traumatic experiences in PTSD, to be sensitive to flumazenil. However, studies

with these patients reported negative results (Coupland et al 2000; Coupland et al 1997; Randall et al 1995).

An important question is whether the panicogenic challenge paradigm can aid in understanding the relationship between PD and major depressive disorder (MDD). Extensive comorbidity between these two conditions (Kessler et al 1998; Roy-Byrne et al 2000), often associated with greater symptom severity and persistence, and poorer treatment response (Roy-Byrne et al 2000; Johnson and Lydiard 1998), has led to debate as to whether PD and MDD are different manifestations of a single disease or distinct diagnostic entities. Neurobiological links between MDD and PD are indicated by shared abnormalities, such as a blunted growth hormone responses to clonidine (Uhde et al 1986), or reduced occipital cortex GABA levels (Sanacora et al 1999; Goddard et al 2001). Both disorders respond to serotonin-enhancing antidepressants (Nutt 2000). Finally, both PD and MDD patients show signs of HPA axis abnormalities, although these are more consistent and pronounced in MDD (see sections 1.2.4.2 and 1.2.4.4 for details on overlap and differences in HPA abnormalities). On the other hand, these conditions seem to have separate familial transmission (Mannuzza et al 1994). Blunted thyroid stimulating hormone responses to TRH and reduced CSF and plasma GABA levels have been found in MDD (Loosen and Prange 1982; Petty 1995), but not in PD (Stein and Uhde 1991; Goddard et al 1996; Rimon et al 1995). In addition, MDD patients generally do not respond to benzodiazepines (Nutt 2000).

In the few pharmacological challenge studies that included MDD patients, these subjects appeared to be insensitive to the panicogenic effects of CO_2 (Kent et al 2001; Perna et al 1995), fenfluramine, or sodium lactate (Targum 1990). Testing of MDD

patients with flumazenil could therefore clarify whether a common diathesis of abnormal $GABA_A$ receptor function contributes to the high comorbidity between PD and MDD. In a placebo-controlled study using a crossover design, flumazenil was not reported to be anxiogenic in nine patients with comorbid MDD and PTSD (Randall et al 1995), but these findings need to be replicated in a larger sample with pure MDD.

The purpose of the current study was to assess the role of biological or cognitive mechanisms in flumazenil-induced panic. Cognitive factors are discussed in chapter 4. The current report focuses on several objectives: 1) to replicate findings of a panicogenic effect of flumazenil in patients with PD; 2) to test the specificity of this effect for PD; 3) to assess whether this effect is consistent with flumazenil being a partial inverse agonist; and 4) to test the reproducibility of flumazenil actions.

The hypothesis was that in PD patients and patients with comorbid PD and MDD flumazenil would cause greater subjective (panic, anxiety and dyspnea) and objective (respiratory, cardiovascular and cortisol) responses than in patients with pure MDD and healthy controls, and greater responses than to placebo. The panicogenic effects of flumazenil were predicted to be reproducible.

3.2. Methods

3.2.1. Subjects

Four groups of subjects were studied, namely patients with pure PD (P), patients with comorbid PD and major depression currently experiencing a major depressive episode (P+D), patients with major depression currently experiencing a major depressive episode (D), and healthy volunteers (HV). Demographic and clinical data of the participants are presented in Table 3.1. The groups did not differ significantly in their age and sex distribution.

Patients were recruited through local media presentations and advertisements, clinical referrals, and notice boards. Healthy volunteers were recruited through notice boards and by referrals from other healthy subjects. After an initial telephone screening, potential participants underwent a screening session. Patients were included in the study if they met the particular diagnostic criteria for one of the three patient groups (P, P+D, D), as determined by the SCID (First et al 1997). Using this structured interview, patients with other significant Axis I psychiatric disorders (with the exception of generalized anxiety disorder) were excluded from the study. Patients with panic disorder (P and P+D groups) had experienced at least 4 panic attacks in the last 4 weeks, patients in the P group did not have a major depressive episode within the last 6 months, and patients in the D group had never experienced a panic attack. The HV group had no current or prior history of Axis I psychiatric disorders, as evaluated by the SCID. All subjects were in the age range 18-50 years. Subjects were excluded from the study if they reported allergy to flumazenil, substance abuse or dependence in the last year, the use of regular benzodiazepines or other GABAergic drugs within last 3 months, intermittent

benzodiazepines within 2 weeks, antidepressants within 2 weeks (5 weeks for fluoxetine), neuroleptics within 4 weeks, or beta-blockers within 1 week. Further exclusion criteria were pregnancy or lactation, or significant physical illness (respiratory, cardiovascular, cerebrovascular, neurological or endocrinological), ruled out by medical history, physical examination, ECG and routine laboratory tests.

Subjects were given verbal and written information about the nature of the study and about possible behavioral and physiological effects of flumazenil, some of which "may resemble some symptoms of panic". They were reassured that the drug is not harmful and that they could withdraw from the study at any time. All subjects gave written informed consent to participate prior to the SCID. The study was approved by the university research ethics committee.

Table 3.1. Demographic and clinical characteristics of the 4 study groups.

Characteristic	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)
Age	36.75 ±11.52[a]	36.91±10.73	34.25±11.22	33.33±11.59
Male sex	8 (40%)[b]	8 (36%)	8 (40%)	11 (52 %)
PD duration (months)	72.0 (12.5 -156.0)[c] 96.2 ± 136.8[a]	36.0 (16.5-165.0) 100 ± 119.2[a]		
Panic frequency/week	3.0 (1.5-3.0) 3.8 ± 3.3	1.0 (1.0-4.0) 2.7 ± 2.3		

Values are [a]means ± SD, [b]number (percentage) of subjects, and [c]medians (25-75th percentiles).

3.2.2. Design and drugs

Flumazenil (Anexate: Roche Products Ltd.) 2mg and 0.9% saline placebo were given as 60 sec 35 ml intravenous infusions in a randomized, double-blind cross-over fashion on two morning sessions. The P and HV groups received both flumazenil and placebo on each test day, each day in a reversed order [i.e., two different orders: flumazenil-placebo-placebo-flumazenil (FPPF) vs placebo-flumazenil-flumazenil-placebo (PFFP)] to assess the reproducibility of the effects of flumazenil (Figure 3.1). The P+D and D groups were challenged with one drug (flumazenil or placebo) on each test day in a randomized order.

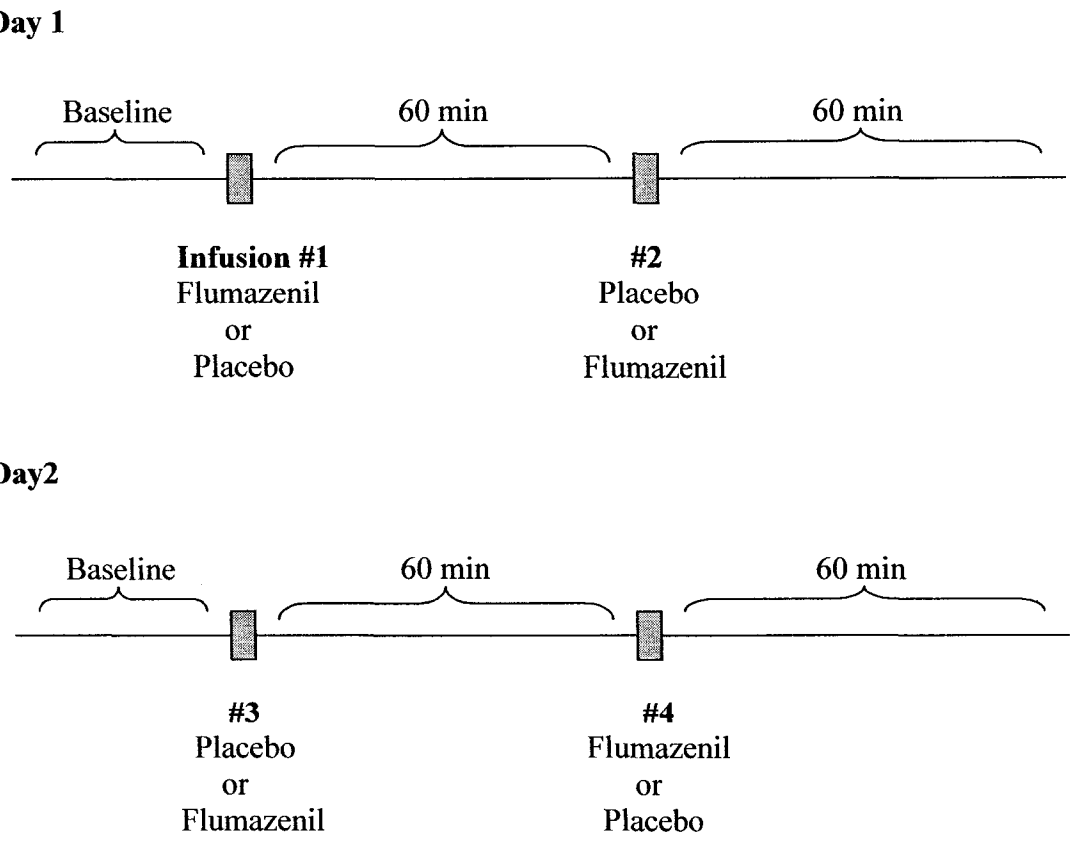

Figure 3.1. Study design for the P and HV groups.

3.2.3. Procedures

Subjects reported to the Psychopharmacology Research Unit at 8:30 am for each test session. A urine sample was taken before the first test to screen for use of illicit drugs or benzodiazepines. A pregnancy test was performed for female subjects. An intravenous catheter was placed in an antecubital vein. Monitoring devices, i.e. impedance plethysmography (Respitrace) bands, ECG electrodes and an automated sphygmomanometer (see physiological measurements for detail), were attached and checked. Subjects then rested in a semi-supine position for 40 min after which baseline measures were made and the continuous recordings started. The test drug was administered at 0 min (9:30-9:40am) and responses were measured for up to 60 min, after which the second drug was administered in the same fashion to the P and HV groups.

3.2.4. Measures

3.2.4.1. Pre-challenge assessment

Self-report questionnaires and scales were administered at the screening session. A measure of the frequency and intensity of anticipatory anxiety (Leon et al 1992) and the PSS (see below), which measures symptoms for the patients' most recent severe panic attack, were administered to the P and P+D groups. The Sheehan Disability Scale, assessing impairment in work, social and family environments (Leon et al 1992), was administered to the P, P+D and D groups. All subjects completed the 21-item Beck Depression Inventory (Beck et al 1961). Other questionnaires completed by the subjects [the ASI, the Agoraphobic Cognition Questionnaire (ACQ) and the Fear Questionnaire

(FQ)] will be described later in this thesis in the section addressing cognitive factors in flumazenil-induced panic.

3.2.4.2. Subjective measures

Panic symptoms were measured using the PSS. Subjects rated the intensity of fear and each symptom at baseline, and the peak response to the infusion for each symptom at 10 min after each infusion on a 0 (symptom not present) to 4 (very severe) scale. In each subject the sum intensity of panic symptoms at these time points was obtained and the increase from baseline was calculated (peak minus baseline scores). Another measure derived from the scale was the number of DSM-IV panic symptoms rated at least 2 (moderate).

The occurrence of a panic attack was assessed at 15 min after each infusion using a semi-structured interview administered by a rater blind to the physiological data. The interviewer started with open and proceeded to direct questions about the subject's experience of the test (without asking directly whether she or he experienced a panic attack) and categorized full panic (sudden severe anxiety with at least four unequivocal DSM IV panic symptoms) and subthreshold panic (sudden moderate anxiety with at least 4 symptoms). Furthermore, using the PSS, the occurrence of a panic attack was defined as an increase from baseline in the subjects' rating of at least 2 points on at least 4 DSM-IV panic symptoms, and an increase in the rating of fear of at least 2 points to severe or very severe (3-4). This additional PSS criterion was used because the interviewer was not blind to the subjects' diagnoses and because it provided for some comparability with previous flumazenil challenge studies that used a similar scoring system (Bell et al 2002).

Subjective anxiety was rated on a 100mm VAS (0mm=not at all anxious, 100mm=the most anxious I have ever felt). Dyspnea was rated on the Borg Scale of Respiratory Exertion (Borg 1982) (see section 2.2.4.2 for details). Subjects rated each of these items at –5 min (baseline) and at 5 (peak responses), 10, 15, 30, 45 and 60 min.

3.2.4.3. Physiological measures

Respiratory: Measures of MV, TV and RR were derived from continuous recording from 5 min before to 15 min after each infusion, using impedance plethysmography (Respitrace: Ambulatory Monitoring, Inc., Ardsley, NY) (see section 2.2.4.3 for details).

Cardiovascular: SBP, DBP and HR were measured by an automated sphygmomanometer (Dinamap: Critikon, Canada) at baseline and then every 20 sec until 5 min after the start of each infusion, then at 10, 15, 30, 45 and 60 min.

Neuroendocrine: Blood samples for serum cortisol were collected through the cannula at baseline (-5 min) and at 15, 30, 45 and 60 min after each infusion. The hormone levels were determined by a direct enzyme immunoassay (Diagnostic Systems Laboratories, Inc., Webster, Tx) with an intra-assay coefficient of variation (CV) of 5.0%, interassay CV of 6.1% and a sensitivity of 0.1ug/dl.

Flumazenil plasma levels: Samples for the estimation of flumazenil were collected as reported in chapter 5 (section 5.2.2).

3.2.5. Data analysis

One-way ANOVAs and t-tests were performed for the pre-challenge scales and questionnaires. Panic attack rates in response to the infusions were compared among the groups using *Chi*-square tests. Panic rates induced by flumazenil and placebo within each group were compared by McNemar binomial tests. Group differences in the number of PSS panic symptoms (data were not normally distributed) were analyzed by Kruskal-Wallis test with *post-hoc* Mann-Whitney tests; Wilcoxon signed ranks tests compared the number of symptoms in response to flumazenil and placebo within the groups.

Baseline values of all the other variables (below) were analyzed by repeated measures ANOVA with group (P, P+D, D, HV) as a between- and drug (flumazenil vs placebo) as a within-subjects factor. The cardiovascular and respiratory data up to 5 min were averaged over 1-min intervals. Repeated measures (sum intensity of panic symptoms, anxiety, dyspnea and cardiovascular parameters) and continuous data (respiratory parameters) were analyzed by repeated measures ANOVA with Greenhouse-Geisser corrections (ε indicated in text), after logarithmic normalization, where indicated. Group was a between subjects factor, drug and time (2 time points for subjective and 6 time points for physiological responses) were within-subjects factors. Group differences in changes from baseline in the subjective measures were also analyzed, using repeated measures ANOVA. Responses to flumazenil and placebo were further compared on the basis of panic status following flumazenil, assessed by the semi-structured interview. Since the rates of panic among the D and HV subjects were very low, only the P and P+D groups were divided into panickers (subjects experiencing both full and sub-threshold panic attacks were included) and non-panickers, and compared with the D and HV

subjects. Significant drug x group or drug x time x group interactions in all the repeated measures ANOVAs were followed by one-way ANOVA, repeated measures ANOVA or *post-hoc* paired t-tests, as appropriate, to assess group effects within each drug and drug effects within each group. The most important statistical results for the subjective and objective physiological measures are presented in tables. All results were considered significant at $p \leq 0.05$ (two-tailed test).

Additional tests performed on the PSS (individual panic symptom analyses and comparison of patients' recent severe and flumazenil-induced panic attacks) will be described in the following section.

3.3. Results

The challenge data of the P and HV subjects (who were given flumazenil and placebo twice) were first analyzed separately to assess effects of order of administration (FPPF vs PFFP) and day (first day = first experience of the infusions vs second day = second experience of the infusions). The order effect (FP vs PF) was also examined in the P+D and D groups. These initial analyses are briefly presented at the beginning of each section. If not indicated otherwise, only the first test day data of the P and HV groups were compared with data of the P+D and D groups, for the following reasons: 1) greater responses were found for some variables on the first test day and 2) data from the second day were missing in 4 P subjects who did not attend. Reasons for non-attendance were: severe panic attack on the first day (n=2), unexpected death in family (n=1), and unexpected change in work commitments (n=1).

3.3.1. Pre-challenge assessment

The mean scores on the pre-challenge questionnaires are given in Table 3.2. Patients with PD (P and P+D groups) did not differ in their anticipatory anxiety scale ratings. *Post-hoc* tests showed that the P+D group scored higher than the P and D groups on the Sheehan Disability Scale. All groups showed significantly different scores on the Beck Depression Inventory (P+D>D>P>HV). The scores indicated a severe and a moderate form of depression in the P+D and D groups, respectively. Group differences in the scores on the ASI, FQ and ACQ will be discussed in the section on cognitive factors in flumazenil-induced panic.

3.3.2. Subjective measures

Panic attacks: In subjects experiencing panic attacks after flumazenil, the symptoms appeared typically within 40 seconds and disappeared within 3-4 min. Using both the semi-structured interview and PSS criteria for a panic attack, the initial analyses of panic rates showed no effect of order of administration (Fisher's exact tests) or day (McNemar tests) for the P subjects. No effect of order was found for the P+D and D subjects. Panic attack rates after flumazenil and placebo are summarized in Table 3.3. Applying the PSS criteria, flumazenil caused significantly more panic attacks in the P and P+D group than in the HV group, and in the P+D group than in the D group. Although no group differences in panic rates after placebo were found (only 1 P subject met the PSS criteria), flumazenil was significantly more panicogenic than placebo only in the P+D group.

The occurrence of *full* panic attacks after flumazenil was significantly higher in the P and P+D groups when assessed by the semi-structured interview, than in the D and HV groups. Placebo did not induce different panic rates across the groups, and flumazenil was significantly more panicogenic than placebo in the P and P+D groups. Since no HV experienced panic, the reproducibility of the panicogenic effect of flumazenil was determined for the P group only. Despite no significant day effect, kappa (κ, a measure of reproducibility of the effect in the same subject, ranges from -1 to 1) was 0, since only two first test day panickers experienced full panic during the second test. The reproducibility was higher when both full and sub-threshold panic attacks were included in the analysis ($\kappa = 0.47$).

Number of panic symptoms (PSS): No day effect was found in the P and HV subjects (Wilcoxon signed ranks test), and no order effect in any of the groups (Mann-Whitney *U*-tests) for the number of panic symptoms. The P and P+D groups showed significantly more symptoms after flumazenil than the HV group. Responses to flumazenil were significantly greater than to placebo in each group and responses to placebo did not differ from each other (Table 3.4).

Table 3.2. Scores on pre-challenge questionnaires of the study groups.

	P (n=18)	P+D (n=22)	D (n=20)	HV (n=21)	t-test	ANOVA			*Post-hoc* tests
Questionnaire	Mean SD	Mean SD	Mean SD	Mean SD	p	F	df	p	
Anticipatory Anxiety Scale (max 110)	60.06 15.20	69.45 16.09			NS				
Sheehan Disability Scale (max 30)	13.0 7.04	20.0 5.68	14.0 8.42			5.9	2,57	0.005	$P+D^{ac} > P = D$
Beck Depression Inventory (max 63)	11.17 8.78	31.73 6.46	25.65 7.62	2.0 4.39		80.54	3,77	<0.001	$P+D^{bcd} > D^{bd} > P^{d} > HV$
ASI (max 64)	30.0 12.7	33.5 10.2	18.7 12.8	10.2 6.2		20.8	3,77	<0.001	P = P+D > D = HV
FQ- total (max 120)	33.6 23.0	42.3 25.3	20.6 16.2	10.0 6.8		11.6	3,77	<0.001	P = P+D > HV P+D > D
- agora (max 40)	8.8 7.7	13.5 11.2	3.2 5.0	1.4 2.3		11.7	3,77	<0.001	P = P+D> HV P+D > D
ACQ (max 54)	14.6 10.3	18.6 10.6	6.7 5.6			9.1	2,57	<0.001	P = P+D > D

[a]p<0.01 vs. P, [b]p<0.001 vs. P; [c]p<0.05 vs. D; [d]p<0.001 vs. HV.

Table 3.3. Rates of panic attacks in the study groups in response to flumazenil and placebo, assessed by the PSS and the semi-structured interview.

	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)	*Chi*-square test			*Post-hoc* tests
	panickers N (%)	panickers N (%)	panickers N (%)	panickers N	χ^2	df	p	
PSS criteria								
Flumazenil	5 (25)	7 (32)[f]	1 (5)	0	11.3	3	0.01	$P^c = P+D^d > HV$ $P+D^a > D$
Placebo	1 (5)	0 (0)	0 (0)	0			NS	
Interview criteria								
Flumazenil	10 (50)[f]	11 (50)[f]	1(5)	0	24.2	3	<0.001	$P^{be} = P+D^{be} > D = HV$
Placebo	2 (10)	3 (14)	0	0			NS	

[a]p<0.05 vs. D, [b]p<0.01 vs. D; [c]p<0.05 vs. HV, [d]p<0.01 vs. HV, [e]p<0.001 vs. HV by *Chi*-square test; [f]p<0.05 vs. placebo by McNemar test.

Table 3.4. Number of PSS panic symptoms of the study groups following flumazenil and placebo.

	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21	Kruskal–Wallis test p	*Post-hoc* tests
Flumazenil	3.0 (1.0-6.25)[d]	4.0 (1.75-7.0)[d]	2.0 (0.25-4.5)[d]	1.0 (0.0-1.5)[c]	0.001	$P^a = P+D^b > HV$
Placebo	0.5 (0.0-1.0)	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.014	NS

Values are median (25^{th}-75^{th} percentiles). [a]p<0.01 vs. HV, [b]p<0.001 vs. HV by Mann-Whitney test. [c]p<0.05 vs. placebo, [d]p<0.01 vs placebo by Wilcoxon test.

The most important repeated measures ANOVA results for the following measures (the PSS sum intensity of panic symptoms, VAS anxiety, and dyspnea measured on Borg scale) are presented in tables at the end of the section on subjective measures. Analyses for the baseline scores are summarized in Table 3.5. Tables 3.6 and 3.7 show the analyses with baseline and post-infusion (peak) scores as repeated measures.

Sum intensity of panic symptoms (PSS): Repeated measures analyses of the P and HV groups found no order or day effect for the baseline sum intensity of panic symptoms and no order or day effects for the change in their sum intensity from baseline. The intra-class correlation coefficient (ICC, range -1 to 1) for changes in the PSS scores after the first and second flumazenil infusions, indicating within-subject reproducibility of the effect, was 0.63. There was no order effect, but a significant day effect for the post-infusion (peak) sum intensity scores (day1 > day2, $F=7.21$, $df=1,33$, $p=0.011$). The ICC for the peak sum intensity scores following flumazenil on days 1 and 2 was 0.853, suggesting a tendency for subjects to score lower on day 2. A significant day effect ($F=12.1$, $d=1,33$, $p=0.001$) was also found for the PSS sum intensity when baseline and peak scores were entered as repeated measures. In the P+D and D groups, no order effects were found for their baseline and peak scores on the sum intensity, or its change from baseline.

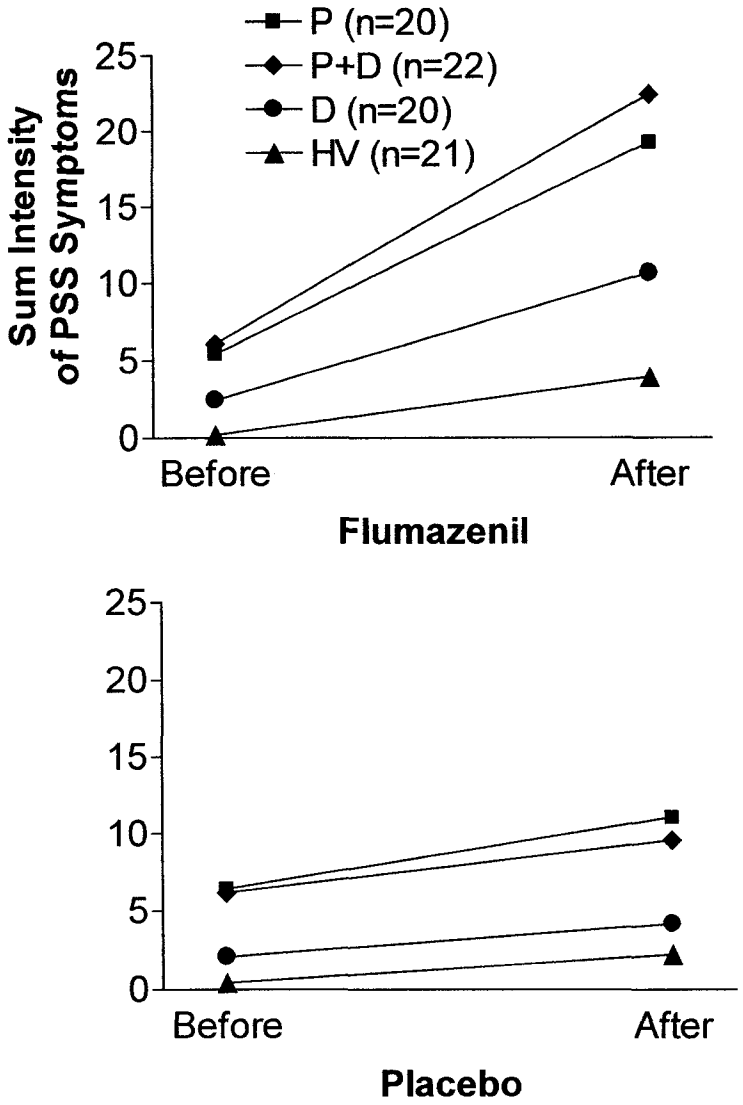

Figure 3.2. Mean sum intensity of panic symptoms of the four study groups before and after flumazenil and placebo.

Figure 3.2 shows the sum intensity of panic symptoms of the four groups at baseline and after flumazenil and placebo infusions. *Post-hoc* analysis of the baseline scores showed that the P and P+D groups scored higher than the HV group and the P+D higher than the D group (Table 3.5). There was a significant effect of group ($F=12.85$, $df=3,79$, $p<0.001$) and drug ($F=53.52$, $df=1,79$, $p<0.001$), and a drug x group interaction ($F=5.58$, $df=3,79$, $p=0.002$) for the peak sum intensity scores. *Post-hoc* testing found that

following flumazenil the P and P+D groups scored significantly higher than the D and HV groups ($p=0.044$ for P vs D; $p<0.001$ for P vs HV; $p=0.002$ for P+D vs D; $p<0.001$ for P+D vs HV). Following placebo, the P and P+D groups also scored higher than the HV group, and the P group higher than the D group ($p=0.005$ for P vs HV; $p=0.021$ for P+D vs HV and $p=0.046$ for P vs D). In all the groups, with the exception of HV, flumazenil caused greater peak responses than placebo ($p=0.008$ for P; $p<0.001$ for P+D and D).

Repeated measures analysis with pre-and post-infusion time points showed significant main effects of group ($F=12.2$, $df=3,79$, $p<0.001$), drug ($F=30.39$, $df=1,79$, $p<0.001$) and time ($F=110.0$, $df=1,79$, $p<0.001$), and significant drug x group ($F=3.52$, $df=3,79$, $p=0.019$), group x time ($F=6.92$, $df=3,79$, $p<0.001$), drug x time ($F=48.34$, $df=1,79$, $p<0.001$) and drug x time x group interactions. The three-way interaction with *post-hoc* tests are given in Table 3.6. Separate analyses for each drug revealed that flumazenil increased the sum intensity significantly more in the P and P+D groups than in the D and HV groups, which was reflected in a significant group x time interaction. The group effect in response to placebo was also significant (P = P+D > HV), but the lack of group x time interaction indicates that this was likely due to baseline differences. Both infusions increased the ratings across the groups, but the responses to flumazenil were higher than responses to placebo in all groups except for the HV group. Consequently, significant drug x time interactions were found in the patient groups.

All the above main effects and interactions were also found when the second test day data of the P and HV groups were entered in the analysis. The drug x time x group

interaction and *post-hoc* tests are presented in Table 3.6. Although the P group still scored significantly higher than the HV group after flumazenil, the difference between the P and D group was no longer significant.

Analysis of changes from baseline of the PSS sum intensity (day 1 data of the P and HV subjects were used) showed a significant effect of group ($F=7.53$, $df=3,79$, $p<0.001$), drug ($F=51.46$, $df= 1,79$, $p<0.001$), and a significant drug x group interaction ($F=5.06$, $df=3,79$, $p=0.003$). The increases after flumazenil were higher in the P and P+D groups compared to the HV group, and in the P+D group compared to the D group ($p=0.002$ for P vs HV; $p<0.001$for P+D vs HV; $p=0.02$ for P+D vs D). The groups did not differ from each other in their responses to placebo. In all groups, flumazenil caused higher increases than placebo (P: $t=3.82$, $df= 19$, $p=0.001$; P+D: $t=4.39$, $df=21$, $p<0.001$; D: $t=4.65$, $df=19$, $p<0.001$; HV: $t=2.09$, $df=20$, $p=0.049$). In addition, increases in the PSS sum intensity in the P and HV groups after flumazenil or placebo, given as the first and second infusion from the two test days (i.e., infusion # 1 or 3, and 2 or 4), were compared separately with PSS increases in the P+D and D groups. These ANOVA results, however, showed similar results as the previously described analysis and are not presented in detail. It should be mentioned that when responses to flumazenil given as the second infusion of the day to the P and HV subjects were entered into the overall analysis, the difference between the P and D groups approached significance ($p=0.051$).

Table 3.8 presents increases in the intensity of individual PSS items induced by flumazenil and placebo. Following flumazenil, both the P and P+D groups experienced

significantly higher increases than the HV group in 5 symptoms (palpitations, shaking/trembling, chest discomfort, paraesthesia, and feeling unreal/detached). The P+D group further showed a higher increase than both the D and HV groups in fear of going crazy/losing control, and the D group showed a higher increase in dyspnea than the HV group. No group differences in increases in dizziness, sweating, nausea, choking feelings, hot flushes/cold chills or fear of dying were found after flumazenil, and no group differences in all the items were found after placebo. In the P group, flumazenil increased all symptoms significantly more than placebo, *except for* palpitations, chest discomfort, choking feelings, hot flushes/cold chills and fear of dying. In the P+D group, increases in all symptoms, *except* fear of dying, were higher after flumazenil. In the D group, flumazenil caused significantly higher increases in dyspnea, dizziness, sweating, feeling unreal/detached, paresthesia and fear of losing control/going crazy. The only significant effects of flumazenil on the HV group were increases in nausea and dizziness.

Comparison of recent severe and flumazenil-induced panic attacks: In P and P+D subjects who met the PSS criteria for panic in response to flumazenil, the severity (i.e., the PSS sum intensity of panic symptoms) of their flumazenil-induced panic attacks (FP) (34.8±11.1) did not differ significantly from the severity of their recent severe panic attacks (SP) (36.6±10.9) ($t=0.7$, $df=11$, $p=0.52$). In addition, the within-subject consistency in ratings was high (ICC=0.83). There were significantly different ratings for two symptoms; dizziness was more severe during FP [median (25^{th}-75^{th} percentiles) FP: 4(3-4), SP: 2(2-3), $p=0.006$], and hot flushes/cold chills during SP [FP: 2(0-2), SP: 3(2-3), $p=0.018$].

Subjects who met criteria for full flumazenil-induced panic attacks according to the semi-structured interview also rated the intensity of these attacks as similar to their severe attacks, although within-subject ratings were less consistent (FP: 31.1.±12.3, SP: 34.7±11.4, t=1.0, df=17, p=0.319, ICC=0.348). Ratings of dizziness were found to be higher during FP (FP: 3 (2-4), SP: 2(1-2.5), p=0.013), while ratings of hot flushes/cold chills [FP: 1(0-1), SP: 2(1-2), p=0.003], sweating [FP: 0(0-1.5), SP: 1(0-2), p=0.025] and fear of dying [FP: 0(0-1), SP: 1(0-4), p=0.012] were higher during SP.

Gender differences in panic attack rates and PSS scores: Within PD subjects, flumazenil induced full panic attacks (assessed by the semi-structured interview) in 62% of females and 31% of males (χ^2=3.6, df=1, p=0.06). No significant difference between genders was found in increases from baseline in PSS sum intensity of panic symptoms. Analyses of individual PSS symptoms showed higher increases in dyspnea in female subjects [median (25^{th}-75^{th} percentiles) females: 1(0-1.5), males: 0(0-1), p=0.05].

VAS anxiety: There were significant day effects in the P and HV groups for their baseline and post-infusion (peak) anxiety scores, with higher scores obtained on day 1 (baseline: F=7.26, df=1,33, p=0.011; peak: F=17.8, df=1,33, p<0.001). No day effect was found for changes of anxiety scores from baseline, and no order effects on any of the above measures were found across the groups.

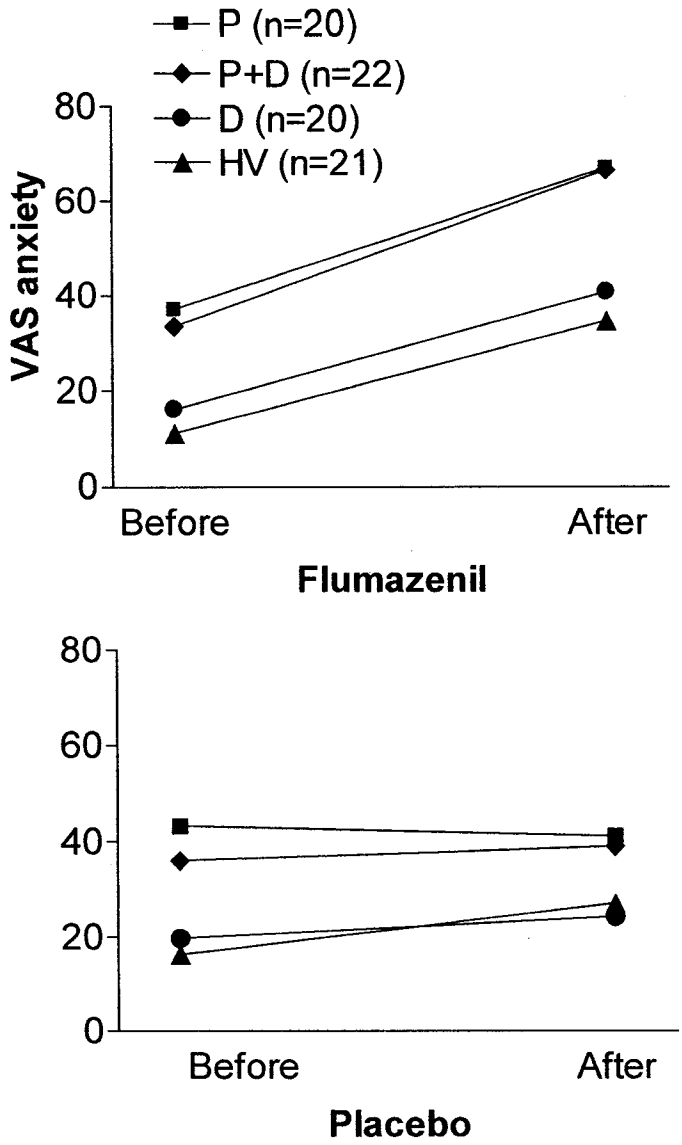

Figure 3.3. VAS anxiety ratings of the four study groups before and after flumazenil and placebo.

Baseline and peak post-infusion anxiety ratings of the study groups are shown in Figure 3.3. Significant effects of group and drug were revealed for baseline anxiety scores (Table 3.5). *Post-hoc* testing found that at baseline the P and P+D groups experienced higher anxiety than the D and HV groups. The drug effect was caused by a higher mean anxiety before placebo. There were significant effects of group (F=9.67,

df=3,79, p<0.001) and drug (F=49.6, df=1,79, p<0.001), and a drug x group interaction (F=2.95, df=3,79, p=0.038) for the peak anxiety scores. As with baseline anxiety, the P and P+D groups scored higher than the D and HV groups following flumazenil (p=0.003 for P and P+D vs D; p<0.001 for P and P+D vs HV). The groups' anxiety responses to placebo were not significantly different. Flumazenil induced higher peak anxiety than placebo in all groups except HV (P: t=3.61, df=19, p=0.002; P+D: t=5.14, df=21, p<0.001; D: t=3.1, df=19, p=0.007).

Repeated measures analysis with the pre- and post- infusion time points showed significant effects of group (F=14.08, df=1,79, p<0.001), drug (F=13.43, df=1,79, p<0.001) and time (F=78.23, df=1,79, p<0.001), and significant drug x time (F=84.64, df=1,79, p<0.001) and drug x time x group interactions (Table 3.6). As demonstrated by *post-hoc* analyses and shown in Figure 3, the P and P+D groups had higher scores than the D and HV groups (p<0.001 for P vs D, P vs HV and P+D vs HV; p=0.001 for P+D vs D), but the lack of a significant group x time interaction indicated that the group differences were already set at baseline. All the groups showed significant drug x time interactions, which demonstrated their higher responses to flumazenil than to placebo. The absence of significantly different responses between the groups was confirmed by the analysis of increases of anxiety from baseline, where a significant drug effect was found, with flumazenil causing higher responses (F=76.027, df=1,79, p<0.001), but no group effect or drug x group interaction.

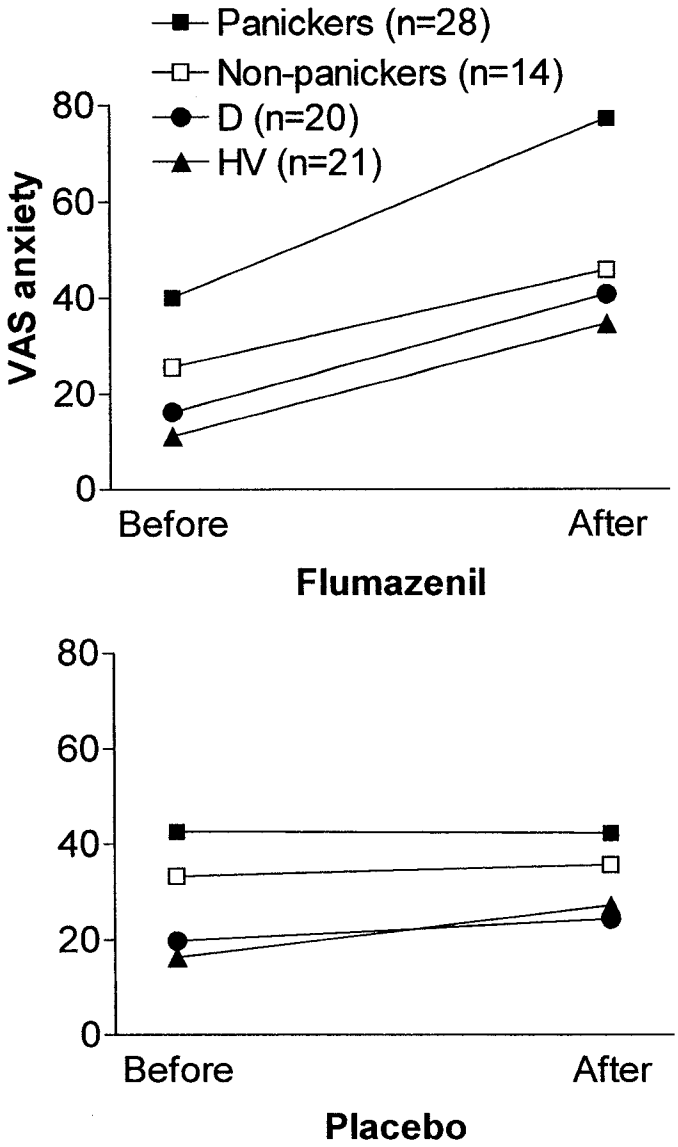

Figure 3.4. VAS anxiety ratings of panickers, non-panickers and the D and HV groups before and after flumazenil and placebo.

Pre- and post-infusion anxiety ratings in PD panickers (subjects experiencing both full and sub-threshold panic attacks) (n=28), non-panickers (n=14) and the D and HV groups are given in Figure 3.4. Repeated measures ANOVA found significant effects of group ($F=19.29$, $df=3,79$, $p<0.001$), drug ($F=9.57$, $df=1,79$, $p=0.003$) and time ($F=69.8$, $df=1,79$, $p<0.001$), and drug x group ($F=3.8$, $df=3,79$; $p=0.014$), drug x time ($F=75.2$,

df=1,79, p<0.001) and drug x time x group interactions (Table 3.7). Further analysis indicated that flumazenil produced higher anxiety in panickers than in all the other groups, causing a significant group x time interaction. Panickers also experienced higher anxiety than the D and HV groups during placebo (p=0.001), but the lack of a significant group x time interaction showed that this was due to their greater baseline anxiety. As presented in Table 3.7, flumazenil induced higher anxiety than placebo in all groups, which was indicated by significant drug x time interactions. However, analysis of changes of anxiety from baseline found that, although responses were greater to flumazenil than to placebo, with a significant drug effect (F=75.2, df=1,79, p<0.001), the groups did not differ significantly in their anxiety responses to the infusions.

Dyspnea (Borg scale 6-20): There were no significant order and day effects for the baseline and peak ratings of dyspnea in the P and HV subjects, and no order and day effects for their changes in dyspnea from baseline. Order effects were not significant for any of the measures in the P+D and D subjects.

Baseline and peak dyspnea scores are presented in Figure 3.5. There was a significant group effect for the baseline scores, due to higher ratings of the P+D group compared to the HV group (Table 3.5). Significant effects of group (F=5.36, df=1,79, p=0.002), drug (F=39.61, df=1,79, p<0.001), and a drug x group interaction (F=4.4, df=3,79, p=0.006) were found for the peak dyspnea scores. Flumazenil induced greater dyspnea in the P and P+D group than in the HV group (p=0.002 for P vs HV; p<0.001 for

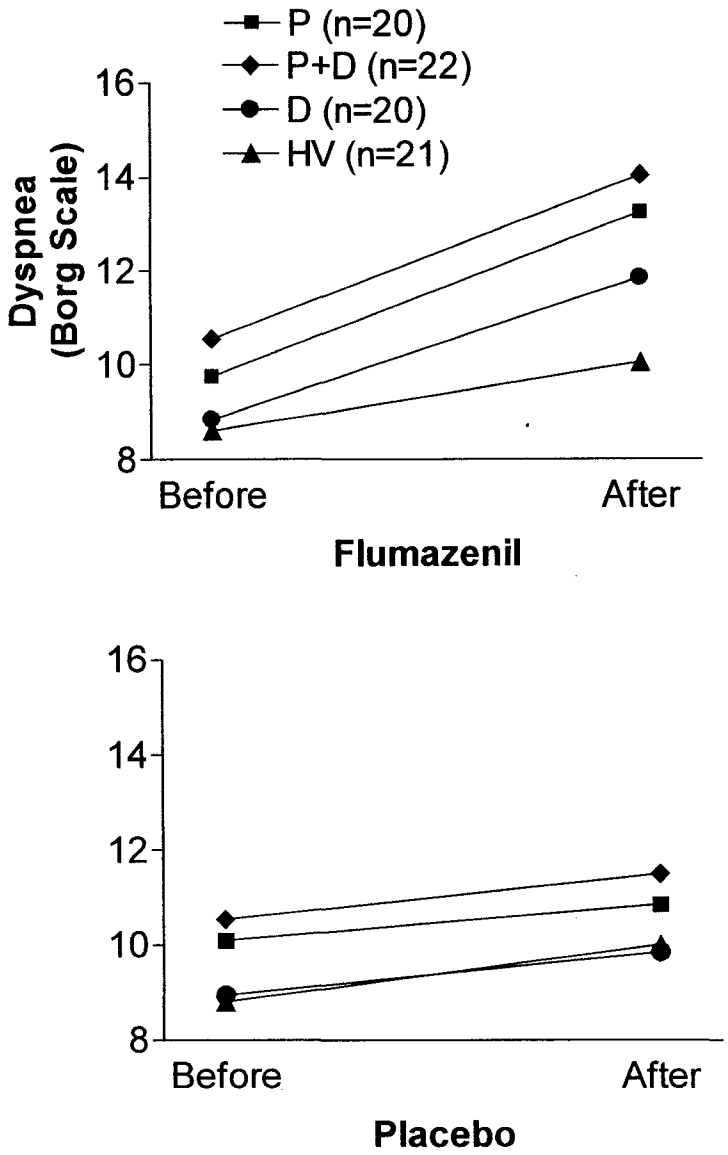

Figure 3.5. Dyspnea ratings (Borg scale) of the four study groups before and after flumazenil and placebo.

P+D vs HV), and greater dyspnea than placebo in all the groups except for HV (P: t=3.13, p=0.005; P+D: t=5.33, p<0.001; D: t=3.37, p=0.003). Responses to placebo were not significantly different among groups. Repeated measures ANOVA (baseline and peak ratings as repeated measures) indicated a significant effect of group (F=4.94, df=1,79,

p=0.003), drug (F=17.04, df=1,79, p<0.001) and time (F=125.11, df=1,79, p<0.001), and significant drug x time (F=48.78, df=1,79, p<0.001) and drug x time x group interactions (Table 3.6). Flumazenil led to higher levels of dyspnea in the P and P+D groups than in the HV group, and in the P+D group than in the D group, which caused a significant group x time interaction. No group differences were found in the subjects' dyspnea following placebo. Although both infusions increased dyspnea, flumazenil induced higher responses than placebo in all the patient groups, where significant drug x time interactions were found.

There was a significant drug effect (F=48.78, df=1,79, p<0.001) and drug x group interaction (F=4.44, df=3, p=0.006) in changes in dyspnea from baseline. Flumazenil caused a significantly greater increase in dyspnea in the P and P+D groups than in the HV group (p=0.022 for P vs HV, p=0.018 for P+D vs HV). Responses to flumazenil were significantly greater than to placebo in each group except for HV (P: t=3.85, p=0.001; P+D: t=4.33, p<0.001; D: t=3.94, p=0.001). Responses to placebo did not differ between groups.

Figure 3.6 presents baseline and peak dyspnea ratings of panickers, non-panickers and the D and HV groups. There was a significant effect of group (F=8.02, df=3,79, p<0.001), drug (F=13.3, df=1,79, p<0.001) and time (F=113.46, df=1,79, p<0.001), and drug x group (F=3.72, df=3,79, p=0.015), drug x time (F=41.95, df=1,79, p<0.001) and drug x time x group interactions (Table 3.7). Panickers had greater dyspnea in responses to flumazenil than all the other groups, as reflected in a group x time interaction. Panickers also felt greater dyspnea than the D and HV groups during placebo

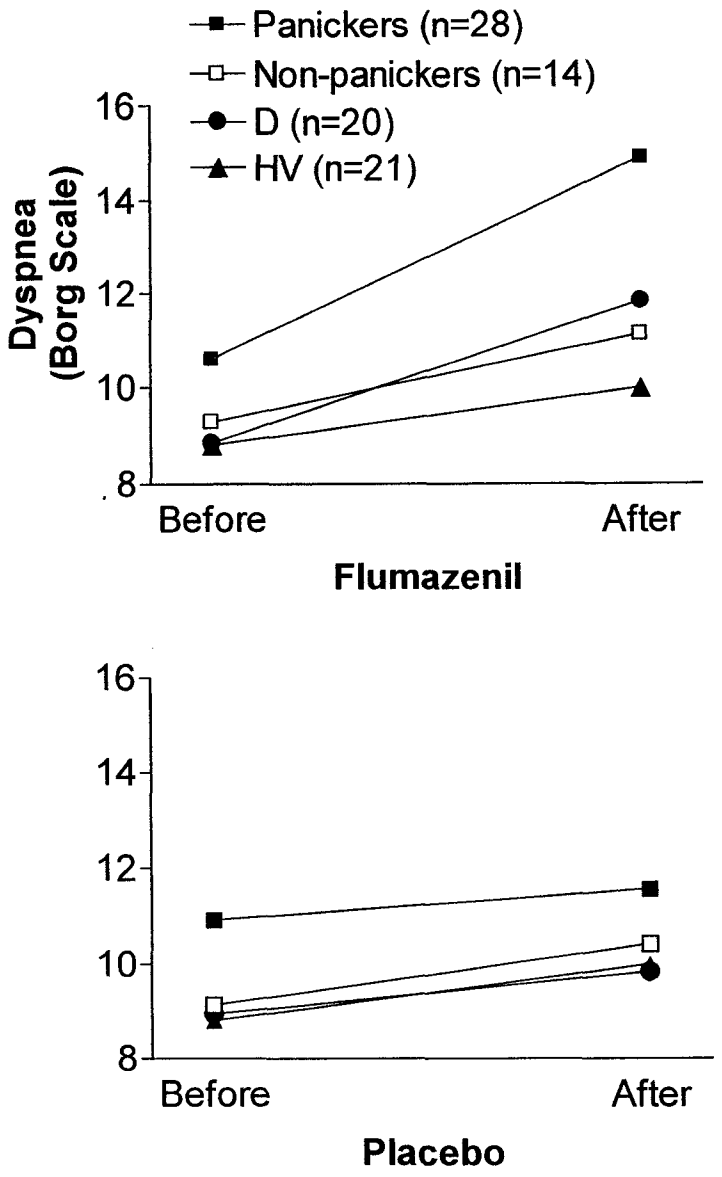

Figure 3.6. Dyspnea ratings of panickers, non-panickers and the D and HV groups before and after flumazenil and placebo.

administration, but a non-significant group x time interaction indicated that this was due to baseline differences. Flumazenil produced more dyspnea than placebo in panickers and the D subjects, where significant drug effects ($p<0.02$) and drug x time interactions were found (Table 3.7). Analysis of changes of dyspnea from baseline showed a significant drug effect ($F=41.95$, $df=1,79$, $p< 0.001$) and drug x group interaction ($F=11.35$, $df=3,79$,

$p < 0.001$). Flumazenil induced greater increases in dyspnea in panickers than in non-panickers and HV ($p < 0.01$) and greater responses than to placebo in panickers and the D subjects ($p < 0.001$). Responses to placebo did not differ among the groups.

Table 3.5. Repeated measures ANOVA results for the subjective measures at baseline before the administration of flumazenil (FL) and placebo (PL).

	Group x Drug		Group		*Post-hoc* tests	Drug		Difference
	(df=3,79)		(df=3,79)			(df=1,79)		
	F	p	F	p		F	p	
PSS sum intensity		NS	7.7	<0.001	$P^{e} = P+D^{e} > HV$ $P+D^{a} > D$		NS	
VAS anxiety		NS	12.8	<0.001	$P^{cf} = P+D^{bf} > D = HV$	4.8	0.031	PL>FL
Borg dyspnea		NS	3.5	0.02	$P+D^{d} > HV$		NS	

[a]p<0.05 vs. D, [b]p<0.01 vs. D, [c]p<0.001 vs. D; [d]p<0.05 vs. HV, [e]p<0.01 vs. HV, [f]p<0.001 vs. HV.

Table 3.6. Repeated measures ANOVA results for the subjective measures with baseline and peak scores as the repeated measures; comparisons between the study groups.

Measure	Group x Drug x Time (df=3,79)		Drug	Group x Time (df=3,79)		*Post-hoc* tests	Group	Drug x Time			Difference
	F	p		F	p			F	df	p	
PSS sum intensity Day1	4.9	0.003	FL	8.4	<0.001	P^{ad} =P+D^{bd}>D=HV	P:	13.0	1,19	0.002	FL > PL
			PL		NS		P+D:	19.4	1,21	<0.001	FL > PL
							D:	21.6	1,19	<0.001	FL > PL
							HV:		1,20	NS	
						P^{d} = P+D^{d} > HV					
Day2	4.4	0.007	FL	8.8	<0.001	P+D^{b} >D	P:	10.5	1,15	0.005	FL > PL
			PL		NS		HV:	19.1	1,20	<0.001	FL > PL
VAS anxiety	3.1	0.031	FL		NS		P:	37.0	1,19	<0.001	FL > PL
			PL		NS		P+D:	26.2	1,21	<0.001	FL > PL
							D:	13.9	1,19	0.001	FL > PL
							HV:	11.2	1,20	0.003	FL > PL
						P^{c} = P+D^{d} > HV					
Borg dyspnea	4.4	0.006	FL	4.0	0.011	P+D^{a} > D	P:	14.8	1,19	0.001	FL > PL
			PL		NS		P+D:	18.8	1,21	<0.001	FL > PL
							D:	15.6	1,19	0.001	FL > PL
							HV:		1,20	NS	

[a]p<0.05 vs. D, [b]p<0.01 vs. D; [c]p<0.05 vs. HV, [d]p<0.001 vs. HV. FL=flumazenil, PL=placebo

Table 3.7. Repeated measures ANOVA results for the subjective measures, with baseline and peak scores as the repeated measures; comparisons between panickers (Panic; n=28), non-panickers (Nonp; n=14) and the D and HV groups.

Measure	Group x Drug x Time (df=3,79)		Drug	Group x Time (df=3,79)		*Post-hoc* tests	Group	Drug x Time			Difference
	F	p		F	p			F	df	p	
VAS anxiety	5.8	0.001	FL	3.0	0.036	Panic[ebc] > Nonp = D=HV	Panic	64.7	1,27	<0.001	FL > PL
			PL		NS		Nonp	9.1	1,13	0.01	FL > PL
							D	Table 6			
							HV	Table 6			
Borg Dyspnea	11.4	<0.001	FL	8.9	<0.001	Panic[dac] > Nonp = D=HV	Panic	53.3	1,27	<0.00 1	FL > PL
			PL		NS		Nonp		1,13	NS	
							D	Table 6			
							HV	Table 6			

[a]p<0.01 vs. D, [b]p<0.001 vs. D; [c]p<0.001 vs. HV; [d]p<0.01 vs. non-panickers, [e]p<0.001 vs. non-panickers. FL=flumazenil, PL=placebo.

Table 3.8. Increases in the intensity of panic symptoms induced by flumazenil (FL) and placebo (PL).

PSS symptom	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)	Kruskal-Wallis test p	*Post hoc* Mann –Whitney tests
Palpitation - FL	1.0 (0.25-2.0)	1.0 (1.0-2.0) [e]	1.0 (0.0-1.0)	0.0 (0.0-0.0)	0.001	$P^b = P+D^c > HV$
- PL	0.5 (0.0-1.0)	1.0 (0.0-1.0)	0.0 (0.0-1.0)	0.0 (0.0-1.0)	0.517	
Dyspnea - FL	1.0 (0.25-2.0) [d]	1.0 (1.0-2.0) [d]	0.5 (0.0-1.0) [d]	0.0 (0.0-0.0)	<0.001	$P^c = P+D^c > HV$ $D^b > HV$
- PL	0.0 (0.0-1.0)	0.5 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.056	
Dizziness - FL	2.0 (1.25-3.0) [f]	2.0 (1.0-3.0) [f]	2.0 (1.0-3.0) [f]	2.0 (1.0-2.0) [e]	0.045	NS
- PL	0.0 (0.0-1.0)	0.5 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.143	
Sweating - FL	0.0 (0.0-2.0) [d]	0.0 (0.0-1.0) [d]	0.0 (0.0-0.75) [d]	0.0 (0.0-0.0)	0.079	
- PL	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.173	
Shaking/trembling - FL	1.0 (0.0-1.0) [d]	1.0 (0.0-2.0) [e]	0.0 (0.0-0.75)	0.0 (0.0-0.0)	0.002	$P^b = P+D^b > HV$
- PL	0.0 (0.0-0.75)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.639	
Nausea -FL	0.0 (0.0-1.0) [d]	0.5 (0.0-1.25) [d]	0.0 (0.0-1.0)	0.0 (0.0-1.0) [d]	0.674	
-PL	0.0 (0.0-0.0)	0.0 (0.0-0.25)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.414	
Chest discomfort - FL	0.0 (0.0-1.0)	0.5(0.0-2.0) [d]	0.0 (0.0-0.75)	0.0 (0.0-0.0)	0.005	$P^b = P+D^b > HV$
- PL	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.06	
Choking - FL	0.0 (0.0-0.0)	0.0 (0.0-1.25) [d]	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.058	
- PL	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.251	
Paraesthesia - FL	1.5 (0.0-2.0) [e]	1.5 (0.0-2.25) [e]	1.0 (0.0-1.0) [e]	0.0 (0.0-0.5)	0.001	$P^b = P+D^b > HV$
- PL	0.0 (0.0-0.75)	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.652	

Values are median (25^{th} -75^{th} percentiles). [a]$p<0.01$ vs. D; [b]$p<0.01$ vs. HV, [c]$p<0.001$ vs. HV by Mann Whitney test. [d]$p<0.05$ vs. PL, [e]$p<0.01$ vs PL, [f]$p<0.001$ vs PL by Wilcoxon test.

Table 3.8. Increases in the intensity of panic symptoms induced by flumazenil (FL) and placebo (PL) – cont'd.

PSS symptom	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)	Kruskal-Wallis test p	*Post hoc* tests
Hot/cold - FL	0.0 (0.0-1.0)	1.0 (0.0-1.0) [d]	0.0 (0.0-0.75)	0.0 (0.0-0.5)	0.203	
- PL	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.014	NS
Unreal/detached - FL	1.0 (0.0-2.0) [d]	1.0 (0.0-3.0) [e]	0.0 (0.0-1.0) [d]	0.0 (0.0-1.0)	0.004	$P^b = P+D^b > HV$
- PL	0.0 (0.0-1.0)	0.0 (0.0-0.25)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.04	NS
Fear of losing control or going crazy - FL	0.5 (0.0-1.0) [d]	1.0 (0.75-2.25) [f]	0.0 (0.0-1.0) [d]	0.0 (0.0-0.0)	<0.001	$P+D^{ac} > D = HV$
- PL	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.759	
Fear of dying - FL	0.0 (0.0-0.0)	0.0 (0.0-0.25)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.291	
- PL	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.649	

Values are median (25^{th}-75^{th} percentiles). [a]$p<0.01$ vs. D; [b]$p<0.01$ vs. HV, [c]$p<0.001$ vs. HV by Mann-Whitney test. [d]$p<0.05$ vs. PL, [e]$p<0.01$ vs PL, [f]$p<0.001$ vs PL by Wilcoxon test.

3.3.3. Physiological measures

Neither flumazenil nor placebo markedly increased any of the objective respiratory, cardiovascular or neuroendocrine variables. Statistically significant findings are described and explained in the following sections, but since they did not follow a consistent pattern, their interpretation is difficult and does not conform in a simple fashion to the study hypothesis. Repeated measures ANOVAs using 6 time points (baseline and post-infusion min 1 through 5) found no differences between the groups for any variable, except for minute volume following flumazenil. The group differences were no longer apparent when the second test day data of the P and HV groups were entered in the analyses. The main results are summarized in Tables 3.9 and 3.10, and in Figures 3.7 -3.20.

Since the results of the above analyses were generally negative, additional ANOVAs were performed to compare changes from baseline in the respiratory and cardiovascular measures between PD panickers and non-panickers, and the D and HV groups at 1 and 2 min time points. Possible group or drug effects, or their interactions, were more likely to be detected during the initial 2-min period, because flumazenil-induced panic attacks typically started within 40 seconds and lasted 3-4 min, and there was a steep decline in flumazenil plasma concentration after 2 min (Zedkova et al 2001 and chapter 5 of this thesis). Since MV and TV in panickers peaked at 3 and 4 min, respectively, in these variables the comparisons were also performed for these time points.

Respiratory: Due to incomplete data in some subjects, the respiratory parameters of only 17 P, 18 P+D, 17 D and 20 HV subjects were analyzed. The main results of repeated measures ANOVA comparing responses between the 4 study groups, and between panickers, non-panickers and the D and HV groups are summarized in Table 3.9. None of these groups differed significantly in their baseline respiratory measures before either flumazenil or placebo.

MV data were log transformed before being analyzed. No day or order effects were found for MV in the P and HV subjects, and no order effect in the P+D and D subjects. Raw data are presented in Figure 3.7. A significant time effect ($F=8.8$, $df=5,340$, $p<0.001$, $\varepsilon=0.737$) and a drug x time x group interaction (Table 3.9) were found for MV. Separate analyses of each drug demonstrated that flumazenil produced significantly higher MV responses in the P group than in the HV group, with a peak at 2-3 min. There were no significant group differences in MV following placebo. P was the only group with a significant drug x time interaction, due to a greater increase in MV after flumazenil.

Repeated measures ANOVA comparing panickers (n=26), non-panickers (n=9) and the D and HV groups found a significant group effect ($F=2.91$, $df=3,68$, $p=0.04$), time effect ($F=7.12$, $df=5,340$, $p<0.001$, $\varepsilon=0.731$), group x time interaction ($F=2.0$, $df=15,340$, $p=0.029$, $\varepsilon=0.731$), and a nearly significant drug x time x group interaction (Table 3.9). Figure 3.8 and *post-hoc* testing revealed that this was caused mainly by significantly higher MV responses to flumazenil in panickers than in HV. A significant

drug x time interaction found only in panickers was caused by their greater MV responses to flumazenil compared to placebo.

Higher MV increases were found in panickers compared with the D and HV groups at 1 min ($p=0.014$ for panickers vs D; $p=0.038$ for panickers vs HV). The lack of a significant drug x group interaction indicated that the groups' responses to flumazenil did not differ significantly from those to placebo. A significant drug x group interaction was found at 3 min ($F=2.8$, $df=3,68$, $p=0.044$). This was due to higher MV increases in panickers than in the D group ($p=0.04$), and due to higher MV increases in panickers after flumazenil than after placebo ($p=0.021$).

To assess whether the effect of flumazenil on MV was reproducible, the second test day data of the P and HV groups were compared with data of the other subjects. The effect of time was the only significant finding (Figure 3.9). Similarly, only a significant time effect was found when panickers ($n=23$) were compared with non-panickers ($n=8$) and the D and HV groups.

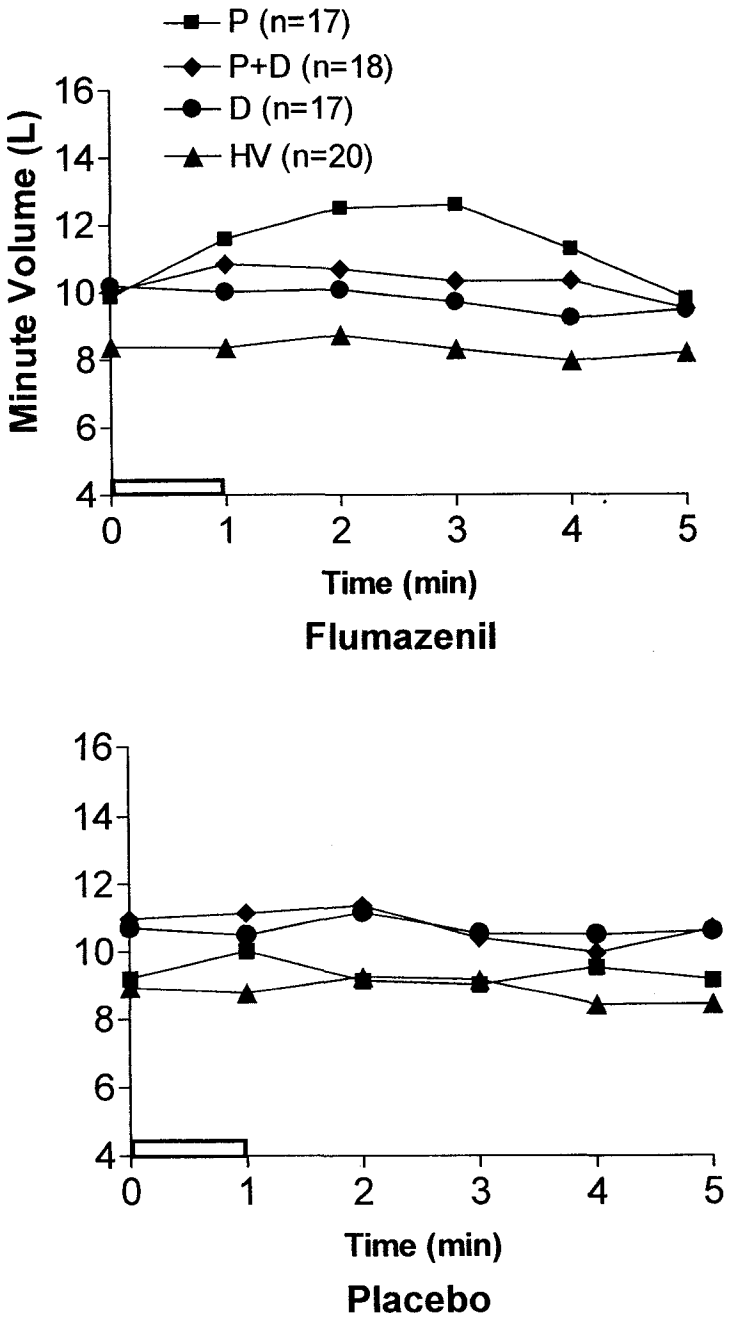

Figure 3.7. Minute volume responses of the four study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

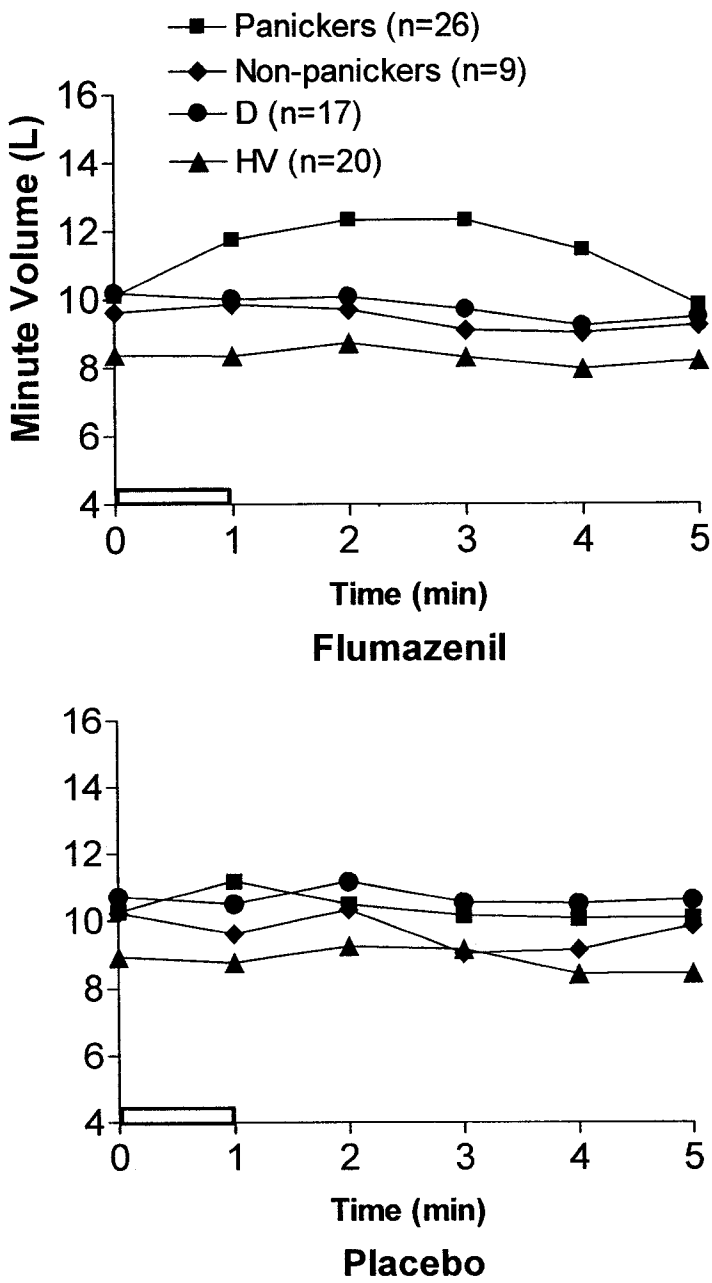

Figure 3.8. Minute volume responses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

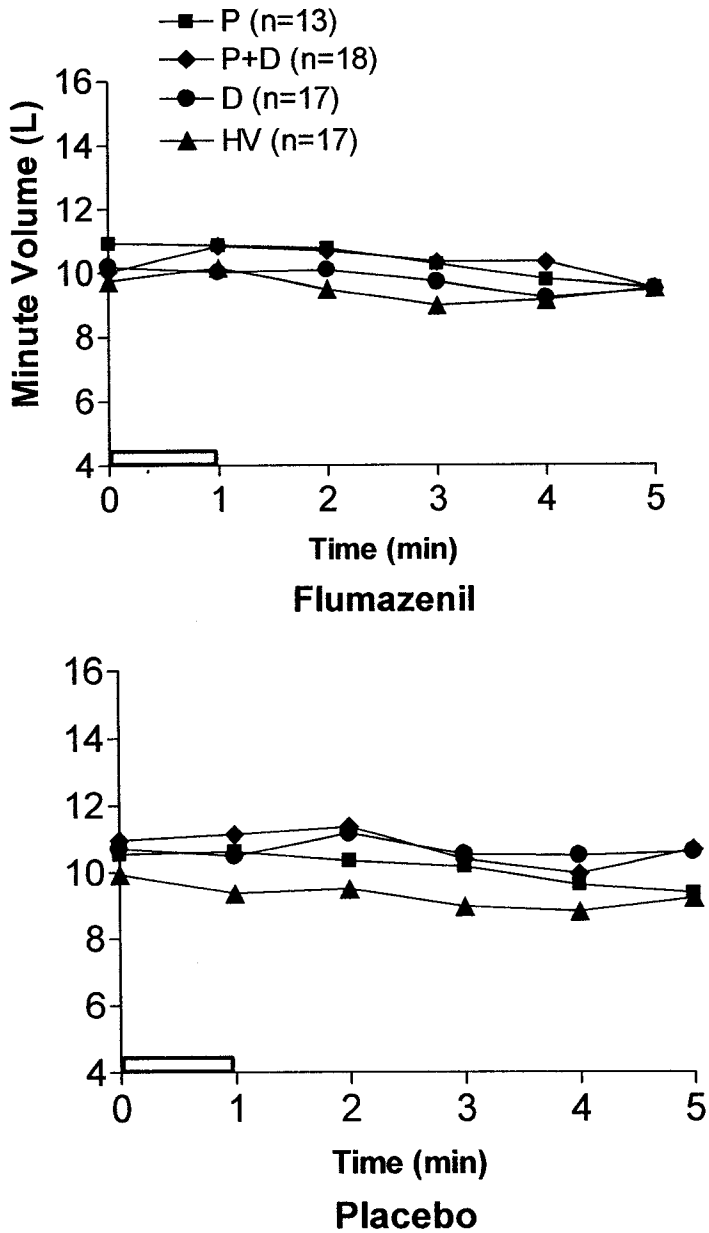

Figure 3.9. Minute volume responses of the four study groups to flumazenil and placebo. Test day 2 data are presented in the P and HV groups. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

Logarithmic transformation was also performed on the TV data. No significant effect of day or order was found in the P and HV groups, and no effect of order in the P+D and D groups. Repeated measures ANOVA revealed a significant time effect ($F=4.88$, $df=5,340$, $p<0.001$, $\varepsilon= 0.766$) and group x time and drug x time interactions (Table 3.9). Figure 3.10 (raw data) indicates that the group x time interaction was caused by a decrease in TV in the D group within 1 min after both infusions, while TV in other groups was rising. When the D group was not included in the comparison, this interaction was no longer significant. Different effects of the drugs on TV over time were apparent primarily in the P group, where flumazenil caused a bigger increase with a peak at 4 min ($F=3.29$, $df=5,80$, $p=0.035$, $\varepsilon=0.529$).

Analysis comparing panickers, non-panickers and the D and HV groups found a significant effect of time ($F=4.15$, $df=5,340$, $p=0.003$, $\varepsilon=0.775$), and group x time and drug x time interactions (Table 3.9). Figure 3.11 shows a decline in TV in non-panickers and the D group in the first minute, while there was an increase in panickers and HV. The drug x time interaction was caused by a higher increase in TV in panickers after flumazenil, lasting 4 min ($F=4.73$, $df=5,125$, $p=0.003$, $\varepsilon=0.693$).

Significantly higher increases in TV were found in panickers than in the D group at 1 min ($p=0.02$), but the drug x group interaction was not significant. Panickers showed higher increases than non-panickers at 4 min ($p=0.002$), but again, the drug x group interaction was not significant.

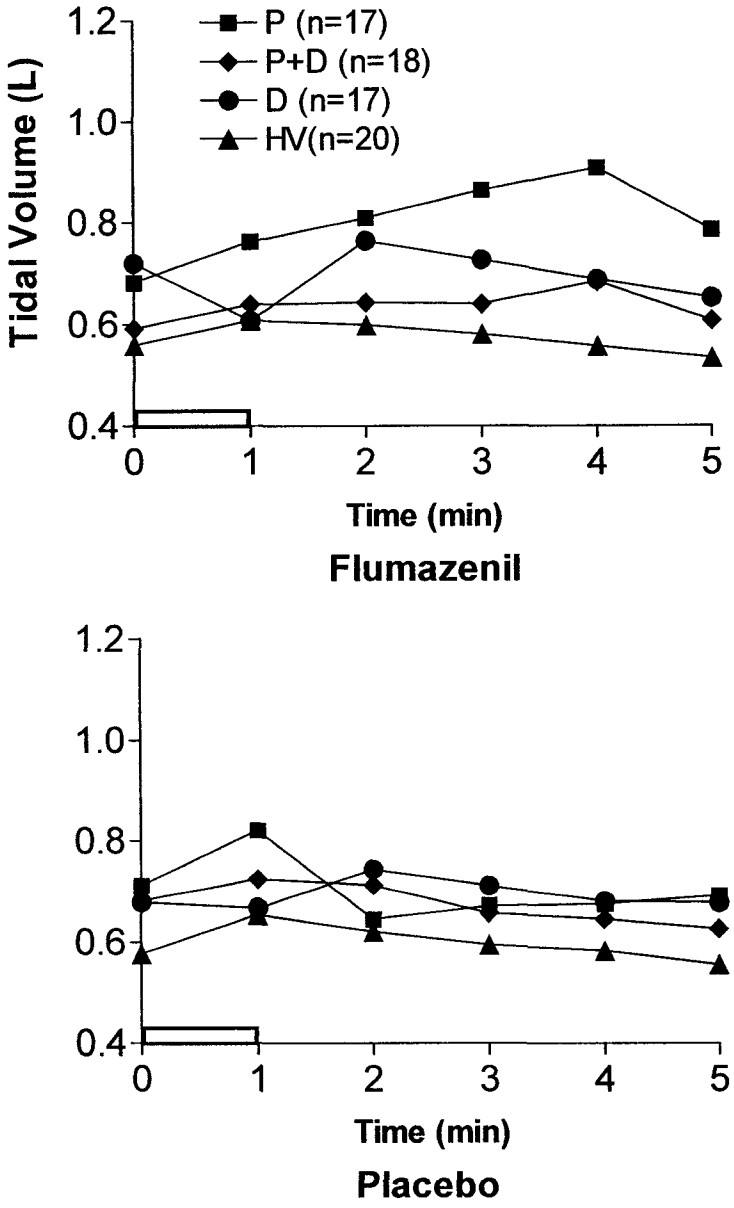

Figure 3.10. Tidal volume responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

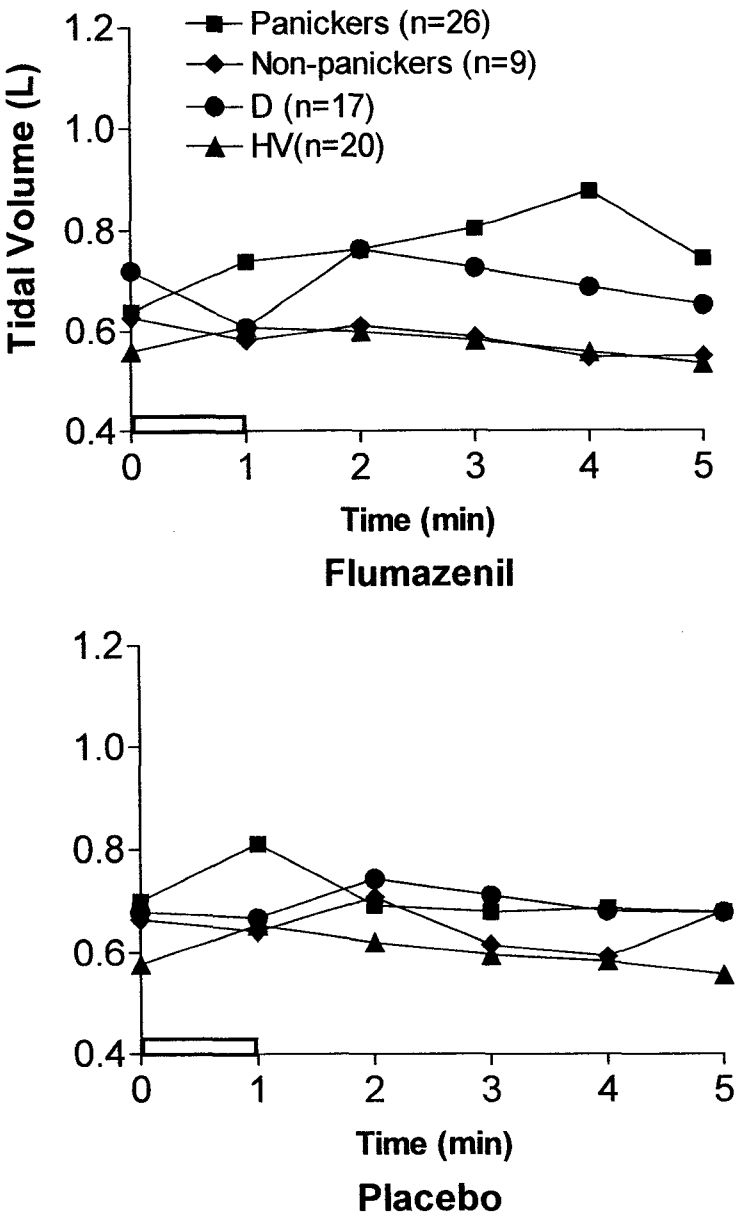

Figure 3.11. Tidal volume responses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

No day or order effects were found for RR in the P and HV groups, and no order effect in the P+D and D groups. Repeated measures analysis detected a significant time effect ($F=3.12$, $df=5,340$, $p=0.019$, $\varepsilon=0.728$) and drug x time interaction (Table 3.9). As Figure 3.12 indicates, flumazenil mildly increased RR in the patient groups with peak at 1 - 2 min, while there was a decrease in RR in all groups within a 3-min interval following placebo. The steeper decline in RR in the D group starting 1 min after flumazenil also contributed to the interaction.

Significant group x time and drug x time interactions were found when panickers and non-panickers were compared with the D and HV groups (Table 3.9). The group x time interaction was caused primarily by different group responses to flumazenil (Figure 3.13); there was an increase in RR in the patient groups versus a decrease in the HV group within the first post-infusion minute, followed by a decrease below baseline in the D group. The drug x time interaction was again due to different effects of the drugs within the first post-infusion minute (flumazenil mildly increased RR in all the groups except HV, while there was a mild decrease in RR after placebo in all the groups), and to the decline in RR in the D group 1 min after flumazenil.

Consequently, a significant drug effect was found for changes in RR from baseline at 1 min ($F > P$; $F=4.2$, $df=1,68$, $p=0.04$); the drug x group interaction was not significant.

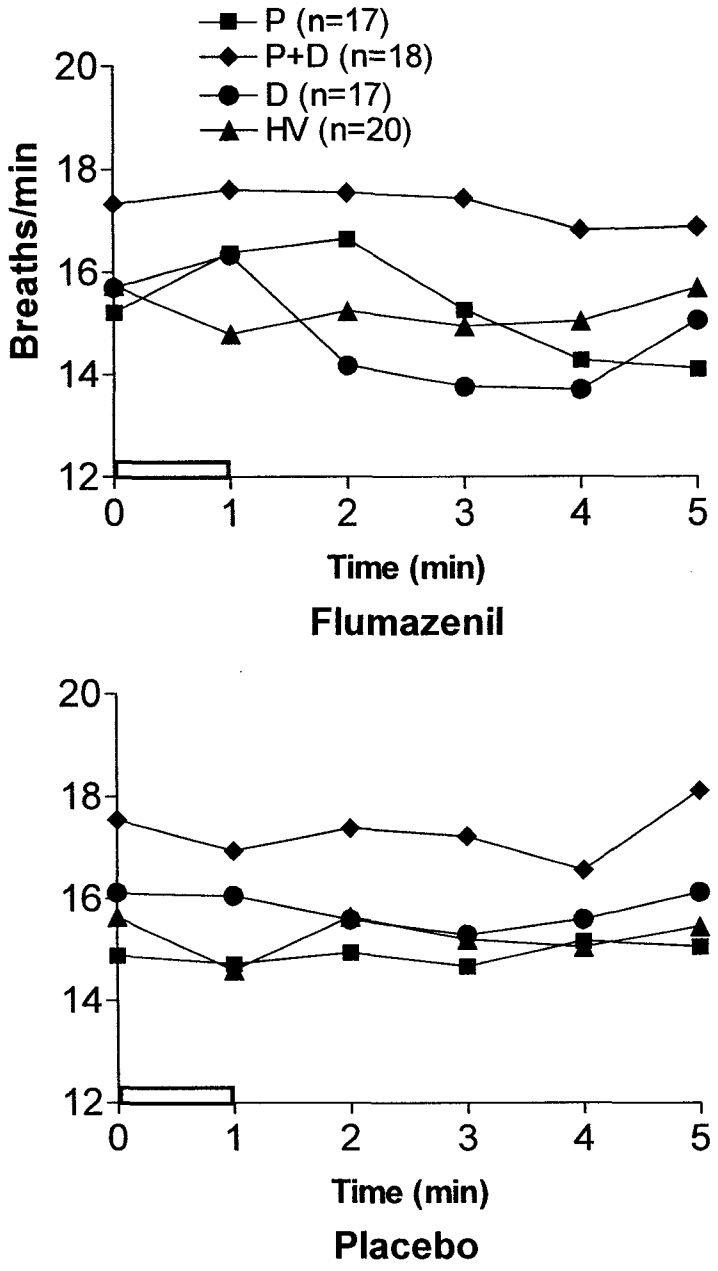

Figure 3.12. Respiratory rate responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

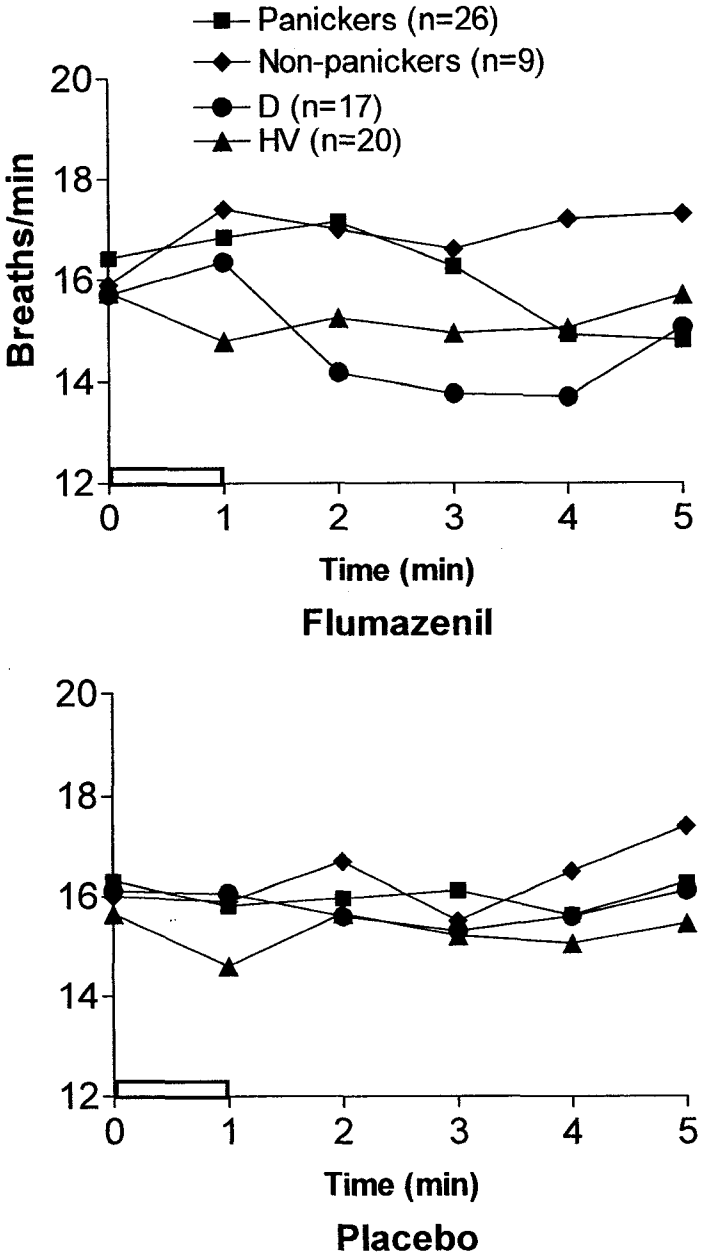

Figure 3.13. Respiratory rate responses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

Table 3.9. Respiratory measures of the study groups (P, P+D, D, HV), and of panickers (Panic), non-panickers (Nonp) and the D and HV groups after flumazenil (FL) and placebo (PL).

Measure	Group x Drug x Time (df=15,340)			Drug	Group x Time (df=15,340)			*Post-hoc*	Group	Drug x Time				Difference
	F	p	ε		F	p	ε			F	df	p	ε	
MV														
P, P+D, D, HV	2.1	0.019	0.798	FL:	1.8	0.05	0.713	P[a] > HV	P:	4.0	5,80	0.021	0.478	FL > PL
				PL:		NS			P+D:			NS		
									D:			NS		
									HV:			NS		
Panic, Nonp, D, HV	1.8	0.052	0.789	FL:	2.2	0.016	0.717	Panic[a] > HV	Panic:	4.3	5,125	0.007	0.615	FL > PL
				PL:		NS			Nonp:			NS		
TV														
P, P+D, D, HV		NS		FL,PL:	1.9	0.036	0.766		All:	4.1	5,340	0.003	0.851	P:FL>PL
Panic, Nonp, D,HV		NS		FL,PL:	2.8	0.001	0.775		All:	2.5	5,340	0.039	0.845	Panic: FL > PL
RR														
P, P+D, D, HV		NS				NS			All:	3.3	5,340	0.01	0.872	See text
Panic, Nonp, D, HV		NS		FL,PL:	2.5	0.005	0.724		All:	2.4	5,340	0.049	0.873	See text

[a]$p<0.05$ vs. *H*

Cardiovascular: Cardiovascular data were missing in one P and one P+D subject. The main results of repeated measures ANOVA comparing responses between the four study groups, and between panickers, non-panickers and the D and HV groups are summarized in Table 3.10. None of the groups differed significantly in their baseline cardiovascular measures before flumazenil or placebo.

The P and HV groups had a significantly higher SBP on the first test day ($F=27.84$, $df=1,32$, $p<0.001$). No order effect for SBP was found in these groups and no order effect in the P+D and D groups. Only a significant effect of time was found over the post-infusion 5 min interval ($F=75.2$, $df=5,385$, $p<0.001$, $\varepsilon=0.873$), caused by a mild increase in SBP in all groups in the first minute after both flumazenil and placebo, followed by a decrease beyond baseline values throughout the test period (Figure 3.14).

Similarly, repeated measures ANOVA comparing panickers, non-panickers and the D and HV groups found only a significant effect of time ($F=74.06$, $df=5,385$, $p<0.001$), due to a mild increase in SBP in all groups lasting one minute after both infusions, followed by a decrease beyond baseline.

No order effect, but a significant day effect, was found for DBP in the P and HV groups (day1>day2: $F=22.23$, $df=1,32$, $p<0.001$). No order effect was found for DBP in the P+D and D groups. As with SBP, the initial mild increase in DBP after both drugs was followed by a decrease below baseline values (Figure 3.15). There was a significant

time effect (F=136.9, df=5,385, p<0.001, ε=0.715) and drug x time interaction (Table 10), due to a bigger decrease in DBP after flumazenil.

Comparison between panickers, non-panickers and the D and HV subjects resulted in a significant effect of time (F=129.0, df=5,385, p<0.001, ε=0.722), and drug x time (F=3.22, df=5,385, p=0.012, ε=0.818) and drug x time x group (Table 3.10) interactions. Figure 3.16 demonstrates that after a 1-minute period, all groups showed a continuous decline in DBP, which was bigger after flumazenil and slightly delayed in panickers. The immediate decline in non-panickers and HV after flumazenil caused significant drug x time interactions in these groups and resulted in the overall drug x group x time interaction.

A significant drug x group interaction was found for changes in DBP from baseline at 2 min (F=5.4, df=3,77, p=0.002), caused by a greater decrease in panickers compared with the HV group following placebo (p=0.01).

No significant day or order effects for HR were found in the P and HV groups, and no order effect in the P+D and D groups. Repeated measures analysis revealed a significant effect of time (F=18.64, df=5,385, p<0.001, ε=0.554) and drug x time interaction (Table 3.10), which was primarily caused by a smaller increase in HR after flumazenil compared to placebo within the first post-infusion min, due to relatively mild responses in the P+D and D groups (Figure 3.17). After the first minute, HR returned to baseline values within 4 min after both infusions.

Analysis comparing panickers, non-panickers and the D and HV groups showed a significant effect of group ($F=2.8$, $df= 3,77$, $p=0.046$), time ($F=16.52$, $df=5,385$, $p<0.001$, $\varepsilon=0.54$), and group x time ($F=2.74$, $df=15,385$, $p=0.007$, $\varepsilon=0.54$), drug x time ($F=3.03$, $df=5,385$, $p=0.034$, $\varepsilon=0.554$) and drug x time x group (Table 3.10) interactions. Figure 3.18 shows that panickers and non-panickers differed the most in their HR responses. However, *post-hoc* analysis of the group effect did not detect any significant group differences. Separate analyses of each drug and group indicated that the above findings were primarily caused by different HR group responses to flumazenil; there was an increase in HR in panickers lasting 2 min, while the other groups showed a mild elevation in the first minute followed by a decrease. Panickers were the only group with a significant drug x time interaction, due to a longer lasting increase in HR after flumazenil. When panickers were excluded from the overall analysis, the time effect was the only finding that remained significant.

A significant drug effect ($F=5.9$, $df=1,77$, $p=0.017$) was found for changes in HR from baseline at 1 min, caused by a higher increase in all the groups following placebo. Panickers showed higher increases in HR than non-panickers and the D group at 2 min ($p=0.041$ for panickers vs non-panickers; $p=0.016$ for panickers vs D); the lack of a significant drug x group interaction indicated that responses to flumazenil were not significantly different form those to placebo in the groups.

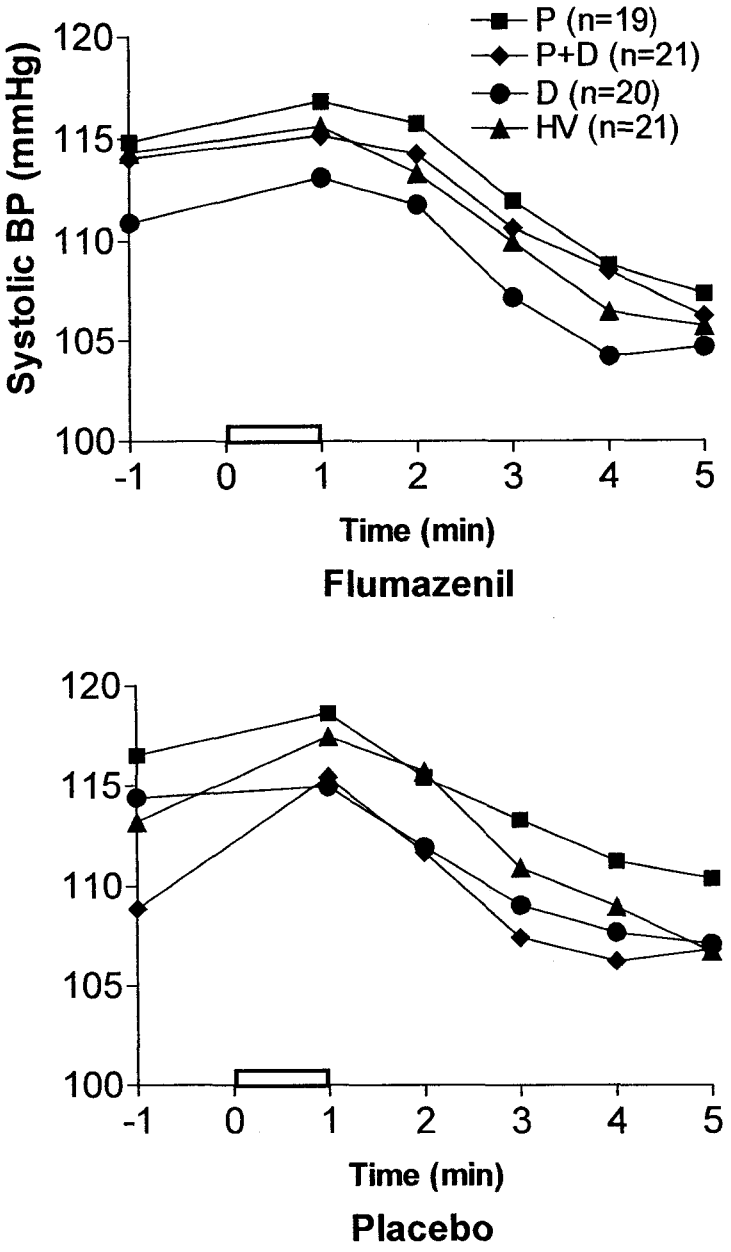

Figure 3.14. Systolic blood pressure responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

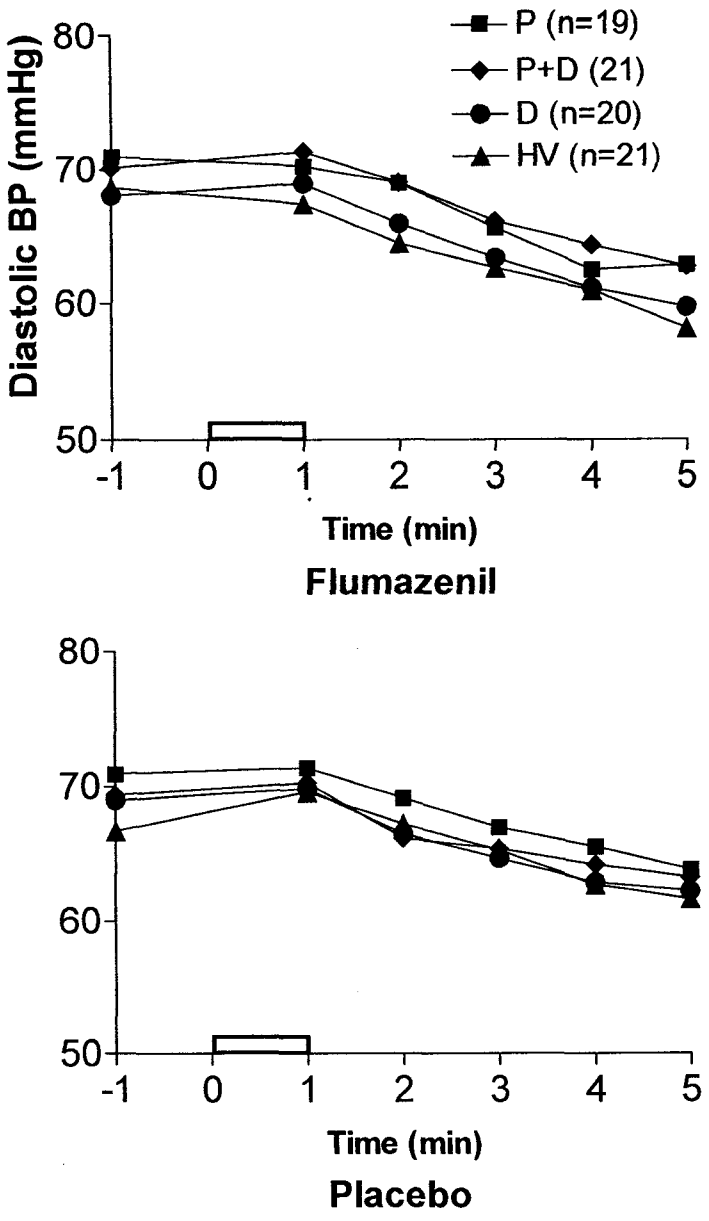

Figure 3.15. Diastolic blood pressure responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

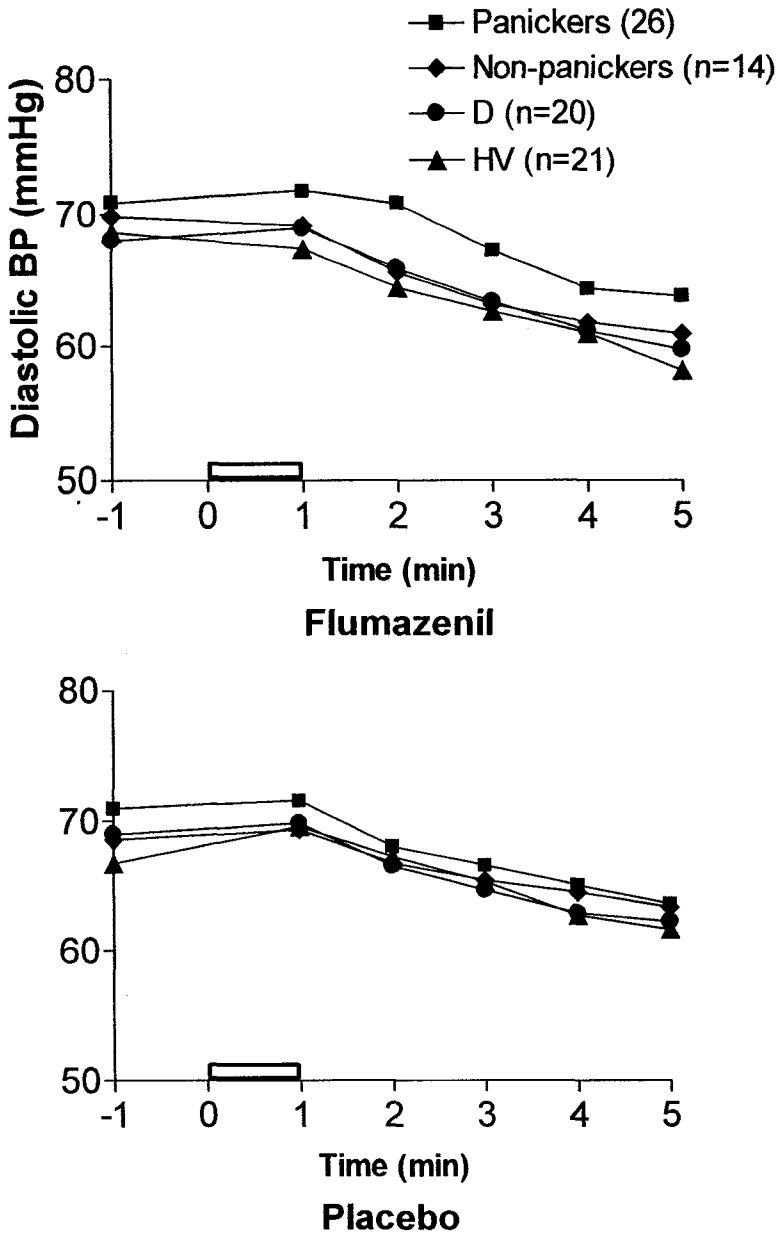

Figure 3.16. Diastolic blood pressure responses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

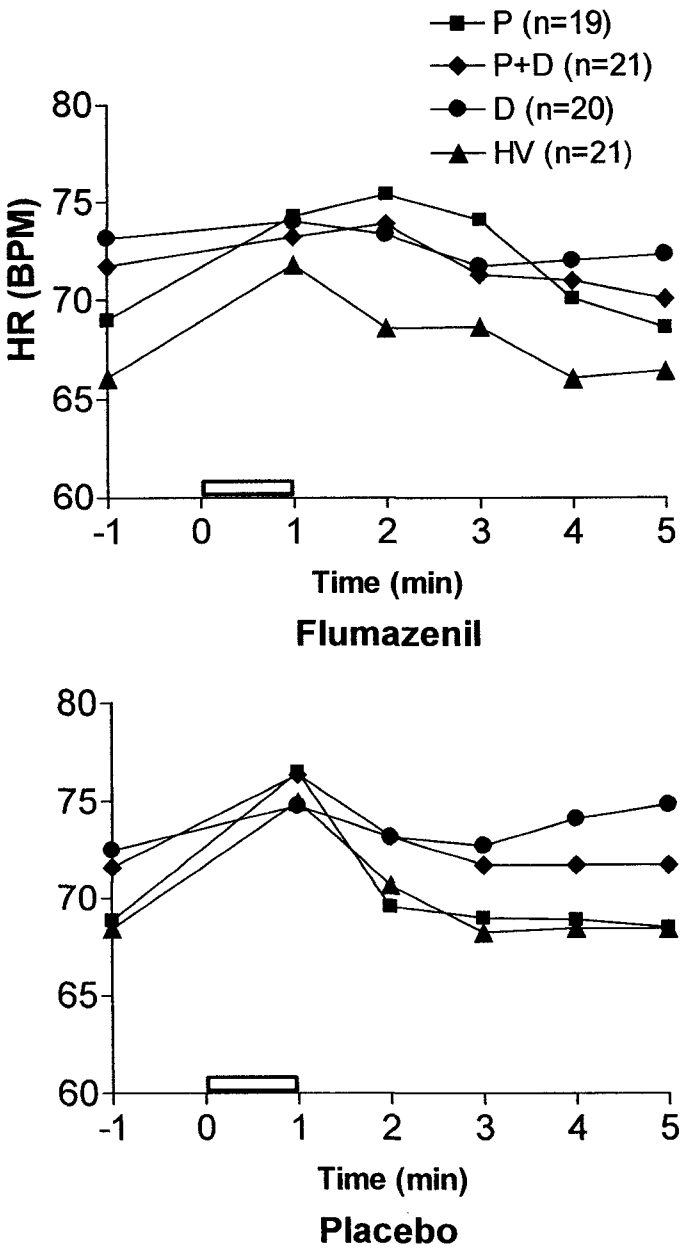

Figure 3.17. Heart rate responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

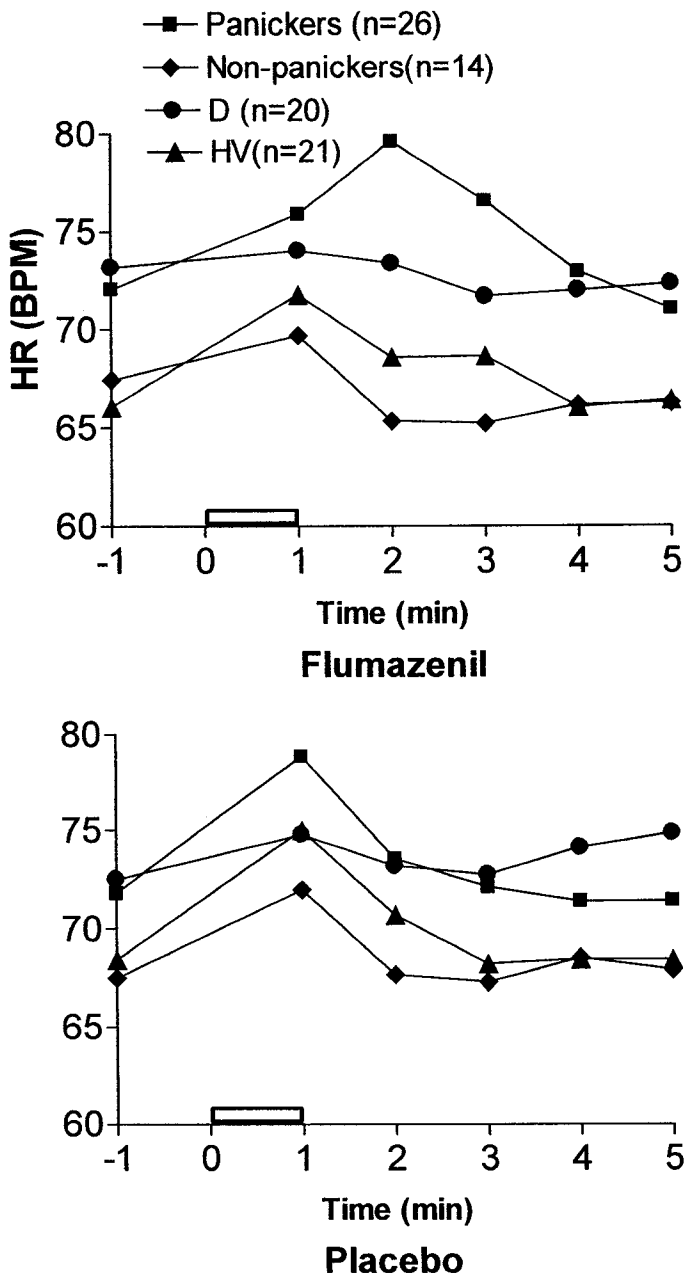

Figure 3.18. Heart rate reponses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

Table 3.10. Cardiovascular measures of the study groups (P, P+D, D, HV), and of panickers, non-panickers and the D and HV groups after flumazenil (FL) and placebo (PL).

Measure	Group x Drug x Time (df=15,385)			Drug	Group x Time (df=15, 385)			Group	Drug x Time				Difference
	F	p	ε		F	p	ε		F	df	p	ε	
SBP													
P, P+D, D, HV		NS				NS					NS		
Panic, Nonp, D, HV		NS				NS					NS		
DBP													
P, P+D, D, HV		NS				NS		All:	2.8	5,385	0.028	0.798	FL < PL
Panic, Nonp, D, HV	1.8	0.039	0.818	FL:		NS		Panic:			NS		
				PL:		NS		Nonp:	2.8	5,65	0.049	0.651	See text
								D:			NS		
								HV:	3.8	5,100	0.012	0.665	See text
HR													
P, P+D, D, HV		NS				NS		All:	4.6	5,385	0.005	0.545	See text
Panic, Nonp, D, HV	2.1	0.038	0.554	FL:	2.7	0.005	0.595	Panic:	5.2	5,125	0.007	0.437	See text
				PL:		NS		Nonp:			NS		
								D:			NS		
								HV:			NS		

Cortisol: Logarithmic transformation was performed on the cortisol data. In the P and HV groups, cortisol data for flumazenil or placebo when given as the first infusions of each test day were used for the analyses, to control for the cortisol circadian rhythm. No significant effect of order was found in the groups. Cortisol responses to flumazenil and placebo are shown in Figure 3.19. The groups did not have significantly different baseline cortisol levels before either infusion. Due to the absence of four P subjects on the second test day and incomplete data due to missing samples for other subjects, only 12 P, 21 P+D, 19 D and 20 HV subjects were analyzed by repeated measures ANOVA. The only significant finding was a time effect ($F=39.9$, $df=4,272$, $p<0.001$, $\varepsilon=0.597$), caused by a mild increase in the P and P+D groups in the first 15 min following both flumazenil or placebo, followed by a continuous mild decrease in all groups during the study period.

Similarly, analysis comparing panickers (n=23), non-panickers (n=10) and the D and HV groups found only a significant effect of time ($F=37.23$, $df=4,272$, $p<0.001$, $\varepsilon=0.596$), caused by a continuous mild decline in cortisol values in all groups except for panickers, who showed a mild increase following both flumazenil or placebo within the first 15 min (Figure 3.20).

Significantly higher increases in cortisol were found in panickers than in non-panickers at 15 min ($p=0.004$), but the lack of a significant drug x group interaction suggested that this was not specific for flumazenil.

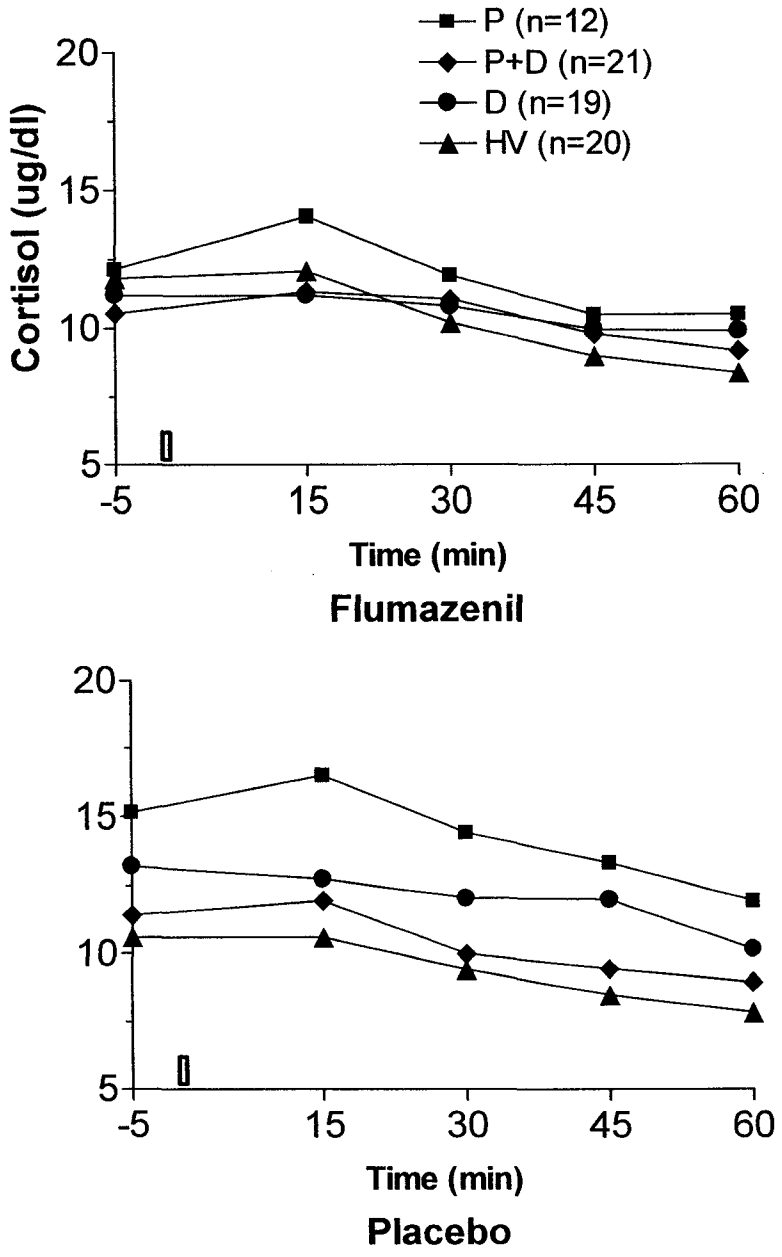

Figure 3.19. Serum cortisol responses of the study groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

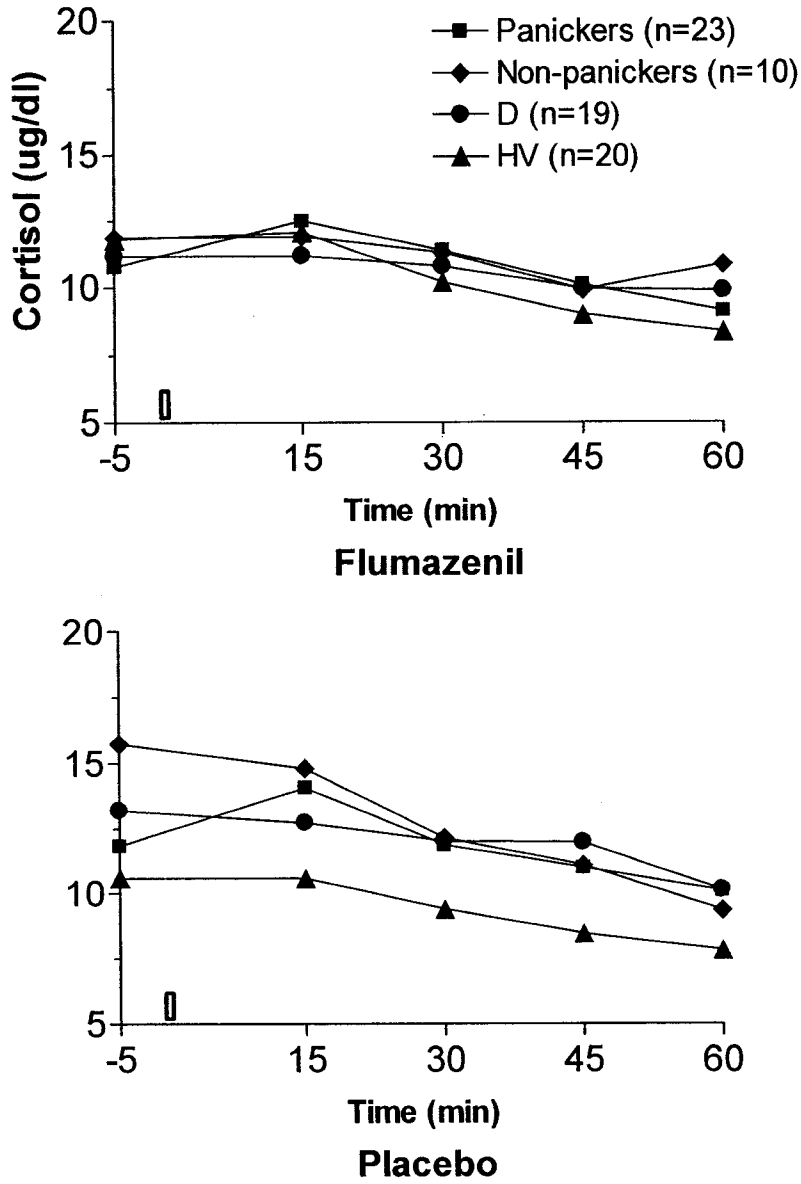

Figure 3.20. Cortisol responses of panickers, non-panickers and the D and HV groups to flumazenil and placebo. Values are group means. The bar on the X-axis indicates the time of the infusion (0-1 min).

3.4. Discussion

The objectives of this study were to replicate the finding that flumazenil has a panicogenic effect in patients with PD, to test the specificity of this effect for PD, and to assess whether this effect is consistent with flumazenil being a partial inverse agonist in PD. In addition, the reproducibility of flumazenil actions was tested.

3.4.1. Panicogenic effect of flumazenil and its specificity

This study replicated previous findings that found flumazenil to be panicogenic in patients with PD (Nutt et al 1990; Woods et al 1991; Bell et al 2002). In patients with pure PD (P) and those with comorbid PD and major depression (P+D), flumazenil provoked higher panic rates and overall panic symptom intensity compared with patients with pure major depression (D) and healthy volunteers (HV). These effects were clearly distinguishable from those of placebo. These results suggest that the neurobiological and/or cognitive abnormality that underlies sensitivity to the panicogenic action of flumazenil is specific for PD. Thus, in addition to CO_2 (Kent et al 2001; Perna et al 1995), fenfluramine and sodium lactate (Targum 1990), flumazenil is another panicogen that, by discriminating PD from the D patients, indicates that although PD and major depression show high comorbidity, they are nevertheless distinct.

However, the specificity of panicogenic actions of an agent may also depend on the dose administered and the criteria used to determine the subjects' sensitivity. For example, while 5% CO_2 appeared to be significantly more panicogenic in panic patients than in depressed patients, this distinction was lost using 7% CO_2, due to increased panic

rates in depressed subjects (Kent et al 2001). The relatively low threshold set to determine the anxiogenic properties of 35% CO_2 in some studies (Battaglia and Perna 1995) may result in an overestimation of PD patients' hypersensitivity. In this study, applying the PSS definition of a panic attack resulted in lower panic rates in PD patients compared with the rates determined by the semi-structured interview, and therefore in the loss of a statistically significant difference between the P and D subjects (although only one D subject experienced panic). Similar results were reported by a recent study, in which objective criteria for a panic attack from a panic symptom inventory (similar to the PSS criteria applied here) determined lower panic rates after flumazenil in PD patients than the patients' own subjective assessment of their experience (Bell et al 2002). Although in the present study flumazenil-induced attacks defined by both criteria did not differ from the patients' most recent severe panic attacks in their overall severity, the PSS-defined attacks and the patients' severe attacks had higher within-subject similarity. This suggests that the PSS panic criterion may be too tight, since it seemed to detect primarily attacks that were close to the patients' severe (rather than typical) panic attacks, which resulted in the lower panic rates and an apparently decreased specificity of the panicogenic action of flumazenil for PD. However, subjects with comorbid PD and depression showed significantly higher panic attack rates than the D subjects, even when this stringent criterion was applied.

In PD subjects (the P and P+D groups), flumazenil caused greater increases in several panic symptoms compared with the HV group, excluding dizziness, sweating, nausea, choking, hot flushes/cold chills, and fear of dying, for which no group differences

were found. With the exception of dizziness, this was due to low scores on these symptoms across the groups. Dizziness was the only symptom in this study that panickers reported to be higher during flumazenil-induced panic than during their natural severe panic. In fact, it was the most prominent symptom in all subjects (median increase of 2 in all groups), which is consistent with previous studies in which volunteers reported dizziness in response to flumazenil (Darragh et al 1983; Nutt et al 1990).

The groups did not differ in their anxiety responses to flumazenil, although these were higher than those to placebo. The P and P+D subjects showed higher post-infusion scores than the D and HV subjects, but this was due to their higher baseline anxiety. These results were unexpected, since PD subjects clearly showed higher sensitivity to panicogenic effects of flumazenil than the other groups, despite similar baseline group differences in the overall panic symptom intensity. One explanation for the relatively small increases in anxiety seen in PD subjects could be a small remaining range of the VAS, preventing already anxious subjects from accurately rating their peak anxiety following flumazenil. However, this was unlikely to be the cause, since peak anxiety ratings generally did not reach the upper 100mm range of the scale (P: mean = 67.0, median = 72.0; P+D: mean = 66.6, median = 73.3). A more likely explanation than a ceiling effect on the scale might be the use of "the most anxious I have ever felt" as the upper anchor of the scale. This comparison with the past experience could have caused relatively low ratings in PD subjects with a history of severe anxiety, and relatively high ratings in subjects with less experience of severe anxiety (the D and HV subjects). Although studies of PD have often used similar types of VAS for anxiety ratings (Bell et

al 2002; Charney et al 1992; Nutt et al 1990) it is possible that a different choice for the upper anchor of the VAS, such as "extreme anxiety" (Miller et al 2000) could have distinguished PD subjects from other groups. Nevertheless, PD patients who panicked in response to flumazenil did show higher anxiety ratings on this scale than PD non-panickers and the D and HV groups, when baseline and peak scores were analyzed as repeated measures. In addition, the PD groups showed higher increases than the HV group ($p=0.003$ for P vs HV; $p < 0.001$ for P+D vs HV by Mann-Whitney test), and PD panickers higher increases than non-panickers and the D and HV groups ($p < 0.001$ by Mann-Whitney test) in the scores on anxiety/fear item of the PSS.

Flumazenil provoked greater dyspnea in PD patients than in HV, and greater dyspnea in the comorbid group than in the D group. In addition, PD panickers experienced higher dyspnea after flumazenil than PD non-panickers. Dyspnea will be discussed in more detail in the context of objective respiratory measures.

3.4.2. Reproducibility

In subjects receiving flumazenil on two occasions (P and HV groups), overall panic attack rates (assessed both by the interview and PSS) and the number of panic symptoms were not significantly different after the two infusions, and independent of the order of administration (i.e. whether flumazenil was administered as the first or second infusion on a test day).

Compared to the first administration, the second administration of flumazenil to the P subjects had a smaller effect on their sum intensity of panic symptoms, and their scores (peak scores, and baseline and peak scores analyzed as repeated measures) were

no longer significantly different from those of the D group, although still significantly higher than those of the HV group. However, the P group also showed lower baseline PSS scores on the second test day, and although the difference was not significant ($p=0.073$), it could have contributed to the lower peak PSS scores. This was confirmed by a non-significant day x time interaction in ANOVA analysis using baseline and peak scores as repeated measures. Accordingly, changes from baseline in the PSS scores were not significantly different after the two flumazenil infusions. The ratings from the first and second test day from the P and HV subjects (peak scores and increases from baseline) showed relatively high internal consistency, as indicated by ICCs. However, a weaker panicogenic action of the second infusion of flumazenil was evident in relation to its ability to reliably induce *full* panic attacks (determined by the interview). Using this criterion, only 2 out of 10 first test day panickers from the P group experienced panic after the second administration of flumazenil. However, the power of this analysis was reduced, since 2 of these 10 patients did not attend their second test. Further, 4 patients had subthreshold panic attacks (i.e., moderate anxiety and 4 symptoms) following the second infusion. One P subject had subthreshold panic after the first and full panic after the second infusion of flumazenil. When both subthreshold and full panic were considered, the reproducibility of panic rates following flumazenil was higher.

Compared with the first flumazenil infusion, the subjects' baseline and peak anxiety was found lower on the second test day before and after flumazenil infusion, perhaps due to the familiarity of the procedure. However, changes from baseline after the

first and second infusion were not different. Ratings of dyspnea were not differentially affected by the repeated administration of flumazenil.

3.4.3. Assessment of an inverse agonistic activity of flumazenil

The suggestion that flumazenil acts as a partial inverse agonist in PD (Nutt et al 1990) led to the hypothesis that in PD patients, flumazenil would cause cardiovascular, respiratory and neuroendocrine activation, as was observed in studies in which inhibitory GABAergic transmission was reduced in animals or humans (Sanders and Shekhar 1995; Shekhar and DiMicco 1987; Dorow et al 1983). However, flumazenil did not produce marked or consistent effects on these physiological measures in any group, including PD patients who panicked in response to the drug.

Physiological responses of PD patients in studies that used flumazenil as a challenge have been rather inconsistent. Flumazenil increased (Nutt et al 1990), decreased (Strohle et al 1999), or did not affect (Woods et al 1991) heart rate and systolic blood pressure, while diastolic blood pressure remained unaffected. Plasma cortisol following flumazenil was decreased (Strohle et al 1999) or unaltered (Woods et al 1991). Although some negative results could have been due to the lack of panicogenic effect of flumazenil (Strohle et al 1999), pharmacokinetic factors may also have played a role. Panicking PD subjects in the study by Woods et al (1991) did not show physiological activation, but in this study an oral dose of flumazenil was used that was approximately 15 times higher than intravenous doses used in other studies (Nutt et al 1990; Strohle et al 1999; Strohle et al 1998). Oral flumazenil has variable bioavailability (Malizia and Nutt

1995), which precluded its development for administration by this route and may have led to differences in the timing of responses, compared with rapid intravenous administration. In addition, possible non GABA-related actions of the high dose of flumazenil, such as potentiation of the depressant effect of adenosine by inhibition of its reuptake (Polc 1988), complicate interpretation of the results. However, in the present study, marked physiological effects were not found despite clear subjective effects.

After the initial 1-min period of no change or a mild increase, there was a decrease in systolic and diastolic blood pressure after flumazenil in most subjects, although this was a little delayed in those who panicked in response to the drug. Given that the pattern of blood pressure decrease occurred after placebo, this was likely to be related to the post-injection relief rather than to the direct effect of the drug. Even lower blood pressure found in the P and HV groups after the second administration of flumazenil indicates that during their first test this variable was likely to be affected by higher anxiety and symptom intensity, and/or the novelty of the situation. It is therefore possible that the flumazenil-induced increase in systolic blood pressure and heart rate seen in PD patients in one study (Nutt et al 1990) may have been related to more severe panic attacks in subjects who were, unlike patients in this study, recruited through clinical referrals.

This is the first report on the objective measures of respiration in response to flumazenil. A mild increase in minute volume in the P subjects and PD panickers after the first administration of flumazenil, which discriminated them from healthy controls, did not occur after its repeated administration, which may have been related to the higher

first test day anxiety and panic symptoms, rather than to the specific pharmacological effects of flumazenil. In addition, a closer inspection of the data revealed that one panicker from the P group, who did not attend the second test, showed markedly increased minute volume compared with other panickers during the first test.

No robust increases in cortisol levels were found in any group; in fact cortisol levels started to decrease after the injection, except for panic patients who showed similar increases after both flumazenil and placebo at the 15 min time point.

These results do not indicate that flumazenil, although panicogenic in half of the PD participants in this study, acts in the same way as systemically-administered inverse agonists in PD. This is consistent with other studies that either did not find flumazenil to be panicogenic in PD subjects (Strohle et al 1999; Strohle et al 1998), or did not find physiological activation during flumazenil-induced panic (Woods et al 1991). Since the intravenous dose of 2mg used in this study occupies up to 75% of $GABA_A$ receptors (Malizia and Nutt 1995), non-GABAergic effects of an oversaturating dose that could have obscured the inverse agonistic action of flumazenil (Polc 1988) were not likely to occur. It would of interest to see if a higher dose of flumazenil, not exceeding the suggested saturating dose of 15 mg (Savic et al 1991), could induce physiological activation in PD patients. However, since flumazenil 5mg i.v. caused some anxiety and EEG changes consistent with a weak inverse agonist activity in HV (Schopf et al 1984), a mild activation following a higher dose could be expected also in control groups.

It should be noted that, although these data do not support the hypothesis of a global shift in the activity of benzodiazepine receptors, as originally proposed (Nutt et al 1990), they may be consistent with a more restricted regional expression of $GABA_A$ receptor subtypes at which flumazenil is not a neutral antagonist. Whereas flumazenil is a neutral antagonist at $GABA_A$ receptors containing α1-3 or 5 subunits, preclinical data have shown that it may act as a partial agonist at $GABA_A$ receptors expressing α4 or α6 subunits in combination with β and γ subunits, which are insensitive to typical benzodiazepines (Knoflach et al 1996; Wafford et al 1996). However the affinity of flumazenil is two orders of magnitude lower at these receptors than at receptors with typical benzodiazepine pharmacology, showing significant effects only at μM concentrations (Knoflach et al 1996; Wafford et al 1996; Smith et al 2001). α4 subunits are also commonly co-expressed in $GABA_A$ receptors with δ subunits (Peng et al 2002; Pirker et al 2000), presumably mostly in extrasynaptic receptors mediating tonic inhibition. Although α4β3δ receptor subtypes may be insensitive to benzodiazepine site ligands (Brown et al 2002), it has very recently been shown that at α4β1δ receptor subtypes expressed in *Xenopus* oocytes, flumazenil acts as an inverse agonist, significantly reducing responses to GABA (Dunn et al 2003). Notably, this effect was obtained at a flumazenil concentration of 100nM, well within the range of plasma flumazenil concentrations achieved in the present study in panickers at 2 min after the infusion (interquartile range = 285 – 1465nM) (Table 5.1). The fast onset of panic attacks and their relatively short duration (2-4 min) was consistent with a rapid and extensive distribution of flumazenil, which was reflected by a steep decline in its plasma levels in the first 4 min (see chapter 5 of this thesis).

It was hypothesized in the present study that if flumazenil acts as an inverse agonist, it might do so in the PAG, since this region is associated with integrated behavioral and cardiorespiratory responses during defensive reactions in preclinical studies (Bandler and Shipley 1994; Graeff et al 1993), and is associated with panic symptoms when stimulated in humans (Nashold et al 1969). Interestingly, the α4, β1 and δ subunits in the rat periaqueductal grey have been shown to undergo parallel changes during the estrus cycle (Griffiths et al 2003, personal communication), which may fit with findings that flumazenil is panicogenic in women with premenstrual dysphoric disorder (Le Melledo et al 2000). It is interesting to speculate that such sex hormone effects might also contribute to higher prevalence of PD in women (Eaton et al 1994). In the present study there was a near significant higher panic rate in female PD participants following flumazenil, but a significant difference in the PSS sum intensity was not found. The PAG has no direct reciprocal connections with hypothalamic nuclei (Bandler and Shipley 1994), which could explain the lack of HPA axis activation in response to flumazenil. In addition, α4 subunits were not found to be present in the paraventricular nucleus of the hypothalamus (Pirker et al 2000), which is also consistent with a lack of a direct inverse agonist effect of flumazenil on the HPA axis in PD patients.

Another example of parallel changes in subunit levels is a downregulation of the α4 and an up-regulation of the γ2 subunit found in the forebrain of mutant δ subunit-deficient mice (Korpi et al 2002; Peng et al 2002). The α4 subunit exhibits a remarkable plasticity (Devaud et al 1997; Gulinello et al 2002; Holt et al 1996; Smith et al 1998a; Smith et al 1998b) and its downregulation may reflect the loss of a subunit, with which it

is frequently co-assembled. The δ and γ2 subunits generally do not occur in the same receptor and may compete with each other during $GABA_A$ receptor subunit assembly (Peng et al 2002; Pirker et al 2000). Therefore, in the δ-knock-out mice, the γ2 subunit has greater access to other subunits with which it may form new receptors (Korpi et al 2002; Peng et al 2002).

If the benzodiazepine insensitive α4β1δ subtype, at which flumazenil acts as an inverse agonist (Dunn et al 2003), is abnormally expressed in PD, the above findings offer several speculative but interesting scenarios that may occur in PD. An increased expression of the δ subunit in PD might result in the downregulation of its competitor, the γ2 subunit, for which fewer subunits would be available. The γ2 subunit is required for $GABA_A$ receptor synaptic clustering, which in turn is critical for normal channel conductance (Essrich et al 1998). In mice heterozygous for the γ2-subunit, the reduced receptor clustering corresponded with a reduced flumazenil binding and, more importantly, resulted in anxiety-like behavior (Crestani et al 1999). Hence, these molecular changes might explain spontaneous panic, reduced sensitivity to benzodiazepines (Roy-Byrne et al 1996), reduced flumazenil binding (Malizia et al 1998), and panic responses to flumazenil (Bell et al 2002; Nutt et al 1990; Woods et al 1991) in PD patients. However, a different order of events is possible. An upregulation of the α4 and δ subunits in the dentate gyrus may be a compensatory mechanism, which via increased tonic inhibition limits increased neuronal excitability in epilepsy (Peng et al 2002). Thus, decreased γ2 subunit levels might be the primary deficit in PD, leading to a protective increased expression of the α4 and δ subunits. In addition, δ subunit-containing receptors have been found to have an increased sensitivity to the positive allosteric

modulation by neuroactive steroids (Brown et al 2002). Therefore, increased levels of neurosteroids recently found in PD (Strohle et al 2002) may represent further compensatory changes that occur in PD.

Future studies are necessary to determine the genetic and/or environmental factors that coordinate the expression of different $GABA_A$ receptor subunits in PD and the mechanisms that regulate their assembly into receptor subtypes. Microenvironmental changes should also be considered. Phosphorylation by protein kinase C of consensus sites on $GABA_A$ receptor subunits, or receptor-associated proteins, regulates receptor internalization and its recycling back to the membrane surface (Chapell et al 1998; Connolly et al 1999). These phosphorylation-dependent processes may be involved in an increased surface expression of the α4 subunit in rat cortex following chronic ethanol exposure (Kumar et al 2002). Interestingly, $GABA_A$ receptor functional changes following chronic ethanol consumption include cross tolerance to benzodiazepines, and sensitization to inverse agonists and neurosteroids (Kumar et al 2002). The α4β1δ subtype tested in Dunn's study (2003) may fit this pharmacological profile, given the preliminary evidence for a shift in the activity of benzodiazepine site ligands towards inverse agonism at this subtype and the possibility of its increased sensitivity to neurosteroids (Brown et al 2002). Thus, these findings indicate that altered $GABA_A$ receptor phosphorylation might contribute to the abnormal expression of this subtype in PD.

Several additional points should be made about the lack of HPA axis activation in response to flumazenil. A weak stimulatory effect of flumazenil on the HPA axis might

have been masked by the tests being performed in the morning, when HPA activation might be offset by relatively high negative glucocorticoid receptor feedback. Furthermore, in some studies HPA activity was found to be related to treatment-seeking behavior and illness severity (Abelson and Curtis 1996; Wedekind et al 2000). A 24-hour secretion of cortisol was found higher in patients who entered a study through clinical referrals compared with those recruited by advertisements (Abelson and Curtis 1996). This was ascribed to higher levels of disability among treatment seeking patients, although this conclusion was based on the authors' assumption rather than an actual assessment of the patients' illness severity. Nevertheless, since the majority of patients in the present study were recruited through advertisement, it is possible that HPA reactivity may have been different if the patients had been referred for treatment. This sample selection could also explain why baseline cortisol levels were not higher in patients with PD or depression compared with HV, as could be expected under the condition of HPA axis hyperactivity (see section 1.2.4.4).

3.4.4. Flumazenil in relation to Klein's criteria for a panicogenic agent

Although the major aim of this study was not to confirm or reject Klein's criteria for a panicogenic agent, some of the results may contribute to the discussion about the validity of his concept of panic (Klein 1993; Klein 1996). Dyspnea and the lack of HPA axis activation during flumazenil-induced panic indicate that, according to Klein's criteria and against his predictions, flumazenil belongs to the group of panicogens that "mimic clinical reality" (section 1.2.4.3) (Klein 1993). However, according to his suffocation alarm hypothesis, fear of suffocation and dyspnea initiate hyperventilation, as

one attempts to lower heightened CO_2 concentrations that activate the alarm system. In the literature, generally, panic-related respiratory symptoms refer to both dyspnea and respiratory activation, as they usually co-occur during panic induced by the above mentioned panicogens. Although more recent neuroanatomical hypotheses of panic disorder (Gorman et al 2000; Sinha et al 2000) propose that a hypersensitivity of brainstem centers, especially those controlling respiration, is not the primary cause of panic as suggested previously (Gorman et al 1989; Papp et al 1993), they still consider hyperventilation as the main component of panic (Sinha et al 2000). It is surprising that dyspnea, rated by patients in the present study as similar to that during their natural severe attacks, did not lead to respiratory activation. However, hyperventilation is no longer believed to be the cause of panic (Papp et al 1993), nor does it seem to invariably co-occur with panic, as suggested by minimal changes in transcutaneous CO_2 during ambulatory monitoring of natural panic attacks (Garssen et al 1996). Interestingly, in the latter study even severe dyspnea attacks did not lead to hyperventilation, which along with the results presented here indicates that subjective experience of respiratory distress is not necessarily reflected in objective respiratory changes.

Recent preclinical research has suggested a physiological mechanism by which flumazenil might induce dyspnea that exceeds the objective changes in minute volume. Microinjection of the GABA-A receptor antagonist, bicuculline, into the dorsal and dorsolateral PAG produced a much greater effect on the tonic neural activity of the diaphragm than on its phasic activity (Hayward et al 2003). The effects of releasing the PAG from tonic GABAergic inhibition differed from those of direct PAG stimulation, which increased phasic neural activity. Phasic, rather than tonic neural activity of the

diaphragm is responsible for changes in mechanical ventilation. If flumazenil acts to disinhibit the PAG in PD, it may therefore produce diaphragmatic contraction (and thereby possibly dyspnea), without a marked change in minute volume. Changes in respiration during spontaneous panic attacks may depend on the occurrence of an excitatory stimulus to the PAG in addition to reduced tonic PAG inhibition.

In two previous studies with flumazenil, panickers reported a lack of respiratory distress (Nutt et al 1990; Bell et al 2002). This is somewhat surprising, considering the higher panic rates and cardiovascular activation found in the study by Nutt and coworkers (1990). Several methodological differences could have contributed to these inconsistencies. On the one hand, verbal reports about dyspnea in the earlier studies could have been less sensitive than ratings on the 15-point Borg scale used in this study (Borg 1982). The accuracy of the information in the present report may have been further increased by a larger number of panickers compared with the Nutt's study (21 vs 8). Repeatedly questioning the present subjects specifically about dyspnea, using the Borg scale, may have increased their attention to respiratory sensations. In the Nutt et al (1990) study, dyspnea was made as just one of a range of symptoms for which repeated ratings were made using VASs. Despite decreased peak anxiety and panic symptom intensity in the P subjects after the second administration of flumazenil, their dyspnea was equally severe, which also suggests that apart from the drug other factors could have contributed to respiratory distress during the challenge procedure.

These data suggest that flumazenil does not meet all Klein's criteria for a true panicogen, as not all studies concur that panickers experience dyspnea provoked by the challenge, and their ventilation does not seem to be significantly altered. Several other

pharmacological agents that have been used to induce panic either did not markedly increase ventilation or activated the HPA system (see section 1.2.4.3). Moreover, challenge agents, whether or not they meet Klein's criteria, induce panic through mechanisms whose relationship to the neurobiology of spontaneous panic is often unclear. Data on respiration and HPA activity during spontaneous panic have also been inconclusive (Bandelow et al 2000; Cameron et al 1987; Garssen et al 1996; Hibbert and Pilsbury 1989), although the inconsistencies may have been in part caused by methodological differences and limitations of monitoring spontaneous panic. Thus, whether panic is indeed characterized by distinct physiology that can be mimicked by some pharmacological agents, but not others, remains to be determined.

Several limitations of the current study that could have affected the results have already been discussed (participation of patients who were not seeking treatment, 4 P subjects not attending their second test, and problems associated with measuring anxiety). Furthermore, a repeated administration of flumazenil to all participants could have provided more accurate information about the specificity and reproducibility of its actions. However, for both financial reasons and insufficient time frame for the completion of the study, a uniform design could not be applied to all the study groups. It should also be noted that the interviewer assessing the occurrence of panic attacks in response to the infusions was not blind to the subjects' diagnoses, which could have reduced the objectivity of the assessment. In addition, family history of psychiatric disorders was not controlled for in HVs. Although increased sensitivity to some panicogens has been reported in healthy first degree relatives of patients with PD (Perna

et al 1995), the absence of panic attacks and the generally mild responses among HVs following flumazenil indicate that "positive" family history was unlikely to confound the results to a great extent. Another limitation of the study is the potential participation of women with a past history of premenstrual dysphoric disorder, since this group has also been found to be sensitive to flumazenil (Le Melledo et al 2000). However, this was unlikely, since symptoms of anxiety and/or depression in female participants had not been confined to their premenstrual phase.

In summary, the present study found panicogenic effects of flumazenil to be specific for PD, although the specificity was somewhat reduced when the PSS criterion for a panic attack, which appeared to detect rather severe attacks, was applied. The severity of flumazenil-induced attacks was comparable to the patients' natural severe panic attacks.

Since these data did not fully support the inverse agonistic activity of flumazenil in PD, it is important to explore other factors that may account for its panicogenic activity. Findings indicating abnormal $GABA_A$ receptor function in PD could also be explained by a higher expression of $GABA_A$ receptors containing the α4 in combination with β and γ2 subunits. As described above, these receptors are insensitive to typical benzodiazepines and show reduced binding affinity for flumazenil. Increased hippocampal expression of α4-containing receptor subtypes (with pharmacological profile identical to that of α4βXγ2 subtypes), induced in rats by a withdrawal from a progesterone metabolite 3α,5α-tetrahydroprogesterone (3α,5α-THP), caused anxiety due to reduced total GABA-gated currents at these receptors (Smith et al 1998a; Smith et al

1998b; Gulinello et al 2002). This mechanism could underlie spontaneous panic and also contribute to the higher prevalence of PD in women, as the expression of the α4βXγ2 receptors could be further increased by the sudden drop in 3α,5α-THP levels during the premenstrual phase.

However, at these subtypes flumazenil does not act as an anxiogenic inverse agonist, but as a partial agonist (Knoflach et al 1996; Wafford et al 1996) . Accordingly, flumazenil showed anxiolytic effects in rats undergoing 3α,5α-THP withdrawal (Gulinello et al 2002). The mechanism via which flumazenil causes dizziness is unknown, but could conceivably involve a partial agonist effect. Perhaps PD patients could panic in response to flumazenil after perceiving a partial agonist effect of dizziness as dangerous. However, dizziness, although rated by panickers higher during flumazenil-induced attacks than during natural attacks, appeared to be strong in all groups, which argues against flumazenil being a partial agonist specifically in PD. Finally, it appears that partial agonist effects require higher (μM) concentrations of flumazenil, and these were unlikely to explain the effects of the drug in patients in the present study (Knoflach et al 1996; Wafford et al 1996).

Although abnormal $GABA_A$ receptor function in PD has been implicated by several independent findings, results presented here did not provide direct evidence that flumazenil causes panic attacks by acting on abnormal receptor subtypes. A reduction in the number of full panic attacks with the repeated infusion of flumazenil indicates that psychological factors may play a role in the mechanism of flumazenil-induced panic. The role of catastrophic misinterpretations of somatic symptoms caused by the challenge and of other cognitive factors is addressed in the following section of this thesis.

3.5. References

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4. COGNITIVE FACTORS IN FLUMAZENIL-INDUCED PANIC

4.1. Introduction

As described earlier in this thesis, a common theme of some cognitive theories of panic has been that PD patients show heightened sensitivity to panicogenic challenges because they associate somatic symptoms related to anxiety (McNally 2002), or produced directly by the challenges (Clark 1993), with threat. Some challenge studies, but not others, have supported this view. PD patients have reported catastrophic ideation during laboratory panic (Rapee et al 1986; Rapee et al 1992). Pre-challenge instructions that reduced patients' negative interpretation of symptoms by providing benign explanations resulted in lower panic rates (Rapee et al 1986; Veltman et al 1998); in other studies, however, instructional manipulations showed negligible panic-reducing effects (Papp et al 1995; Welkowitz et al 1999).

Similarly, high anxiety sensitivity (see section 1.3.2) predicted fearful or panic responses to challenges in PD subjects (Rapee et al 1992; Rassovsky et al 2000) and non-clinical subjects (Holloway and McNally 1987; Rapee et al 1992; McNally and Eke 1996; Sturges et al 1998) in some studies, but not in others (Koszycki and Bradwejn 2001; Koszycki et al 1996; Veltman et al 1998; Aluoja et al 1997; Koszycki et al 1993). These inconsistent reports indicate that the cognitive theories need further testing.

Other cognitive factors that have been found or considered to modulate reactions to challenges include panic expectancy (Margraf et al 1989), perceived control (Sanderson et al 1989; Veltman et al 1998) and perceived safety (Carter et al 1995; Rapee et al 1991).

After examining possible biological mechanisms of flumazenil-induced panic, a further aim was to assess cognitive factors that may mediate or modulate responses to this challenge. This is important because all studies using flumazenil as a challenge have focused on biological aspects of its panicogenic mechanism (Nutt et al 1990; Strohle et al 1999; Strohle et al 1998; Woods et al 1991).

The hypothesis was that if panic attacks following flumazenil are triggered by somatic sensations that are associated with danger, these attacks should be accompanied by strong catastrophic ideation. It was also hypothesized that if any other factors play a significant role, they should be detected as strong predictors of panic occurrence or intensity.

4.2. Methods

4.2.1. *Subjects, design, drugs and procedures*

Four groups of subjects participated in the study; 20 patients with PD (P), 22 patients with comorbid PD and major depression currently experiencing a major depressive episode (P+D), 20 patients with major depression currently experiencing a major depressive episode (D), and 21 healthy volunteers (HV). Subjects, design, drugs and procedures were described in detail earlier in the thesis in the section on subjective and physiological responses to flumazenil in patients with panic disorder, major depression and comorbid panic disorder and major depression (section 3.2).

4.2.2. Measures

4.2.2.1. Pre-challenge assessment

At the screening session, participants completed the ASI (Reiss et al 1986). This 16-item questionnaire measures fear of harmful consequences of anxiety symptoms (subscales are related to physical concerns, mental incapacitation concerns and social concerns); each item is rated on a 0 to 4 point scale. Participants were also administered the 15-item FQ (Marks and Mathews 1979), which contains subscales measuring agoraphobia, social fear and fear of blood/injury. Items are rated on a 0-8 point scale. The total scores and the agoraphobia subscale scores were assessed in this study. Finally, the P, P+D and D subjects completed the ACQ, a 14-item measure evaluating how strongly one believes each of a list of catastrophic cognitions when one experiences anxiety (Chambless et al 1984). Each item is rated from 0="did not believe at all" to 4="totally believed".

Before each infusion, subjects were asked to give subjective ratings on a number of variables identified by Clark (1993) as potential cognitive predictors of panic responses to challenge tests. These were panic expectancy ("How likely are you to panic during this test?"), perceived safety ("How safe do you think you will feel during this test?") and perceived control ("How much control will you have over your reactions to this test?"). Each question was rated by the subjects on a 100mm VAS. At baseline, subjects also rated their anxiety on a 100 mm VAS, and the intensity of their panic symptoms on the PSS (see section 3.2.4.2).

4.2.2.2. Challenge assessment

The peak sum intensity of panic symptoms was rated by subjects on the PSS 10 min after each infusion. Increases in ratings (peak minus baseline ratings) were calculated. The occurrence of a *full* panic attack was assessed 15 min after each infusion by a semi-structured interview based on DSM-IV criteria (see section 3.2.4.2).

Cognitions following the infusions were rated using the mACQ (Chambless et al 1984). Subjects were asked to describe how strongly they had believed each of a list of catastrophic thoughts after the injection. The items were taken from the ACQ.

4.2.3. Data analysis

The first aim was to characterize the four study groups in terms of their scores on pre- and post-challenge cognitive measures. ANOVAs were performed on the ASI, FQ and ACQ. Baseline scores on VAS cognitive predictors were analyzed by repeated measures ANOVA with group (P, P+D, D, HV) as a between- and drug (flumazenil and placebo) as a within-subjects factor. Scores on the post-infusion mACQ were not normally distributed and were compared by Kruskal-Wallis test and *post-hoc* Mann-Whitney tests, where indicated. To assess the association between panic responses and catastrophic beliefs, the P and P+D subjects were divided into those who scored at least 3 (i.e., "strongly believed" or "totally believed") on at least one of the items of the mACQ, and those who did not. Increases in the sum intensity of panic symptoms and panic rates were compared between these two groups, using a t-test and *chi*-square test, respectively.

Additional analyses of individual items of the mACQ will be described in the Results section.

The second aim was to evaluate if any of the pre-challenge data, i.e. scores on questionnaires administered at the screening session (the ASI, FQ and ACQ), or baseline measures (VAS cognitive predictors and anxiety, and PSS sum intensity of panic symptoms), were associated with behavioral and cognitive responses to flumazenil. This was performed in several ways. First, the P and P+D subjects (who had the highest panic rates) were divided into those who panicked (panickers) and those who did not panic (non-panickers) in response to flumazenil, and their pre-challenge data were compared with those of the D and HV groups using one–way ANOVA. Significantly different results between panickers and non-panickers determined variables that were likely to predict the occurrence of a panic attack. The identification of *independent* predictors of panic attacks was then achieved using a logistic regression analysis, with panic/no panic as an outcome variable. Pearson correlation coefficients were calculated for each pre-challenge variable to reveal its unique relationship with increases in the PSS sum intensity of panic symptoms. A multiple regression procedure was used to determine predictors of increases in PSS scores, with pre-challenge measures as independent variables and increases in PSS scores as the dependent variable. The ability of the variables to predict a high score on at least one mACQ item (at least one item rated 3+ vs 2- was the dichotomous outcome variable) was also assessed by logistic regression. The correlation and regression analyses included data from the P, P+D and D subjects.

All results were considered significant at $p \leq 0.05$ (two-tailed test). Where appropriate, a Bonferroni correction of the probability level was applied for multiple comparisons.

4.3. Results

Baseline anxiety, cognitive predictors and sum intensity of panic symptoms were initially analyzed separately in the P and HV groups for the effect of day and order, and separately in the P+D and D groups for the effect of order (see sections 3.2.2 and 3.3. for the description of the study design and this procedure) by repeated measures ANOVA. Non-parametric statistics (Wilcoxon and Mann-Whitney tests) were used to assess day and order effects for the scores on the mACQ. None of the analyses detected significant main effects of day or order. This report focuses on the first test day data of the P or HV groups. Results of several additional analyses that included the second test day data of the P group are presented in less detail.

4.3.1. Pre-challenge cognitive assessment

The mean scores on the ASI, FQ, ACQ and VAS cognitive predictors (panic expectancy, and perceived control and safety) are summarized in Table 4.1, together with ANOVA statistics and results of *post-hoc* pair-wise comparisons. PD patients (P and P+D groups) scored significantly higher on the ASI than did the D and HV groups. These patients also scored higher than the HV group on the total and the agoraphobia subscale score of the FQ (the P+D group also higher than the D group), and higher than the D group on the ACQ.

Table 4.1. Scores on pre-challenge questionnaires, VAS cognitive predictors and anxiety, and baseline PSS sum intensity of panic symptoms of the study groups.

Rating Scale		P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)	ANOVA group			*Post-hoc* tests
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	df	p	
ASI (max 64)		30.0±12.7	33.5±10.2	18.7±12.8	10.2±6.2	20.8	3,79	<0.001	$P^{bf} = P+D^{cf} > D = HV$
FQ - total (max 120)		33.6±23.0	42.3±25.3	20.6±16.2	10.0±6.8	11.6	3,79	<0.001	$P^{e} = P+D^{f} > HV$ $P+D^{b} > D$
- agora (max 40)		8.8±7.7	13.5±11.2	3.2±5.0	1.4±2.3	11.7	3,79	<0.001	$P^{d} = P+D^{f} > HV$ $P+D^{c} > D$
ACQ (max 54)		14.6±10.3	18.6±10.6	6.7±5.6		9.1	2,57	<0.001	$P^{a} = P+D^{c} > D$
Panic expectancy	FL	47.9±25.4	40.7±19.8	17.9±16.6	14.9±18.2	18.0	3,79	<0.001	$P^{cf} = P+D^{bf} > D = HV$
	PL	47.4±22.5	39.2±24.4	19.1±14.4	14.4±13.4				
Perceived safety	FL	63.7±25.3	78.1±22.9	73.3±28.7	80.3±22.9	3.1	3,79	0.032	$P^{d} < HV$
	PL	58.5±26.0	68.2±28.4	79.0±19.9	76.7±19.3				
Perceived control	FL	48.9±22.5	58.0±26.2	66.1±30.1	78.8±18.0	8.8	3,79	<0.001	$P^{f} = P+D^{e} < HV$ $P^{a} < D$
	PL	45.1±23.5	51.7±25.7	68.0±24.4	76.7±21.4				
Anxiety	FL	37.1±21	33.5±22	16.2±15.1	11.2±12.8	12.8	3,79	<0.001	$P^{cf} = P+D^{bf} > D = HV$
	PL	43.3±22.4	36.1±22.5	16.2±3.6	16.4±13.5				
PSS sum intensity	FL	5.4±6.2	6.1±5.9	2.5±4.2	0.2±0.4	7.6	3,79	<0.001	$P^{e} = P+D^{e} > HV$
	PL	6.4±7.5	6.2±8.4	2.1±3.1	0.5±0.6				

$^{a}p< 0.05$ vs D; $^{b}p<0.01$ vs D; $^{c}p<0.001$ vs D; $^{d}p< 0.05$ vs HV; $^{e}p<0.01$ vs HV; $^{f}p<0.001$ vs HV. FL=flumazenil, PL=placebo.

Significant group effects were found for baseline VAS cognitive predictors, but no drug effects or drug by group interactions. *Post-hoc* tests showed that the P and P+D groups had significantly higher expectancy of a panic attack than the D and HV groups. Perceived safety during the test was significantly lower in the P group than in the HV group. Perceived control over reactions to the test was significantly lower in the P and P+D groups than in the HV group, and significantly lower in the P group than in the D group. Scores on baseline anxiety and the sum intensity of panic symptoms are also presented in Table 4.1, because these variables were considered as possible predictors of responses to flumazenil. These results were already described in section 3.3.2.

4.3.2. Challenge cognitive assessment: mACQ

Table 4.2 shows post-infusion ratings of the groups on the mACQ. After both placebo and flumazenil the P and P+D groups showed significantly higher scores on catastrophic beliefs than the D and HV groups, but scores after flumazenil were significantly higher across the groups.

Of the 42 PD patients in the study, 22 (52%) rated 3 or more (strongly believe or totally believe) on at least one mACQ item (mACQ3+). When the 22 mACQ3+ patients were compared with PD patients who did not rate at least one item 3+ (mACQ2-), they showed significantly higher panic rates (mACQ3+:82%, mACQ2-:15%, χ^2=18.7, p<0.001) and higher increases in the sum intensity of panic symptoms (mACQ3+: 20.9±12.0, mACQ2-: 9.2±6.7, t=4.0, df=40, p<0.001). Conversely, the proportion of PD panickers (n=21) with at least one strong (3+) belief was significantly higher than that of PD non-panickers (n=21) (86% and 19%, respectively, χ^2=18.7, p<0.001). As Table 4.3

indicates, the item "I am going to pass out" was the most common strong belief among PD subjects. They also had this belief more frequently (36%) than other groups (D: 10%, HV: 0%), although dizziness, a symptom that could have been related to this belief, was strong in all the groups (Table 3.8.).

Table 4.2. Scores on the mACQ after flumazenil (FL) and placebo (PL).

	P (n=20)	P+D (n=22)	D (n=20)	HV (n=21)	Kruskal–Wallis test p
FL	8.0 (2.0-12.8)[abd]	7.0 (1.8-12.3)[abd]	0.5(0.0-5.3)[c]	0.0 (0.0-1.0)[c]	0.001
PL	1.5 (0.0-7.8)[ab]	1.0 (0.0-7.5)[ab]	0.0 (0.0-0.8)	0.0 (0.0-0.0)	<0.001

Values are median (25^{th} -75^{th} percentiles). [a] $p<0.005$ vs D, [b] $p<0.001$ vs HV by Mann-Whitney test. [c] $p<0.05$ vs PL, [d] $p<0.01$ vs PL by Wilcoxon signed ranks test.

To assess how higher scores on *individual* mACQ items were associated with behavioral responses, the data of PD subjects who scored 3+ on individual items were compared with those who did not (Table 4.3). To increase the discriminative power of these analyses, the same comparisons were then performed only within the mACQ3+ subgroup (Table 4.4). Table 4.3 indicates that PD subjects who scored 3+ on individual items showed significantly higher increases in the sum intensity of panic symptoms regardless of the item in question (with the exception of "I am going to throw up"; Mann-Whitney tests). Panic rates were *not* significantly different whether or not the subjects scored 3+ on beliefs "I am going to throw up", "I am going to have a stroke" and "I am going to babble or talk funny". Table 4.4 indicates that within the mACQ3+ subjects,

those who scored 3+ on items "I will be paralyzed by fear", "I am going to throw up", "I am going to pass out", "I am going to babble or talk funny " and "I am going to have a stroke" did *not* show higher increases in the sum intensity of panic symptoms than those who did not score high on these items, although the differences in the last three items approached significance. Panic attacks were not significantly different between these mACQ3+ subgroups, suggesting that none of the catastrophic beliefs was specifically associated with the occurrence of a panic attack.

In PD panickers, the scores on the ACQ obtained during the screening session were compared with the scores on the mACQ, to evaluate how catastrophic cognitions associated with "real life" anxiety corresponded with cognitions following flumazenil challenge (Table 4.5). Although the median total score on the ACQ was higher than the total score on the mACQ, the difference was not significant. Comparisons of individual items revealed that beliefs "I will have a heart attack", "I am going to have a stroke" and "I will have a brain tumor" were rated significantly higher at the screening session, whereas the belief "I am going to pass out" was rated higher following flumazenil.

Table 4.3. Increases in the sum intensity of panic symptoms and panic rates in 42 PD subjects who did and did not score 3+ on individual items of the mACQ.

mACQ item	Item rated 3+ n (%)	Increase in the sum intensity of panic symptoms: Item rated 3+ Yes median (25^{th} -75^{th} percentiles)	Increase in the sum intensity of panic symptoms: Item rated 3+ No median (25^{th} -75^{th} percentiles)	Mann-Whitney test U	Mann-Whitney test p	Panic rates: Item rated 3+ Yes n (%)	Panic rates: Item rated 3+ No n (%)	χ^2 test χ^2	χ^2 test p
1. I am going to pass out	15 (36)	18.5 (13.0-33.0)	7.0 (4.25-13.5)	70.5	0.001	12 (80)	9 (33)	8.4	0.004
2. I will not be able to control myself	11 (26)	22.0 (16.0-39.0)	8.0 (5.0-19.0)	57.5	0.001	11 (100)	10 (33)	14.9	<0.001
3. I will be paralyzed by fear	8 (19)	27.5 (13.8-37.8)	11.0 (5.0-19.0)	54.0	0.007	7 (88)	14 (41)	5.6	0.018
4. I am going to act foolishly	7 (17)	33.0 (16.0-39.0)	12.0 (5.0-19.0)	36.0	0.002	7 (100)	14 (40)	8.4	0.004
5. I am going to go crazy	5 (12)	34.0 (24.5-41.5)	12.0 (5.5-19.0)	16.5	0.001	5 (100)	16 (43)	5.7	0.017
6. I am going to scream	5 (12)	34.0 (20.5-43.0)	12.0 (5.5-19.0)	16.0	0.001	5 (100)	16 (43)	5.7	0.017
7. I will choke to death	4 (10)	36.5 (33.3-42.8)	12.5 (5.8-19.0)	3.5	<0.001	4 (100)	17 (45)	4.4	0.035
8. I will have a heart attack	4 (10)	33.5 (23.3-37.8)	12.5 (5.8-19.0)	14.5	0.004	4 (100)	17 (45)	4.4	0.035
9. I am going to babble or talk funny	3 (7)	39.0 (16.0-41.5)	13.0 (6.0-20.0)	16.0	0.036	3 (100)	18 (46)	3.2	0.072
10. I am going to throw up	3 (7)	19.0 (-1.0-31.5)	13.0 (6.0-21.0)	50.5	0.712	3 (100)	18 (46)	3.2	0.072
11. I am going to have a stroke	2 (5)	36.5 (34.0-39.0)	13.0 (6.0-19.8)	4.0	0.021	2 (100)	19 (48)	2.1	0.147

Table 4.4. Increases in the sum intensity of panic symptoms and panic rates in 22 mACQ3+ subjects who did and did not score 3+ on individual items of the mACQ.

mACQ item	Item rated 3+	Increase in the sum intensity of panic symptoms		t-test		Panic rates		Fisher's exact test
		Item rated 3+				Item rated 3+		
		Yes	No	(df=20)		Yes	No	
	n (%)	mean±SD	mean±SD	t	p	n (%)	n (%)	p
1. I am going to pass out	15 (68)	24.2±12.4	13.9±8.0	2.0	0.06	12 (80)	6 (86)	1.0
2. I will not be able to control myself	11 (50)	26.1±12.6	15.7±9.3	2.2	0.04	11 (100)	7 (64)	0.09
3. I will be paralyzed by fear	8 (36)	26.3±13.0	17.9±10.7	1.6	0.118	7 (88)	11 (79)	1.0
4. I am going to act foolishly	7 (32)	29.4±12.2	16.9±10	2.5	0.019	7 (100)	11 (73)	0.263
5. I am going to go crazy	5 (23)	33.2±10.6	17.3±10.0	3.1	0.006	5 (100)	13 (77)	0.535
6. I am going to scream	5 (23)	32.2±11.5	17.6±10.3	2.7	0.013	5 (100)	13 (77)	0.535
7. I will choke to death	4 (18)	37.5±5.1	17.2±9.8	4.0	0.001	4 (100)	14 (780	0.554
8. I will have a heart attack	4 (18)	31.5±8.1	18.6±11.6	2.1	0.049	4 (100)	14 (78)	0.554
9. I am going to babble or talk funny	3 (14)	33.0±14.9	19.0±10.8	2.0	0.06	3 (100)	15 (79)	1.0
10. I am going to throw up	3 (14)	20.7±22.5	20.9±10.6	0.04	0.971	3 (100)	15 (79)	1.0
11. I am going to have a stroke	2 (9)	36.5±3.5	19.4±11.5	2.1	0.052	2 (100)	16 (80)	1.0

Table 4.5. Median (25^{th}-75^{th} percentiles) scores on the ACQ and mACQ in 21 PD panickers.

	ACQ	mACQ	Wilcoxon test p
I am going to pass out	2.0 (0.0-3.0)	3.0 (2.0-4.0)	0.012
I will not be able to control myself	3.0 (1.0-3.0)	3.0 (2.0-3.5)	0.144
I will be paralyzed by fear	1.0 (0.0-2.5)	2.0 (0.0-3.0)	0.186
I am going to act foolishly	1.0 (0.0-3.0)	2.0 (0.0-3.0)	0.977
I am going to go crazy	1.0 (0.0-4.0)	0.0 (0.0-2.5)	0.121
I am going to scream	1.0 (0.0-2.0)	0.0 (0.0-2.5)	0.502
I will choke to death	0.0 (0.0-2.0)	0.0 (0.0-1.5)	0.739
I will have a heart attack	2.0 (0.0-3.0)	0.0 (0.0-2.0)	0.029
I am going to babble or talk funny	1.0 (0.0-2.5)	0.0 (0.0-2.0)	0.07
I am going to throw up	1.0 (0.0-3.0)	0.0 (0.0-1.5)	0.117
I am going to have a stroke	1.0 (0.0-3.5)	0.0 (0.0-0.0)	0.005
I must have a brain tumor	0.0 (0.0-2.0)	0.0 (0.0-0.0)	0.011
I am going blind	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.655
I will hurt someone	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.102
Total score	18 (11.0-28.0)	12.0 (9.0-20.0)	0.073

4.3.3. Predictors of responses to flumazenil

Measures initially considered as possible predictors of behavioral and cognitive responses to flumazenil included the ASI (and scores on the physical concerns subscale and fear of fainting item of the ASI), the agoraphobia subscale of the FQ, the ACQ, baseline (i.e., pre-flumazenil) VAS cognitive predictors and anxiety, and baseline PSS sum intensity of panic symptoms.

Comparisons between the scores on these measures from PD panickers and non-panickers, and the D and HV groups are presented in Table 4.6. Despite diagnosis-related group differences found in all measures except for perceived safety, significant differences between panickers and non-panickers were found only for the agoraphobia subscale of the FQ and for baseline anxiety. Due to relatively high inter-correlations between the ASI and ACQ scores (0.77) and baseline anxiety and PSS scores (0.72), only the ASI and anxiety were included with other variables in a logistic regression analysis, in order to obtain more accurate information on their independent contribution to the panic/no panic outcome. These specific variables were chosen from the two pairs since both have predicted panic responses in previous studies (e.g., Rapee et al 1992; Coplan et al 1998; Goetz et al 2001). However, none of the six entered variables was found to be a significant predictor of panic in response to flumazenil.

In patients (P, P+D and D subjects), significant Pearson correlations were found between increases in the PSS sum intensity of panic symptoms and the ASI ($r=0.30$, $p=0.003$), the FQ agoraphobia subscale ($r=0.35$, $p=0.007$), the ACQ ($r=0.45$, $p<0.001$), baseline anxiety ($r=0.31$, $p=0.013$), panic expectancy ($r=0.49$, $p<0.001$) and (negative) for perceived control ($r=-0.27$, $p=0.031$). However, a multiple regression analysis found

only panic expectancy to be a significant independent predictor of increases in the PSS sum intensity (Beta=0.42, t=2.3, p=0.025). When only patients with PD (P and P+D) were considered, none of the above predictors was significant. However, the intensity of the PD patients' recent severe panic attacks (assessed by the PSS) was a significant predictor when entered into the model (Beta=0.45, t=2.7, p=0.011).

Logistic regression further determined that the ASI score was the only significant predictor of a high score on at least one mACQ item [Wald=4.65, p=0.03, Exp (B)=1.08)].

4.3.4. Cognitive factors associated with the second infusion of flumazenil

The proportion of P subjects having at least one strong catastrophic thought (rated 3+ on the mACQ) following the second infusion of flumazenil (38%) was reduced compared to the first infusion (55%), but not significantly (p=0.33 by Fisher's exact test). However, only 4 subjects had at least one catastrophic thought on both test days (κ = 0.15).

The ACQ scores and baseline PSS scores were again excluded from the regression analyses for their shared variance with the ASI scores and baseline anxiety (r=0.75), respectively. No variable was identified as a significant predictor of the occurrence of panic attacks by a logistic regression analysis. Modest correlations were found between increases in the PSS sum intensity and the ASI (r=0.32, p=0.015) and the agoraphobia subscale of the FQ (r=0.31, p=0.019), however regression procedure did not find any significant predictor of this outcome measure. As with the first flumazenil infusion, the ASI scores predicted strong catastrophic beliefs (a high score on at least one item of the mACQ) [Wald=5.0, p=0.025, Exp (B)=1.08)].

Table 4.6. Identification of predictors of flumazenil-induced panic; comparisons between PD panickers (Panic) and non-panickers (Nonp), and the D and HV groups.

Rating Scale	Panickers (n=21)	Non-panickers (n=21	D (n=20)	HV (n=21)	ANOVA group			*Post-hoc* tests
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	df	p	
ASI (max 64)	33.4±11.6	30.2±11.1	18.7±12.8	10.2±6.2	20.7	3,79	<0.001	Panic^{cf} = Nonp^{bf} > D = HV
- physical concerns subscale	18.1±6.6	14.4±6.3	9.7±8.8	4.4±4.4	16.0	3,79	<0.001	Panic^{cf} = Nonp^{f} > HV Panic^{c} > D
-fear of faintness (ASI item)	2.6±1.3	2.4±1.0	1.3±1.2	0.9±1.1	9.8	3,79	<0.001	Panic^{bf} = Nonp^{af} > D = HV
FQ - agora (max 40)	14.8±11.2	7.6±6.8	3.2±5.0	1.4±2.3	14.6	3,79	<0.001	Panic^{hf} > Nonp^{d} > HV Panic^{c} > D
ACQ (max 56)	18.2±9.8	15.2±11.3	6.7±5.6		8.6	2,57	0.001	Panic^{c} = Nonp^{a} > D
VAS - Anxiety	42.4±23.2	28.1±16.9	16.2±15.1	11.2±12.8	13.2	3,79	<0.001	Panic^{hf} > Nonp^{d} > HV Panic^{c} > D
-Panic expectancy	50.0±25.5	38.2±18.2	17.9±16.6	14.9±18.2	14.6	3,79	<0.001	Panic^{cf} = Nonp^{be} > D = HV
-Perceived safety	67.3±28.5	75.1±20.6	73.3±28.7	80.3±22.9	0.9	3,79	0.426	
-Perceived control	56.7±25.8	50.6±23.7	66.1±30.1	78.8±18.0	7.5	3,79	<0.001	Panic^{d}= Nonp^{e} < HV
PSS sum intensity	7.5±6.8	4.1±4.8	2.5±4.2	0.2±0.4	9.1	3,79	<0.001	Panic^{f} = Nonp^{d} > HV Panic^{b} > D

$^{a}p< 0.05$ vs D; $^{b}p<0.01$ vs D; $^{c}p<0.001$ vs D; $^{d}p< 0.05$ vs HV; $^{e}p<0.01$ vs HV; $^{f}p<0.001$ vs HV; $^{h}p<0.05$ vs Nonpanic.

4.4. Discussion

4.4.1. Pre-challenge cognitive assessment

This study primarily investigated cognitive mechanisms that may predict or influence responses to flumazenil. The initial pre-challenge assessment of the four study groups confirmed previous findings that patients with PD (the P and P+D groups) have higher anxiety sensitivity than patients with major depression (the D group) and healthy volunteers (HV) (Rapee et al 1992; Taylor et al 1996); their total ASI scores were within the same range as those of PD patients in previous studies (Rapee et al 1992; Taylor et al 1992; Taylor et al 1996). As expected, PD patients exhibited a higher tendency to have anxiety-related catastrophic thoughts compared with the D group, as indicated by the ACQ scores, and more severe agoraphobic avoidance than the D and HV groups, as indicated by the agoraphobia subscale of the FQ.

4.4.2. Challenge cognitive assessment: mACQ

PD patients also showed higher total scores on the post-injection mACQ compared with other study subjects. Cognitive models predict that panic in response to flumazenil is triggered primarily by catastrophic beliefs (Clark 1993; Rapee 1995). Since high total scores on the mACQ may also be caused by lower scores on a higher number of items (which may not be critical for triggering panic), further analysis focused on subjects who had at least one strong catastrophic belief after the flumazenil infusion (i.e. "strongly or totally believed" at least one of the thoughts listed in the mACQ). These subjects were selected from the P and P+D groups, because only one (D) subject outside these groups experienced a panic attack. These subjects showed higher increases in panic

symptom intensity and higher panic rates than PD subjects without a strong catastrophic belief. Furthermore, a significantly higher proportion of PD panickers had strong negative beliefs compared with PD non-panickers, suggesting an association between catastrophic cognitions and panic responses. However, a more precise interpretation of these results is complicated by the uncertainty of the origin of these thoughts and the nature of their association with panic attacks.

One possibility is that the thoughts were based on a catastrophic misinterpretation of physical symptoms produced by flumazenil, as suggested by Clark (1986; 1993). The most frequent strong belief among PD subjects was "I am going to pass out", which could have been linked to dizziness, the strongest flumazenil-induced symptom in all the groups (Table 3.8). Although dizziness was experienced by all subjects, the proportion of PD subjects who strongly believed that they were going to pass out was higher than the proportion of the D and HV subjects. Further arguments that may be in favor of dizziness triggering panic via catastrophic cognitions are that in PD panickers, dizziness was stronger during flumazenil-induced panic attacks than during their recent severe attacks. In fact, although the median increase in the intensity of dizziness on the PSS was 2 in all the groups (Table 3.8), the peak intensity reached 3 (i.e. severe) only in the P and P+D groups. On the other hand, 43% of panickers did *not* have a strong "passing out" belief. Furthermore, panic rates and increases in the PSS sum intensity of symptoms among PD subjects having catastrophic ideation did not differ between those who did and did not have a strong "passing out" belief.

In addition, the links between somatic symptoms and cognitions are not as straightforward as it might appear. In previous studies, fear of passing out appeared to be the most common cognition even if dizziness was *not* the strongest somatic sensation during panic attacks (Rachman et al 1987; Westling and Öst 1993). Further, the association between a combination of somatic symptoms and cognitions was found to be even stronger than that between single symptoms and cognitions (Rachman et al 1987). Although in PD patients in the present study the scores on the "passing out" belief correlated significantly with increases in the intensity of dizziness following flumazenil ($r=0.76$; $p<0.0001$), these scores still significantly correlated with increases in the sum intensity of panic symptoms even after *excluding* the dizziness item ($r=0.62$; $p<0.0001$). The correlation between the "passing out" beliefs and the intensity of dizziness could be interpreted in two ways. It is possible that misinterpretation of some initial symptoms of dizziness (i.e., the "passing out" belief) led to apprehension and further amplification of dizziness, as part of a vicious circle escalating into panic (Clark 1986, see section 1.3.1). The other possibility is that subjects who felt more dizzy during their already full blown panic attacks were more likely to believe that they could pass out. One argument that could favor cognitive amplification of dizziness as a mechanism of panic would be if panickers, or patients with strong "passing out" beliefs, had shown more negative attitudes to fainting as a trait characteristic. However, additional analyses found that scores on the ASI item "it scares me when I feel faint" were not significantly different between PD panickers and non-panickers, or between subjects with or without strong "passing out" beliefs.

Panic disorder patients, and particularly panickers, rated several other *somatic* panic symptoms significantly higher following flumazenil than did controls, including those related to cardio-respiratory activation (i.e., dyspnea, palpitation, chest discomfort; Table 3.8). Links between these somatic symptoms and corresponding cognitions are even more difficult to trace, as other frequent strong beliefs, such as "I will not be able to control myself", "I will be paralyzed by fear", "I am going to act foolishly" or "I am going to go crazy", signifying mental/social catastrophe, could have been triggered by various somatic sensations (Rachman et al 1987; Westling and Öst 1993). A problem with a purely cognitive interpretation of flumazenil-induced panic is that these somatic symptoms were not induced to a meaningful extent in healthy subjects. Although, in theory, a misinterpretation of initially mild sensations by PD patients could result in their amplification (Clark 1986), in practice only dizziness could be a credible trigger for catastrophic cognitions in relation to flumazenil.

It is possible that the negative thoughts in some subjects reflected their fears of anxiety-related autonomic arousal, rather than a firm conviction that sensations experienced during the test are signs of a catastrophe. Indeed, high anxiety sensitivity appeared to be a strong significant predictor of strong negative beliefs. But since subjects with high anxiety "are especially prone to catastrophic misinterpretations" (McNally 1994), the relative contribution of these two cognitive factors to post-infusion ideation is difficult to determine. The close relationship between the two cognitive factors was also suggested by Cox (1996), who described catastrophic misinterpretation as a cognitive

process (a state characteristic) of a panic attack, and high anxiety sensitivity as a trait characteristic predisposing some individuals to such interpretations.

While a number of interview (Hibbert 1984; Ottaviani and Beck 1987), self-report (Rachman et al 1987; McNally and Foa 1987; Clark et al 1997; Chambless et al 2000) and self-monitoring (Westling and Öst, 1993) studies focused on the causal relationship between symptoms and cognitions, there has been little investigation of the association between cognitions and panic attacks; in most studies the sequence of events during panic attacks (i.e., symptoms – cognitions – panic) was assumed, but not assessed. When directly asked, patients in one study reported that cognitions preceded their natural panic attacks (Ley 1985). However, in another study patients reported the opposite order (Wolpe and Rowan 1988). Following epinephrine challenge, the information was also mixed (Veltman et al 1998). Thus, due to the inconsistency of these reports (which is not surprising given their retrospective nature and the short latency between the events), whether negative thoughts precede and cause panic, or whether they are concomitants of panic caused by other mechanisms is unclear.

In the current study, 14% of PD panickers did not report any strong catastrophic beliefs. This is in agreement with previous reports that not all natural and laboratory panic attacks are accompanied by catastrophic thoughts (Rachman et al 1987; Westling and Öst 1993; Aronson et al 1989). Several explanations of "non-cognitive" panic attacks, which could apply to the present results, have been suggested (Rachman et al 1987; Westling and Öst 1993). First, these panickers could have had catastrophic cognition(s) that did not appear in the mACQ, such as fear of dying. However, the PSS

ratings of these subjects indicated that the intensity of this symptom was mild, or that the symptom was not present. The subjects were also asked to describe any thoughts that were not listed in the questionnaire. Second, it has been proposed that the cognitive process of misinterpretation in these subjects may be automatic, unconscious, and therefore non-detectable (Clark 1988). However, as mentioned in the Introduction to this thesis, Clark's explanation makes his cognitive theory difficult to falsify. The third possible explanation of non-cognitive panic is that catastrophic cognitions are not always necessary for panic to occur. This is supported by findings that instructional manipulation designed to reduce negative misinterpretation did not invariably reduce panic responses to CO_2 (Papp et al 1995; Welkowitz et al 1999). On the other hand, 19% of non-panickers in this study reported at least one strong catastrophic belief, again arguing against oversimplifying the relationships between beliefs and symptoms. Thus, regardless of whether catastrophic thoughts resulted in panic attacks in some patients, as in previous studies, they were neither necessary (Rachman et al 1987; Westling and Ost 1993; Aronson et al 1989; Papp et al 1995) nor sufficient (Rachman 1987) for panic to occur.

4.4.3. Predictors of responses to flumazenil

Although baseline anxiety and the severity of agoraphobia were found to be stronger in PD panickers than PD non-panickers, these variables were not identified as strong independent predictors of panic occurrence by a logistic regression analysis that included both PD and D subjects. In previous studies, baseline anxiety has been found higher among panickers (Roth et al 1992; Yeragani et al 1988), or has been identified as an independent predictor of panic (Coplan et al 1998; Goetz et al 2001) in response to

lactate and CO_2. The extent to which anticipatory anxiety is associated with panic responses may vary with different panicogenic agents (e.g., their route and duration of administration) and laboratory settings.

More powerful analyses were performed to assess the association between pre-challenge variables and quantitative outcome measures within the PD and D subjects. Increases in the sum panic symptom intensity were positively correlated with the scores on all pre-challenge questionnaires (the ASI, the FQ agoraphobia subscale and the ACQ), baseline anxiety and panic expectancy, and negatively correlated with perceived control over responses. Apart from the correlation between panic expectancy and symptom intensity, these correlations were relatively low.

In agreement with these analyses, a multiple regression analysis found only panic expectancy to be a significant independent predictor of increases in panic symptom intensity across the patient groups (P, P+D, D). In a previous report, PD patients whose expectancies of panic were experimentally increased by describing hyperventilation challenge as a "panic attack test" experienced greater subjective anxiety and panic symptoms than a PD group undergoing a "fast paced breathing task" (Margraf et al 1989). It has been argued that experimenter demand effects on subjects' responses (i.e., compliance with instructions) may play a role in studies manipulating panic expectancy (Rapee 1995). However, the significance of this cognitive variable was confirmed in a natural setting where prediction of panic attacks, unlike anxiety, sense of control and sense of threat or danger, appeared to be the only significant cognitive precursor of panic attacks during a one week-self-monitoring study (Kenardy et al 1992). Several authors

have suggested that expectancy of intense fear causes excessive fear as well as its maintenance. In addition, expectancy of fear often leads to further heightened expectancy and avoidance (Kirch 1985; Kenardy et al 1992; Whittal and Goetsch 1997), which implicates its role in the onset and course of PD and agoraphobia. However, it should be emphasized that within patients with PD (P and P+D), expectancy was not found to be a significant predictor of panic responses in the present study.

Apart from the tendency to associate bodily symptoms with threat, low perceived control over threat is considered the key factor in panic disorder and in the affective responses to challenges by some theorists (Barlow 1988; Rapee 1995). PD patients have scored lower on questionnaires assessing perceived control over threat compared with healthy controls, although other anxiety disorder patients showed similar results (Rapee 1997). The illusion of control over CO_2 concentration reduced panic in response to this challenge in PD subjects (Sanderson et al 1989). In the present study, prior to flumazenil administration PD patients expected less control over their reactions than did healthy controls, but these low scores did not significantly predict either panic or increases in panic symptom intensity following flumazenil. Since none of the subjects expected to have control over the drug administration, the restricted range of similar baseline ratings within patients may have reduced the predictive value of this variable (data from the HV group were not included in regression analyses). However, in another study with CO_2, the possibility of reducing the gas concentration with an operative dial did not affect panic rates (Welkowitz et al 1999). Similarly, a manipulation of perceived control did not influence panic responses to epinephrine (Veltman et al 1998). Finally, sense of control was not found to be a significant precursor of natural panic attacks (Kenardy et al 1992).

Thus, the importance of this cognitive variable in natural and challenge-induced panic has yet to be clarified.

Low expected safety during the flumazenil procedure was not associated with panic responses. Low pre-challenge safety scores increased the likelihood of panic attacks in some studies (Rapee et al 1991), but not others (Veltman et al 1998). Similarly, studies assessing the effect of manipulation of perceived safety during CO_2 inhalation on subjects' reactions showed inconsistent results (Rapee et al 1991; Carter et al 1995). To date, evidence that perceived safety is critical for responses to laboratory panicogens has been inconsistent.

Although high anxiety sensitivity predicted strong catastrophic cognitions, it did not predict panic outcome or increases in the sum intensity of panic symptoms following flumazenil. Elevated anxiety sensitivity in non-clinical subjects has been associated with anxious responses to hyperventilation (Carter et al 2001; Holloway and McNally 1987; Rapee and Medoro 1994; Sturges et al 1998), CO_2 (McNally and Eke 1996; Forsyth et al 1999) or caffeine (Telch et al 1996), but not to CCK-4 (Koszycki et al 1993; Aluoja et al 1997). The relatively few studies that assessed the relevance of this variable in behavioral responses to challenges in PD subjects also reported inconsistent data. Anxiety sensitivity predicted responses to hyperventilation and CO_2 (Rapee et al 1992; Rassovsky 2000; Shipherd et al 2001), but not to pentagastrin (van Megen et al 1994), CCK-4 (Koszycki et al 1996), or epinephrine (Veltman et al 1998). In addition, in one study anxiety sensitivity failed to enhance behavioral responses to CO_2 (Koszycki and Bradwejn 2001).

Several factors may have contributed to the inconsistency of these reports. The predictive power of anxiety sensitivity may vary with qualitatively and quantitatively different sensations produced by different challenges or different doses. However, if the level of fear of somatic symptoms (rather than direct pharmacological action of an agent) determines responses to panicogens, it is surprising that anxiety sensitivity did not mediate responses to CCK-2R agonists and epinephrine, which all produce strong somatic symptoms and objective autonomic arousal (Abelson and Nesse 1994; Bradwejn et al 1992; Veltman et al 1998). The role of cognitive factors is even more likely (Nutt and Lawson 1992) given that the sympathomimetic epinephrine does not cross the blood brain barrier and the CCK-2 agonists may also, in part, produce their symptoms via peripheral actions (McCann et al 1994).

Another source of the discrepancy may be the choice of a statistical analysis. For example, while some studies used multiple regression analyses to determine independent predictors of responses to a challenge, other studies reporting high importance of anxiety sensitivity merely compared responses between individuals with high and low scores on the ASI (Holloway and McNally 1987), or assessed correlations between the ASI scores and outcome measures (Shipherd et al 2001). In the present study, while the ASI scores significantly correlated with increases in panic intensity, they were not identified as significant predictors of this measure by regression analysis. Furthermore, in order to identify truly independent predictors and to avoid type I errors, a simultaneous entry method was chosen for regression analyses, instead of a step-wise method used in most studies. Due to this more conservative approach the probability of obtaining null results was increased.

The clinical characteristics of study subjects may also play a role. As the above studies of the relevance of anxiety sensitivity in response to panicogens indicate, the proportion of positive results appeared to be higher in studies with non-clinical subjects. Perhaps this could be attributed to a higher range of their ASI scores relative to that of PD subjects, who are likely to have similar high scores. McNally (2002) has argued that in clinical studies, the restricted range could have contributed to negative results when only PD subjects were included (e.g., Koszycki et al 1996; Koszycki and Bradwejn 2001). It could equally be argued that inclusion of non-PD subjects inflates the relationship between ASI scores and responses to panicogens. In this case, the ASI scores could predict the response to the panicogen simply due to their association with the diagnosis of PD. This interpretation might be partly born out by the present study. When the analyses included both PD and D subjects, whose scores were significantly different, the ASI correlated with PSS responses to flumazenil (i.e., changes in the sum intensity of panic symptoms) but did not significantly predict them. When the regression analysis was limited to the PD patients, the severity of usual panic, but not the ASI, was a significant predictor of PSS response. In fact, although the ASI scores significantly correlated with the severity of usual panic ($r=0.42$, $p=0.01$), they did not correlate with the PSS response ($r=0.23$, $p=0.16$).

Unlike some other recent studies (Koszycki and Bradwejn 2001; Koszycki et al 1996; Rassovsky et al 2000), the planned analyses in the present study did not assess relative contributions of the three ASI subscales (i.e., Physical concerns, Mental Incapacitation concerns and Social concerns). However, exploratory analyses using the Physical Concerns factor did not show any major differences from the findings with the

total ASI scores, with which the Physical Concerns factor was very strongly correlated (r=0.94).

In summary, the results are consistent with previous studies that found high anxiety sensitivity to be associated with cognitive panic symptoms, but not with other panic symptoms or with the occurrence of panic attacks (Veltman et al 1998; Koszycki et al 1993; Koszycki and Bradwejn 2001).

4.4.4. Cognitive factors associated with the second infusion of flumazenil

Given the use of a repeated flumazenil challenge, it was of interest to examine how cognitive factors predicted responses on the second occasion. Within the P subjects, no effects of day were found for baseline anxiety, panic expectancy, perceived control and perceived safety.

During the second session, the proportion of P subjects having at least one strong catastrophic thought following flumazenil was not significantly reduced compared with the first session. However, the ability of flumazenil to trigger these thoughts reliably in the same subjects was low. The power of this analysis was reduced since 2 out 11 subjects who had catastrophic ideation on the first test day did not attend their second test. Nevertheless, 5 of the remaining 9 subjects did not have catastrophic thoughts after their second infusion. Of the 4 subjects with catastrophic ideation on both test days, 2 had full and 2 had sub-threshold (sudden moderate anxiety with at least 4 symptoms) panic following the second flumazenil challenge. All full panic attacks within the P group were accompanied by at least one catastrophic thought. When the second test day data of the P

subjects were entered in the logistic regression analysis, anxiety sensitivity was again found to be the only significant predictor of strong negative beliefs within the patient (PD and D) groups, further confirming its importance for catastrophic ideation. These results suggest that negative thoughts were associated with the panic experience, but again do not provide direct evidence of a causal relationship between the thoughts and panic.

Neither anxiety sensitivity nor any other variable predicted the occurrence of full panic attacks. Although high anxiety sensitivity along with the agoraphobia subscale of the FQ was modestly correlated with increases in panic symptom intensity, no strong predictors of this measure were detected across the patient groups. Compared with the first test day, within PD subjects the predictive power of recent panic severity for the second test day PSS responses to flumazenil was somewhat lower (Beta=0.29; t=1.4; p=0.174).

It is important to note that the accuracy of regression analyses in detecting predictors of responses to the repeated flumazenil infusion was limited because they included second test day data of the P group and first test day data of the P+D and D groups (who were given flumazenil only once).

In summary, flumazenil-induced panic was repeatedly associated with strong catastrophic beliefs. While this ideation could have been attributed to high anxiety sensitivity and/or direct catastrophic misinterpretation of somatic symptoms produced by flumazenil, it is unclear whether these resulted in panic attacks, as predicted by cognitive models. Thus, the possibility that the thoughts were concomitants of a full blown panic caused by other mechanisms cannot be ruled out. The finding that the thoughts were

neither necessary nor sufficient for the development of panic further adds to the argument that flumazenil-induced panic is not mediated purely by cognitive mechanisms.

It has been demonstrated several times (twice in this study) that, during challenge tests, high anxiety sensitivity is predictive of catastrophic ideation, but neither of the occurrence of panic, nor of non-cognitive components of panic responses (Veltman et al 1998; Koszycki et al 1993; Koszycki et al 1996; Koszycki and Bradwejn 2001). Further research will be necessary to determine whether anxiety sensitivity is the primary factor that mediates responses to panicogenic challenges in PD, especially considering that in the study where anxiety sensitivity predicted responses to CO_2 and hyperventilation the amount of explained variance was relatively low (Rapee et al 1992).

Panic expectancy was found to be the best predictor of amplification of panic symptoms in response to the first flumazenil infusion across the patient groups (PD and D). However, neither panic expectancy, nor any of the variables initially entered in the regression analyses were predictive when only PD patients were considered. Prediction was considerably improved when the severity of the patients' recent attacks was included in the model.

Familiarity with the test procedure appeared to be associated with a reduction in catastrophic cognitions and the frequency of full panic attacks (section 3.3.2), although the sample size was possibly too small for this difference to be significant. Although there were no "non-cognitive" panic attacks on the second test day, the nature of the relationship between catastrophic thoughts and panic remains unclear. The low predictive value of other cognitive factors for response to flumazenil again suggests that flumazenil may activate panic symptoms more directly than via cognitive mechanisms.

4.5. References

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5. A RAPID HIGH-PRESSURE LIQUID CHROMATOGRAPHIC PROCEDURE FOR DETERMINATION OF FLUMAZENIL IN PLASMA

5.1. Introduction

Flumazenil (Ro 15-1788), ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4]benzodiazepine-3-carboxylate, is a water-soluble imidazobenzodiazepine. In the CNS it binds to $GABA_A$ receptor complex and competes for a binding site with benzodiazepines. While in healthy volunteers flumazenil shows no or minimal intrinsic activity of its own (Darragh et al., 1983; Schöpf et al., 1984; Higgitt et al., 1986), in patients with PD it has provoked panic attacks (Nutt et al., 1990; Woods and Charney, 1991). The mechanism of its panicogenic action is still being investigated.

Flumazenil is rapidly and extensively metabolized by the liver, and less than 0.2% of unchanged flumazenil is excreted in the urine (Klotz et al., 1984). The principal metabolites are carboxycyclic flumazenil acid (and the corresponding glucoronide), N-desmethylflumazenil and N-desmethylflumazenil acid, all pharmacologically inactive (Klotz and Kanto, 1988). Methodologies that have been used to measure flumazenil concentrations in plasma include high performance (pressure) liquid chromatography (HPLC) (Timm and Zell, 1983; Klotz et al., 1984; Roncari et al., 1986) and gas chromatography with nitrogen-sensitive detection (Zell and Timm, 1986). The present report describes a rapid, simple HPLC method used to determine flumazenil in plasma of PD patients participating in a placebo-controlled study investigating the role of biological or cognitive mechanisms in flumazenil-induced panic (see chapters 3 and 4 of this thesis). The relevance of the pharmacokinetics of the drug in its panicogenic effects was assessed.

5.2. Methods

5.2.1. Materials

Flumazenil was purchased from Tocris (Ballwin MO, USA). Lamotrigine, used as internal standard, was a gift from Glaxo Wellcome Research and Development (Hertfordshire, UK). Sodium bicarbonate, ethyl acetate, ammonium acetate and acetonitrile were provided by Fisher Scientific (New Jersey, USA). For the preparation of plasma standards for a calibration curve, blood of healthy volunteers free of medication was collected into vacutainer tubes with EDTA as anticoagulant and centrifuged at 3000g for 10 min. The plasma was separated and stored at –80°C until analyzed.

5.2.2. Subjects

Flumazenil was determined in the plasma of 37 PD patients (36±10.6 years, 15 males, 22 females), of whom 18 had comorbid major depressive episode. Subjects, design, drugs, procedures and subjective measures were described in detail earlier in the thesis (section 3.2.). Blood samples for the estimation of flumazenil were collected into vacutainers with EDTA at baseline and at 2, 4, 7.5, 15, 30, 45 and 60 min after the infusion. The blood was centrifuged, and the plasma separated and stored at –80°C until analysis.

5.2.3. Extraction

The plasma (1ml) was added to a screw cap tube and mixed with 3μg of internal standard (IS) (30μl of 100μg/ml lamotrigine stock solution). The sample was diluted with 1ml of distilled water and basified by the addition of solid sodium bicarbonate. After

addition of 5ml of ethyl acetate, the sample was shaken vigorously for 5 min and centrifuged at 1000g for 3 min. The organic phase was transferred to a different tube and taken to dryness in a SAVANT evaporator. The residue was reconstituted in 200µl of degassed mobile phase (75% 10mM ammonium acetate buffer, 25% acetonitrile, adjusted to pH 5.4 with acetic acid). The sample was then vortexed, centrifuged briefly to remove any particulate matter and transferred to a microfuge for HPLC analysis.

5.2.4. Chromatography

An HPLC system consisting of a Waters model 510 pump, a Waters WISP 710B autosampler and a Waters Lambda Max Model 481 UV detector linked to a Hewlett Packard model 3392a integrator was used. A SPHERISORB octadecylsilane (ODS) (250 x 4.6mm, 5µm particle size) column (Phenomenex, Torrance CA, USA) coupled to a guard column with the same packing were used. The flow rate of the mobile phase was 1ml/min and the amount of each sample injected was 20µl. Detection was performed using UV absorbance at 250nm and range 0.002.

5.2.5. Calibration and data analysis

A calibration curve was created during each assay run via a parallel analysis of a series standards added to blank plasma (1ml). The standards contained 1000, 500, 250, 100, 50, 25, 10, 5, 2.5 or 0 ng of flumazenil and 3µg of IS. The curve was obtained by a linear regression of peak height ratios of flumazenil/IS versus flumazenil concentrations. The amount of flumazenil in each analyzed sample was determined by comparing the peak height ratio of flumazenil/IS in the sample to the ratios in the calibration curve.

Mann-Whitney *U* tests were used for the comparison of plasma concentrations of flumazenil between panicking and nonpanicking patients at 2 and 4 min after flumazenil administration. Spearman correlation coefficients were calculated to assess the relationship between plasma concentrations at 2 min and increases in the sum intensity of panic symptoms.

5.3. Results and discussion

Under the operating conditions used, the retention times of flumazenil and the IS were 13.1±0.1 min and 15.5±0.3 min respectively (Figure 5.1). Calibration curves were linear within the 2.5-1000ng range, with r^2 values consistently over 0.99 (Figure 5.2). The limit of detection was < 2.5ng /ml. The CVs (n=6) at a flumazenil concentration of 250 ng/ml were 3.5% (intra-assay) and 6.9% (inter-assay). Recovery of the same concentration from plasma was 92%.

In the present study, plasma concentrations of flumazenil were measured in all the subjects only in samples collected at 2 and 4 min, since all panic responses occurred within this time period (Table 5.1). Plasma concentrations in samples collected also at 7.5 and 15 min were measured in 6 subjects (Figure 5.3), and at 30, 45 and 60 min in 1 subject (Figure 5.4).

As in previous studies reported in the literature, there was a steep decline in the plasma concentration-time profile during the first 4 min (Figures 5.3 and 5.4), due to an extensive and rapid distribution, which is completed within 5 minutes. This phase is followed by a fast hepatic elimination ($t_{1/2}$=0.8-1.4 hours) (Klotz et al., 1984; Klotz et al., 1985; Roncari et al., 1986; Amrein and Hetzel, 1990). This explains the rapid onset of the

antagonistic action of flumazenil – within 1-2 minutes following i.v. administration (Geller et al., 1985; Lauven et al., 1985; Carter et al., 1990; Savid et al., 1991) and the rapid onset of panic attacks in our study (within 30-40 seconds). After the distribution phase, the decline in concentrations was slower (Figures 5.3 and 5.4); in one study reported in the literature flumazenil was still detectable 3 hours after an i.v. dose of 2.5 mg (Klotz et al., 1984). The method described here is rapid, replicable, and convenient for the determination of flumazenil in plasma.

At 2 and 4 min time points, flumazenil concentrations did not differ significantly between "panickers" (n=24) and "nonpanickers" (n=13) (Mann-Whitney, 2min: $p=0.12$, 4min: $p=0.19$) (Table 5.1). In addition, the concentrations at 2 min did not correlate with increases in the sum intensity of panic symptoms (Spearman $\rho=-0.24$, $p=0.16$). This indicates that the pharmacokinetics of the drug is not the major determinant of the responses.

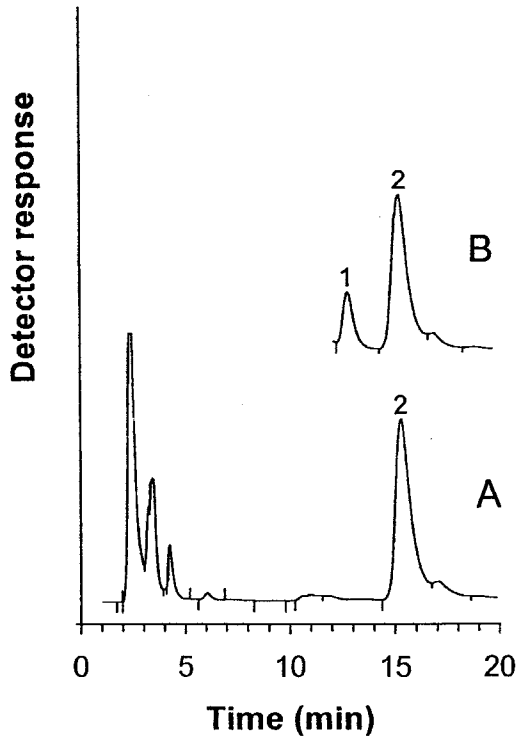

Figure 5.1. Typical HPLC traces from plasma samples taken from the same subject at baseline (A) and 2 min following flumazenil injection (B). Traces from samples in which no lamotrigine (IS) was added contained no peak at the position of the IS. (Peak 1: flumazenil; peak 2: IS)

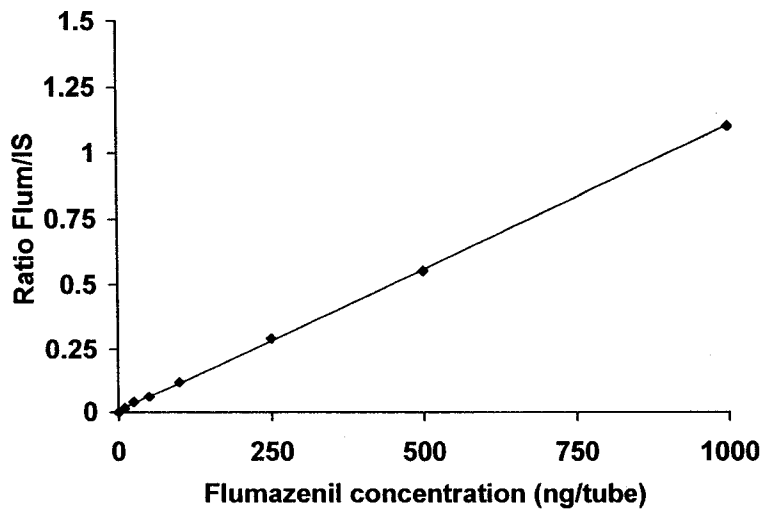

Figure 5.2. A typical calibration curve from the assay procedure.

Table 5.1. Plasma flumazenil concentrations (ng/ml) in panickers (Panic) and non-panickers (Nonp) following a single intravenous dose of 2 mg.

Panic/ subject no.	2min	4min	Nonp/ subject no.	2min	4min
1	102.33	52.95	1	322.73	78.18
2	283.32	158.73	2	214.4	58.57
3	914.23	75.88	3	155.41	56.52
4	151.49	22.68	4	236.85	86.79
5	91.41	33.29	5	322.81	126.55
6	77.99	23.87	6	824.16	492.65
7	65.44	46.95	7	373.34	95.29
8	499.46	81.69	8	411.14	67.14
9	705.56	64.52	9	173.05	57.5
10	41.67	7.59	10	520.09	160.07
11	76.33	9.08	11	1533.85	158.19
12	29.46	6.73	12	105.24	15.09
13	123.62	71.56	13	462.54	62.25
14	253.85	131.47	**Median**	**322.81**	**78.18**
15	191.63	92.41		**(193.7-491.3)**	**(58.0-142.4)**
16	191.11	61.3			
17	256.5	82.75			
18	436.53	61.72			
19	704.73	140.71			
20	592.98	167.94			
21	241.7	58.18			
22	171.82	69.45			
23	157.71	38.0			
24	694.89	154.81			
Median	**191.37**	**63.12**			
	(94.1-483.7)	**(34.5-90.0)**			

Median values (25^{th} - 75^{th} percentiles).

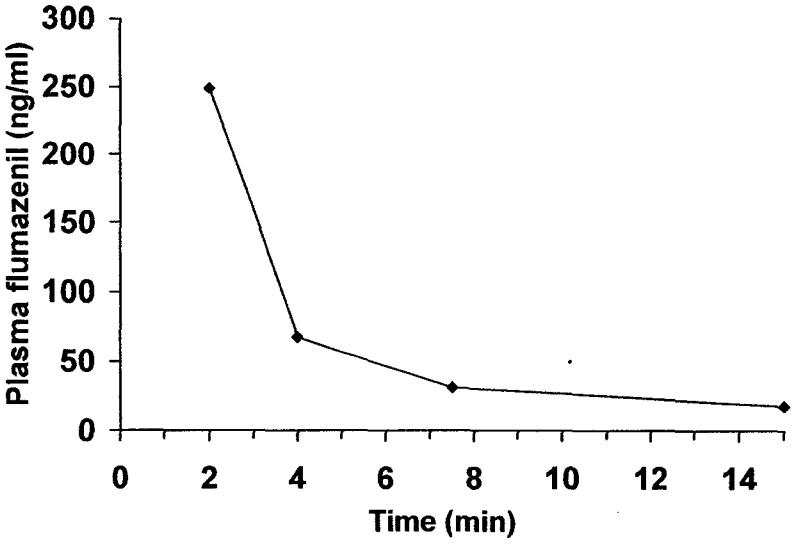

Figure 5.3. Plasma concentration time profile of flumazenil in 6 subjects (median values).

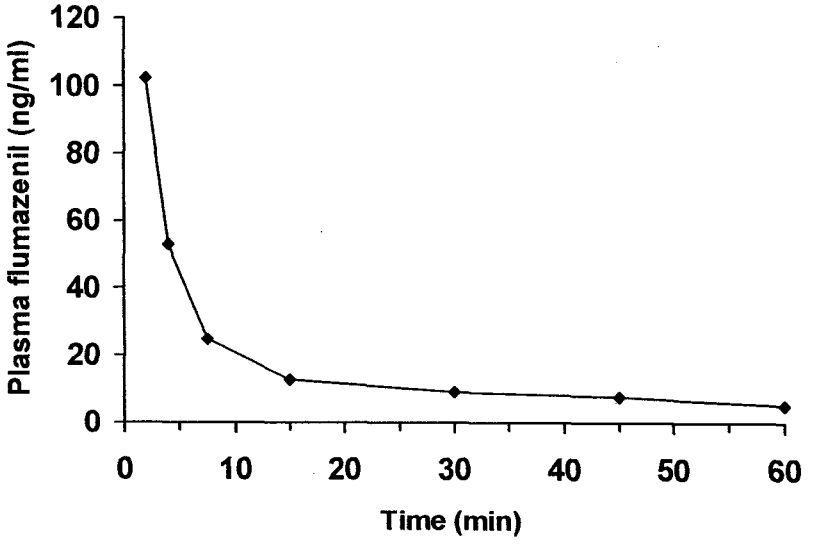

Figure 5.4. Plasma concentration time profile of flumazenil in 1 subject.

5.4. References

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6. GENERAL DISCUSSION

The main aim of all three studies presented in this thesis was to investigate the mechanism of the panicogenic action of flumazenil, a competitive antagonist at the benzodiazepine site of the most common $GABA_A$ receptor subtypes.

In the major study, presented in chapters 3 and 4, flumazenil was administered to 4 groups of subjects (P, P+D, D, HV), and a full range of behavioral, physiological, and cognitive measures was collected and thoroughly analyzed. In addition, the reproducibility of the effects of flumazenil was assessed. This study represents the most systematic evaluation of responses to flumazenil to date. Thus, despite several methodological limitations discussed in previous sections, the probability of obtaining comprehensive and objective information was relatively high. In the first study with healthy volunteers (chapter 2), doses of TRH and flumazenil were determined which, if given to PD patients, may help to clarify whether flumazenil has specific panicogenic effects that are not explained solely by negative interpretations of somatic symptoms. In the final study (chapter 5), comparisons of plasma flumazenil levels between PD panickers and non-panickers determined the relevance of the pharmacokinetics of the drug in its actions.

The results indicated that flumazenil-induced panic involves both biological and cognitive mechanisms.

6.1. Mechanisms of flumazenil-induced panic: general comments

Recently a theory has been proposed that panic is generated by a hypersensitive fear network centered in the amygdala, and that different panicogenic agents trigger panic by *nonspecifically* activating this network, including projections from the amygdala to brainstem centers, the periaqueductal grey and hypothalamus (Gorman et al 2000; Sinha et al 2000). These brain regions have been implicated in the generation of panic in a number of animal studies (Brandao et al 1986; Shekhar and DiMicco 1987; Graeff et al 1993; Milani and Graeff 1987; Sanders and Shekhar 1995). The theory further posits that a differential "strength" of the efferent pathways from the amygdala that develops over time may account for the inconsistent autonomic and neuroendocrine activation observed during panic attacks (Gorman et al 2000). While this may apply to natural panic, the suggestion is unlikely to explain why physiological activation varies with different laboratory panicogens. The observation that physiological responses to individual challenge agents tend to be consistent (whether or not activation occurs) indicates that these challenges are likely to exhibit their effects by targeting *specific* brain areas, rather than nonspecifically activating the above fear network. This does not preclude the possibility that the target areas are common for some challenges, or that the initial regional actions result in a more widespread activation within the network.

The role of biological factors in flumazenil-induced panic was indirectly supported by the results of the first study in this thesis (chapter 2). TRH, which is not panicogenic in PD patients (Stein and Uhde 1991; Tancer et al 1990), induced equal or greater subjective and objective physiological responses than flumazenil in healthy

controls, indicating that cognitive response to somatic symptoms may not be the primary or the only mechanism of flumazenil-induced panic. The other indirect evidence for biological mechanisms was the finding that catastrophic cognitions, although frequently associated with responses to flumazenil, were neither necessary nor sufficient for the occurrence of panic. The final argument for a direct panicogenic effect of flumazenil was the lack of strong cognitive predictors of panic responses in PD patients (chapter 4).

The idea that flumazenil induces panic by acting as an inverse agonist due to a global shift in the activity of benzodiazepine receptors in PD (Nutt et al 1990) was neither supported by the results of this, nor of other studies (Strohle et al 1999; Strohle et al 1998; Woods et al 1991). However, as discussed earlier in the thesis, a more restricted regional expression of $GABA_A$ receptor subtypes at which flumazenil has an inverse agonist activity is possible. Such a subtype has recently been discovered (Dunn et al 2003), and this finding will certainly stimulate further research on altered $GABA_A$ receptor function in PD.

Nevertheless, cognitive factors, particularly catastrophic cognitions, may also play a role in responses to flumazenil, although it is unclear whether these are the causes or concomitants of panic. A common limitation of studies of cognitive mechanisms mediating responses to challenges is the focus on one or two variables. In addition, since the manipulation of one variable of interest can affect other variables, the relative contribution to the panic outcome may be difficult to determine. A strength of the study presented in chapter 4 was a conservative assessment of a variety of variables, including the effect of familiarity on these variables

6.2. Validity of flumazenil challenge as a model of panic

Although the criteria for an "ideal" panicogen (see section 1.2.1) have not yet been systematically evaluated with flumazenil, several comments can be made. The drug is safe for humans; no significant side effects of an intravenous dose of 2 mg were observed in patients with PD or other psychiatric disorders (Coupland et al 2000; Nutt et al 1990; Randall et al 1995; Strohle et al 1998), or of intravenous doses up to 100 mg in volunteers (Darragh et al 1983). The present study (chapter 3) and previous studies (Nutt et al 1990; Woods et al 1991; Bell et al 2002) have demonstrated that flumazenil causes affective, cognitive and physical symptoms of a panic attack. Furthermore, the panicogenic effect is specific for PD patients; patients with PTSD (Randall et al 1995); social phobia (Coupland et al 2000), MDD and HV (the present study and Nutt et al 1990) are significantly less vulnerable.

Whether flumazenil meets the remaining criteria for an ideal panicogen remains unclear. First, it has not been determined whether antipanic drugs antagonize its panicogenic effects. Testing is limited to non-benzodiazepine drugs due to the possibility of flumazenil-precipitated benzodiazepine withdrawal. In a recent study, only 1 out of 14 PD patients who had recovered on the SSRI paroxetine panicked in response to flumazenil, while seven of them panicked when serotonin activity was reduced via tryptophan depletion (Bell et al 2002). This finding suggests that treatment with paroxetine reduces the panicogenic effect of flumazenil, but it needs to be confirmed by a comparison of responses to flumazenil before and after treatment.

Second, although the overall intensity of severe spontaneous and flumazenil-induced panic attacks (rated on the PSS) was not significantly different in this study, the

average similarity between flumazenil-induced panic (assessed by the semi-structured interview) and usual panic, rated by subjects on a 100 mm VAS (0mm = not at all similar; 100mm = exactly the same), was only 55.7±26.7 (median = 62.0). Furthermore, although overall panic rates did not differ significantly on day 1 and day 2 in subjects who received flumazenil twice, the occurrence of panic attacks was not highly reproducible in each subject. The sum intensity of panic symptoms showed better reproducibility.

These results and previous reports on the lack of panicogenic activity of flumazenil (Strohle et al 1999; Strohle et al 1998) may raise doubts about whether or not flumazenil challenge is a suitable tool for studying the etiology of panic disorder. Nevertheless, the finding that flumazenil, but not placebo, triggered abnormal responses in a subgroup of PD patients in 4 out of 6 studies clearly points to biological and/or cognitive abnormalities that need to be elucidated.

It is unclear why a significant proportion of PD patients is not sensitive to flumazenil, but several explanations, that are not mutually exclusive, come to mind. The proposed shift in the benzodiazepine site activity may not generalize to all PD patients, which points to the possible existence of PD subtypes. Further, if flumazenil causes panic by revealing abnormalities in the $GABA_A$ system triggered by primary defects elsewhere (e.g., by replacing an endogenous agonist at the benzodiazepine site, section 1.2.2.5), these compensatory changes may not yet have taken place in some patients undergoing flumazenil challenge. Finally, as the major study indicated (chapter 4), increases in panic

symptomatology in response to flumazenil may be associated with the severity of recent natural panic attacks.

6.3. Future research

Since the role of GABA and $GABA_A$ receptors in PD remains unresolved, at this stage it is difficult to assess the clinical significance (i.e., implications for the pathophysiology and therapy of PD) of the studies presented in this thesis. Understanding the nature of $GABA_A$ receptor abnormalities in PD may result in the development of antipanic drugs which will target these proposed dysfunctional receptors.

Neuroimaging and recombinant receptor studies may be the most promising research strategy for future work in this area. The former can provide information on areas activated during flumazenil-provoked panic attacks. The lack of hyperventilation during these attacks may reduce the probability that hypocapnia-induced vasoconstriction will obscure results by affecting cerebral blood flow during neuronal activation. In addition, the development of subtype-specific radioligands may determine regions with theoretically aberrant expression of $GABA_A$ receptors at which flumazenil acts as an inverse agonist. Another important task for future research will be to find mechanisms that regulate $GABA_A$ receptor gene expression. Flumazenil challenge, with a thorough assessment of cognitive variables, is likely to remain a valuable tool for gathering information, especially if TRH is used as a direct comparator. Finally, interactions between GABA and other neurotransmitters that may play a role in flumazenil-induced panic, such as serotonin (Bell et al 2002), should also be addressed in future studies.

6.4. References

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