

# On the role of the human amygdala. Mapping functions and individual variations using fMRI.

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## LIST OF PAPERS

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1) Ousdal OT, Jensen J, Server A, Hariri AR, Nakstad PH, Andreassen OA. *The human amygdala is involved in general behavioral relevance detection: evidence from an event-related functional magnetic resonance imaging Go-NoGo task*. *Neuroscience* 156 (2008): 450-455.

2) Ousdal OT, Reckless GE, Server A, Andreassen OA, Jensen J. *Effect of relevance on Amygdala activation and association with the Ventral Striatum*. *Neuroimage*. *In press*

3) Ousdal OT, Brown AA, Jensen J, Nakstad PH, Melle I, Agartz I, Djurovic S, Bogdan R, Hariri AR, Andreassen OA. *Associations between variants near a monoaminergic pathways gene (PHOX2B) and amygdala reactivity: A genome-wide functional imaging study*. *Twin Research and Human Genetics*. *In press*

## ABSTRACT

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The classic view of the amygdala argues that this collection of nuclei located deep within the temporal lobe is part of a neural circuit essential for fear and fear-related learning. A surge of work in animals and humans during the past ten years has modified this view, demonstrating that amygdala is involved in a number of processes of both emotional and nonemotional nature. However, the precise operating characteristic of this brain area is still not known, and the aim of the current thesis was therefore to gain more knowledge about amygdala's functional specialization.

One intriguing theory put forward by Sander and colleagues (2003) assessed the amygdala as a "relevance detector", responding to stimuli and events that are of central importance for the individual. This hypothesis integrates the diverse findings from animal and human studies, but few attempts had been made to directly operationalize and test this putative function of the amygdala. The first goal of this thesis was to test this hypothesis by using functional Magnetic Resonance Imaging (fMRI) and tasks encompassing high and low relevant stimuli. We demonstrated that amygdala responses reflected instructed behavioral relevance of a set of neutral letter and number stimuli. We found increased amygdala responses to behavioral relevant letter and number stimuli as compared to less relevant letter stimuli.

Equivalently, in a second experiment, amygdala responses to a neutral stimulus – response task varied according to the relevancy of the task. While relevance was manipulated based on instruction in paper 1, a more sophisticated design was used in paper 2, linking the event's relevancy to the achievement of a monetary reward. Thus, significant greater amygdala responses was observed in high relevance conditions where reception of a monetary reward



was contingent upon subject's task performance compared to low relevance conditions where the reward was received unrelated to this task. The data replicated and extended the findings from paper 1, supporting that amygdala encodes or calculates relevance. By using reward to manipulate relevance and not only instructions, the central importance of the highly relevant events' became more explicit in the second experiment. In addition, functional connectivity analysis in paper 2 indicated that information regarding relevancy may be transferred to the ventral striatum, which subsequently initiate actions.

Importantly, amygdala activation to emotional and nonemotional stimuli demonstrates considerable variation across individuals. Interestingly, such interindividual variation in amygdala responsivity has been linked to both personal traits and vulnerability to psychopathology. Our second goal was to search for biological mechanisms, i.e. gene variants, related to such variations. We combined genome-wide data with functional imaging phenotypes to discover genetic variants which affect amygdala activation to emotional stimuli. We found a genome-wide significant association with a regulatory region upstream of a monoaminergic pathway gene, possibly affecting this gene's expression. Thus, the present finding is in line with substantial evidence indicating how genetic variants within monoamine signaling pathways influence amygdala structure and function.

The current results demonstrate that encoding or calculation of events' relevance is an important amygdala function. Further, the individual's response to such relevant events may depend on genetic variation within monoaminergic signaling pathways.

## ABBREVIATIONS

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**BOLD:** Blood Oxygen Level Dependent

**CS:** Conditioned Stimulus

**EPI:** Echo Planar Imaging

**fMRI:** functional Magnetic Resonance Imaging

**FDR:** False Discovery Rate

**FOV:** Field Of View

**FWE:** Family-Wise Error

**GLM:** General Linear Model

**GWA:** Genome-wide association

**Hb:** Hemoglobin

**HRF:** Hemodynamic Response Function

**ITI:** Intertrial Interval

**LTP:** Long Term Plasticity

**MRI:** Magnetic Resonance Imaging

**PPI:** Psychophysiological Interaction

**ROI:** Region Of Interest

**SPM:** Statistical Parametrical Mapping

**TE:** Echo Time

**TR:** Repetition Time

**US:** Unconditioned Stimulus

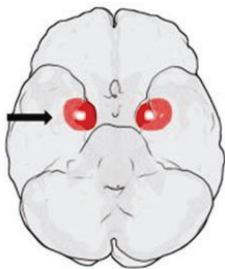
# INTRODUCTION

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## 1.1 DEFINITION AND HISTORY OF THE AMYGDALA

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It was not until the beginning of the 19<sup>th</sup> century that the anatomist K.F. Burdach first used the name “amygdala” to describe a subcortical grey matter located anterior to the hippocampus deep within the temporal lobe. The term “amygdala” is derived from Greek and probably refers to the resemblance of an almond that the amygdala has in gross anatomy slices (Davis and Whalen, 2001; LeDoux, 2007). It very soon became apparent that the amygdala was not a homogenous structure, but rather a collection of nuclei, and thus in the 1920s the contemporary partition of the amygdala into basolateral, centromedial and cortical divisions was formally expressed by anatomist J.B. Johnston (Johnston, 1923). Our current understanding of the amygdala reflects a long and important research tradition which was initiated by anatomists of the 1800s. The pioneer lesion studies in nonhuman primates of Kluver and Bucy in the 1930s initiated the debate regarding the functions of the amygdala. They demonstrated that temporal lobectomi in primates resulted in approaching previous



feared objects, hyperorality and hypersexuality (Klüver and Bucy, 1937). Later, Weiskrantz (Weiskrantz, 1956) hypothesized that amygdala was the structure in the temporal lobe eliciting these behavioral changes, based on lesion work in monkeys. This was followed by a series of electrical amygdala stimulation studies and

concomitant EEG studies in cats by the Norwegian scientists Kaada and Ursin (Ursin and Kaada, 1960) and subsequently in humans by Gloor and colleagues (Gloor et al., 1982).

**Figure 1:** Localization of the amygdala from below (Joseph E. LeDoux (2008), *Scholarpedia*, 3(4):2698.

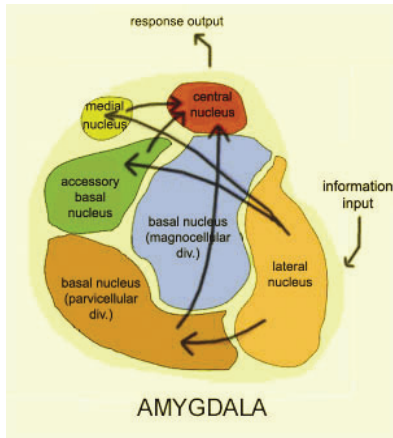
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Kaada and Ursin's important discovery of an alertness response of the cat under mild amygdala stimulation (Ursin and Kaada, 1960) sparked off numerous of studies exploring amygdala's role in attention and alertness processes. Our modern understanding of the amygdala builds upon the studies investigating amygdala's essential role in Pavlovian fear conditioning in rats by the scientists such as Kapp, LeDoux, McGaugh and Davis' in the 1980s (Kapp et al., 1979; Davis, 1986; LeDoux et al., 1988). The essential role of amygdala in fear resulted in the belief that amygdala was a specialized fear module/threat detector (Ohman and Mineka, 2001) and initiated numerous of studies investigating the link between amygdala and concepts of emotion. Due to accumulating diverging results, this specialized function of amygdala has recently been questioned. Thus, an aim of the current thesis was to further characterize amygdala function with emphasis on the broad range of motivating stimuli engaging the amygdala.

## 1.2 ANATOMICAL ORGANIZATION AND NOMENCLATURE OF THE AMYGDALA REGION

---

The amygdala is a relatively small brain structure with an altogether diameter of around 15 mm (Zald, 2003) in humans. As described above, the amygdala structure is not homogenous, but rather a conglomeration of nuclei, which can be separated according to trajectory of fibers, chemical signature and histological appearance (LeDoux, 2007). There is still a great deal of controversy on how the amygdala should be subdivided and further how these subdivisions relate to other brain areas.



**Figure 2.** Some of the different nuclei of the amygdala (Canadian Institutes of Health Research. Institute of Neuroscience ©)

Though amygdala consists of at least 13 nuclei, and even more subnuclei (Sah et al., 2003), it is common to group these nuclei into regions. One longstanding theory is that amygdala encompasses a phylogenetically older part, the centromedial and the

cortical division, consisting of the cortical, medial and central nuclei, which relates to the olfactory system (Johnston, 1923; Davis and Whalen, 2001). However, some scientists prefer to assign these nuclei together as the centrocorticomedial division (LeDoux, 2007), or even consider them as a continuum of the basal forebrain (Heimer, 2003). Contrary to these, the basolateral complex is of a phylogenetically newer origin, including the lateral, basal and accessory basal nuclei relating to the neocortex (Johnston, 1923; Davis and Whalen, 2001). Within the primate amygdala, the lateral nucleus is often regarded as the major input gate of the amygdala, receiving afferent information from all sensory modalities by means of thalamic and cortical fibers (Davis and Whalen, 2001; LeDoux, 2007). The lateral nucleus relays this information to one of the major output regions, the central nucleus, both directly and indirectly via the basal and accessory basal nuclei (Pitkanen et al., 1997; LeDoux, 2007). When activated, the central nucleus is essential for controlling emotional behavior and related physiological responses via its projection to the brainstem and the hypothalamus (Davis and Whalen, 2001). These connections are reciprocal and travel mainly within the ventral amygdalofugal pathway and the stria terminalis (Amaral et al., 1982). However, controversies regarding this serial model exists, as some scientist prefer to look at the centromedial and basolateral regions as separate systems working in parallel (Balleine and

Killcross, 2006). Indeed, the basal nucleus sends projections to several cortical areas thus completing independent loops of information flow between the amygdala and neocortex in addition to subcortical areas like the striatum (Price, 2003).

Along a rostralcaudal line, the Anterior Amygdaloid Area (AAA) and the Amygdalohippocampal Area (AHA) are nuclei defining the most rostral (i.e. AAA together with Nucleus of the Lateral Olfactory Tract) and caudal (i.e. AHA) areas of amygdala, respectively (Sah et al., 2003). In addition, the intercalated cell masses defines clusters of neurons located within the fiber bundles separating the different amygdala nuclei, and their functional specificity are largely unknown (Sah et al., 2003). The remaining nuclei will not be described in detail here, all of which are more or less described in primates. One important notion is that the current spatial resolution of functional neuroimaging in humans does not allow one to separate individual nuclei within the amygdala. Thus, in neuroimaging studies, the focus on amygdala is more at a macrolevel than the microlevel evident in rodent and nonhuman primate studies. However, with the emergence of more focused high-resolution fMRI, this may change in the future.

### 1.3 CELLULAR ACTIVITY

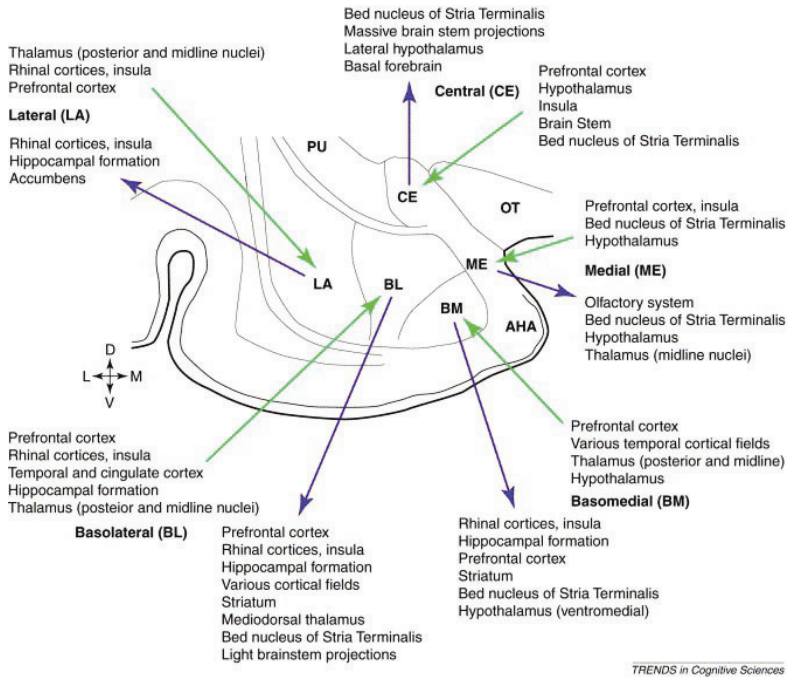
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The amygdala consists of several types of neurons (Sah et al., 2003). The principal neurons (also called the projection neurons as they project out of the amygdala) are both inhibitory (projecting from the central nucleus) and excitatory (projecting from the basolateral complex) in nature (Sun and Cassell, 1993; Sah et al., 2003). The principal neurons receive excitatory inputs from glutamatergic cortical and subcortical afferents, which also form synapses with local amygdala interneurons (Sah et al., 2003). The interneurons are mainly responsible for the communication between the different nuclei in the amygdala, many of them being

inhibitory using GABA as a transmitter (McDonald, 1985). However, interneurons also connect to principal neurons, which results in feedforward inhibition (Woodson et al., 2000). It is speculated that such mechanisms causes the observed habituation of amygdala activity to repeated stimuli presentations. In addition to the projection neurons and the interneurons, a third cell type referred to as the intercalated cells have been described. These cells are small, and show firing patterns between those of projection neurons and interneurons (Sah et al., 2003). As for the interneurons, these cells are also GABAergic. The amygdala is considered to be a rather “silent” area of the brain due to its strong inhibitory network keeping spontaneous cellular activity low. This is mostly due to GABAergic mechanisms (i.e. interneurons and intercalated cells) controlling amygdala projection neurons (Woodson et al., 2000; LeDoux, 2007).

Information flow through the amygdala is modulated by various neurochemical systems. Several neurotransmitters, including noradrenalin, dopamine, serotonin and acetylcholine are released within the amygdala and influence the interaction of excitatory and inhibitory neurons. These neuromodulators are released in a diffuse manner, thus the individual amine’s receptor profile determines its specific effect within amygdala (LeDoux, 2007). In addition, hormones reaching the amygdala through the bloodstream and different peptides released locally from axons within the amygdala influence its activity (LeDoux, 2003).

## 1.4 CONNECTIONS



**Figure 3.** Amygdala main connections. Afferents are represented by a green arrow, efferents in blue. For simplicity, some of the projections to neuromodulatory systems of the brainstem and the basal forebrain are not demonstrated. AHA, amygdalohippocampal area; OT, optic tract; PU, Putamen; D, dorsal; V, ventral; L, lateral; M, medial. ((Pare et al., 2002). Printed with permission)

To understand the actions amygdala takes part in, its vast intrinsic and extrinsic connections must be appreciated. Due to amygdala's close relations to autonomic functions and arousal, the earliest studies focused on subcortical connections. Amygdala has bidirectional connections to the hypothalamus, and takes part in a central circuit regulating the autonomic nervous system (Kapp et al., 1982). In addition, amygdala is interconnected with the basal forebrain (including Nucleus basalis of Meynert) and several brain stem nuclei like the serotonergic raphe nucleus, the dopaminergic ventral tegmental area and the noradrenergic



locus coeruleus, thus allowing the amygdala to initiate neuroendocrine responses to received information (Davis and Whalen, 2001). As observed in early amygdala stimulation studies in cats, reflexive behavioral changes occurred as a response to amygdala stimulation, and these are possibly mediated through the connections with periaqueductal gray, pons and the medulla (Ursin, 1960).

Amygdala is able to influence both the memory of facts and events and those of habits and skills, due to its connections with the hippocampal formation and the striatum, respectively (Packard and Teather, 1998). The connections to striatum are also implicated in goal directed behavior in response to rewards and their predictors (Cardinal et al., 2002).

All of our sensory modalities project to the amygdala, and hence amygdala receives sensory information from the visual system (via the inferior temporal lobe, especially area TE) as well as auditory (superior temporal lobe, especially area TA), somatosensory (insula), olfactory (olfactory lobe), gustatory and visceral (Nucleus tractus Solitarius and Parabrachial nucleus) systems (Davis and Whalen, 2001; Price, 2003). Classically, the amygdala auditory afferents were separated into a high-route (cortical afferents) and a low-route (direct thalamic afferents) of information, based on findings in rodents (LeDoux, 2000). However, the existence of a low-route in humans is still debated (Pessoa et al., 2002; Pessoa and Ungerleider, 2004). Among these sensory inputs, the olfactory, gustatory and visceral systems have direct inputs from early processing stages, while the other modalities reach the amygdala via higher order sensory association areas. The projections are reciprocal, and importantly, the efferent connections are more widespread than the afferent, hence extending to more primary sensory areas of the different modalities (Price, 2003). This has led to the

understanding that amygdala modulates information processing within different sensory modalities (Phelps and LeDoux, 2005; Vuilleumier, 2005).

In addition to sensory cortex, amygdala is heavily connected to some prefrontal areas, which are implicated in decision making (the Orbitofrontal cortex and ventromedial cortex) (Bechara et al., 2003), processing of painful stimuli (insula) (Bornhovd et al., 2002) and regulating amygdala neural activity (for example the anterior cingulate cortex and indirectly lateral prefrontal cortex) (Phillips et al., 2003a).

## 1.5 AMYGDALA'S FUNCTIONAL PROFILE

---

The functional specialization of the amygdala has been a theme of discussion since its earliest recognition. Below, the current knowledge of the amygdala is briefly summarized. Though our knowledge has increased vastly the last decades, there are still aspects that are uncertain related to both functional specialization and individual variations in functional aspects. The focus of this Ph.d. thesis was therefore to add knowledge about amygdala's functional profile, and to discover biological sources of individual variation in amygdala activity.

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### 1.5.1 EMOTIONAL LEARNING

---

The proposal that memory is composed of several distinct systems is not new. Milner demonstrated that a simple hand-eye coordination skill could be learned by the severely amnesic patient H.M despite his lack of memory of having practiced the task before (Milner et al., 1968). This finding was a breakthrough as it demonstrated that our memory is not a uniform entity, but rather should be considered as several heterogeneous processes involving several brain areas. Cases like H.M. together with a vast of animal research lead to our

current understanding of long time memory systems and brain areas associated with these. Often, memory is divided into declarative and nondeclarative systems, where the latter is an umbrella term referring to several additional memory systems (Squire, 2004). The perhaps best studied of implicit memories is fear conditioning, also called Pavlovian fear conditioning, which is a memory form highly conserved across species involving the amygdala (LeDoux, 2003).

#### *1.5.1.1 FEAR CONDITIONING*

In classical fear conditioning, the animal learns to predict aversive events by means of association learning. Basically, an initial neutral stimulus (Conditioned stimulus (CS)), often a visual or auditory stimulus acquires emotional properties as it is paired temporally with a biological significant event (Unconditioned stimulus (US)), often an electrical shock. After this US-CS pairing has been presented several times, the CS can illicit fear responses such as freezing behavior and related physiological changes (Conditioned response (CR)), when presented alone. This indicates that an association is learned, and a long lasting memory is formed (LeDoux, 2000; Ohman and Mineka, 2001).

An influential study by Kapp and colleagues introduced amygdala as a part of the neural circuit underlying fear conditioning by demonstrating that lesions to the central nucleus impaired heart-rate conditioning in rabbits which is part of the conditioned physiological response (Kapp et al., 1994). This was followed by the work of LeDoux and colleagues on rodents, which set the ground for our current understanding of the neurophysiologic underpinning of conditioned fear responses (LeDoux et al., 1988; LeDoux, 1993). Based on current knowledge, amygdala is an essential part of the neural circuit acquiring and expressing conditioned fear. Information about the CS and US reach the amygdala by two

parallel pathways in the rat, which were named the “high road” and the “low road”. The low road involves a fast subcortical direct transmission of information from the thalamus to the amygdala and was demonstrated by LeDoux for the auditory domain (LeDoux et al., 1990). The “high road” is regarded as a slower road which includes cortical part of the brain, passing sensory cortices before entering the amygdala (Romanski and LeDoux, 1993). There is an ongoing discussion if a similar dichotomy can be found in humans, however so far no direct anatomical evidence exist (Pessoa and Adolphs, 2010). Both information about the CS and the US terminate in the lateral (La) nucleus of the amygdala where plasticity and hence memory storage has been proposed to occur (LeDoux, 2000). Information entering the La is transmitted via direct and indirect routes to the central nucleus (Pitkanen et al., 1997). Therefore, the central nucleus is often regarded as a major output region, initiating the observed behavioral, autonomic and endocrine responses as the learning progresses (LeDoux, 2000). A thorough description of the molecular mechanisms underlying fear conditioning is beyond the scope of this introduction, but so far evidences support that mechanisms parallels those observed in the hippocampus, i.e. Long Term Plasticity (LTP) in which protein-synthesis strengthens CS-input synapses (Blair et al., 2001; Schafe et al., 2001).

Fear conditioning has been studied in numerous species, including rodents, nonhuman primates and humans. In animals, conditioned fear is often measured with freezing (stop of current activity) or fear potentiated startle (augmented startle reflex), as these responses have proven reliable measures of fear learning. In humans, such physiological responses are measured in terms of skin conductance responses (sympathetic induced skin response) and often combined with a neuroimaging tool to simultaneously obtain a neurological correlate. Briefly, functional Magnetic Resonance Imaging (fMRI) studies have reported that fear conditioning leads to increased fMRI-responses in the amygdala, and that the strength of

these conditioned responses measured with skin conductance responses correlated with the amygdala fMRI-response (Hugdahl et al., 1995; LaBar et al., 1995). Parallel to the findings in nonhuman primates, humans suffering from bilateral amygdala lesions fail to develop conditioned fear responses, but are able to explicitly recall the association between the CS and the US which most likely relies on the hippocampal formation (Bechara et al., 1995; LaBar et al., 1995).

Though amygdala's role in fear learning is widely accepted, the last decades have seen a great number of works linking amygdala to other functions than just fear. The notion that amygdala responds to reward and reward-predictive cues as well as positive affect (Baxter and Murray, 2002; Murray, 2007), accelerated this debate.

#### *1.5.1.2 REWARD LEARNING*

---

A considerable amount of research has assigned the amygdala to reward-related learning as well (Baxter and Murray, 2002; Murray, 2007). Amygdala neurons are responsive to reward-predictive cues (i.e. CS) (Paton et al., 2006), consistent with the idea that a cue-reward association is learned parallel to that observed in fear conditioning. Indeed, a strengthening of thalamo-amygdala synapses has been observed as learning progress (Tye et al., 2008), also similar to fear conditioning (McKernan and Shinnick-Gallagher, 1997). Though evidence indicate that a conditioned response to the rewarding CS can be learned without an intact amygdala (contrary to fear conditioning) (Gallagher et al., 1990), lesions of the amygdala may impair flexible use of the CS-US association in new learning (i.e. second order conditioning) (Hatfield et al., 1996) and revalue the CS when the current value of the US is changed (i.e. reinforcer revaluation) (Malkova et al., 1997). The reason for the apparent discrepancy to fear learning is currently under investigation. One way to interpret this is to

consider the association between CS-US to consist of several parallel associations, in which some rely on sensory features of US and other more general motivational features (Balleine and Killcross, 2006). Some of these associations depend on a subset of amygdala nuclei (mainly the centromedial or the basolateral complex), where each subset of nuclei takes part in separate parallel loops. This parallel view of the amygdala (Balleine and Killcross, 2006), has now received some support in fear conditioning as well (Pare et al., 2004). Ultimately, some aspects of the cue-reward association depend on different amygdala-circuits (i.e. approach behavior to the cue is presented depend on the central nucleus etc), while others are unrestrained of an intact amygdala during their formation (Balleine and Killcross, 2006). Others have suggested that some types of stimulus-reward association learning can be learned by simple visual discrimination, hence bypassing the amygdala (Baxter and Murray, 2002).

The observation that amygdala was involved in parts of reward learning as well, was difficult to reconcile with the view that amygdala was a fear module/threat detector. Thus, it sparked off a number of studies that linked amygdala to other emotional qualities like sadness (Yang et al., 2002) and surprise (Kim et al., 2004), as well as emotional neutral stimuli of social relevance (Adolphs, 2003). Consequently, a more generalized role in emotional association learning was proposed, in which amygdala parcel out the emotional significance of stimuli and events, subsequently adjusting related behaviors (Zald, 2003).

---

### *1.5.1.3 EMOTIONAL FACES*

Both fear and reward conditioning are examples of emotional learning where a sensory stimulus (the CS) predicts a negative or positive event (the US). However, in humans emotional facial expressions of con-species may serve as CSs. Most likely, such emotional faces have predicted significant (both positive and negative) events in our past, and thus these

experiences are used to respond appropriately when facing emotional expressions at present. As the field of neuroimaging (i.e. positron emission tomography (PET) and functional magnetic resonance imaging (fMRI)) evolved, the interest in the neural substrate of emotional face perception among humans grew tremendously. Based on the important role of the amygdala in fear learning in the animal literature, the amygdala soon became a candidate region for this network. Neuropsychological research had documented that patients with selective amygdala lesions had problems recognizing fearful expressions (Adolphs et al., 1994), and that they judged such faces more trustworthy (Adolphs et al., 1998). From the 1990s numerous of neuroimaging studies examined the neural correlates of emotional facial processing and reported increased amygdala responses to fearful relative to happy facial expressions (Morris et al., 1996; Morris et al., 1998). Further, these responses were also found when the fearful face was not the focus of attention (Vuilleumier et al., 2001), and even when it did not reach awareness due to very brief presentation (backward masking) (Whalen et al., 1998), or striatal cortex damage (Morris et al., 2001). However, other scientists reported significant amygdala hemodynamic responses to all types of facial emotions (Fitzgerald et al., 2006; Sergerie et al., 2008), even neutral faces if relevant for the task (Wright and Liu, 2006), thus questioning this superiority of fear. Also, the amygdala responses to such emotional faces were sensitive to context (Kim et al., 2004) and even current goals (Cunningham et al., 2008), indicating that other factors influenced amygdala neuronal firing as well. Based on this, it was argued that amygdala's domain of specificity had to be of a more general art (Fitzgerald et al., 2006; Wright and Liu, 2006), however redefining its role based on newer data was challenging.

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## 1.5.2 MODULATION OF OTHER BRAIN AREAS

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To assess amygdala's domain of specificity, its role in modulating other brain areas should be appreciated. Amygdala exhibits extensive connections with much of the cerebral cortex in addition to numerous subcortical regions allowing it to modulate cognitive processes like attention and memory (Phelps and LeDoux, 2005; Vuilleumier, 2005). Importantly, the amygdala is not essential for these processes, but act modulatory, meaning that for those events that engage the amygdala, memory and attention increase (Phelps and LeDoux, 2005; Vuilleumier, 2005). By combining neuroimaging and psychological tests, this enhancement of attention and memory orchestrated by the amygdala has been demonstrated for emotional events (Cahill et al., 1996; Lim et al., 2009). However, there are data indicating that such modulation occurs for a broader array of motivational stimuli than just emotional ones (Ferguson et al., 2001; Adolphs and Spezio, 2006).

### *1.5.2.1 PERCEPTION AND ATTENTION*

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As our sensory systems often receive several competing stimuli simultaneously, amygdala inputs may help guiding which stimuli that preferentially should be processed. There are several ways in which amygdala can affect perception, but perhaps most important here is the enhancement of neuronal responses to attended stimuli (Kastner and Ungerleider, 2000).

Data relating amygdala to modulation of sensory cortex responses comes from both animal and human research. During fear conditioning, amygdala influence plasticity in specific sensory-processing systems in rodents. To demonstrate such, auditory-frequency-receptive fields of single cells were recorded in the auditory thalamus or auditory cortex during fear conditioning. The CS was a frequency not optimal for the particular cell. After repeatedly pairing the CS with the US, the cell's frequency response had attuned to the specific CS-



frequency at the expense of other sequences. This change then lasted for weeks, hence facilitating perception of the relevant frequency (Edeline, 1999; Phelps and LeDoux, 2005). To support that these changes were mediated by the amygdala, temporal recordings demonstrated that the plasticity changes occurred in amygdala before those in sensory cortices (Quirk et al., 1997). Secondly, lesions of the amygdala prevented these changes in sensory systems to occur (Maren et al., 2001). The second line of evidence linking amygdala to modulation of sensory systems, comes from neuroimaging studies in humans. A number of studies have demonstrated that amygdala-visual cortex activity covaries according to both the stimuli's emotional intensity (Morris et al., 1998; Winston et al., 2003; Sabatinelli et al., 2005) and valence (Anders et al., 2004). Evidence from lesion (Vuilleumier et al., 2004) and functional connectivity studies (Herrington et al., 2011) indicate that such modulation may be driven by the amygdala, and that these top-down projections from amygdala to visual cortex may be a neural substrate for the emotional modulation of attention (Vuilleumier, 2005).

There are at least three routes by which amygdala can influence visual sensory processes. As demonstrated by Amaral and colleagues in primates, amygdala has direct monosynaptic feedback projections to the visual cortex (Amaral, 1986). These monosynaptic projections reach all cortical stages along the ventral visual stream in a topographically organized manner (Amaral et al., 2003a), including the primary visual cortex. They terminate primarily in cortical layers I-II and V-VI, which is a typical pattern of feedback-type connections (Freese and Amaral, 2005). Secondly, amygdala's widespread connections with other parts of the cortex allow other indirect routes to serve the same purpose. Amygdala exhibits bidirectional connections with the orbitofrontal cortex, which both directly and indirectly communicates with the visual cortices (Vuilleumier, 2005; Ghashghaei et al., 2007). Indeed, functional

connectivity analysis in neuroimaging supports the latter (Lim et al., 2009). Thirdly, the central nucleus may influence visual cortex activity by its projections to basal forebrain neurons, which release excitatory acetylcholine onto sensory neurons upon stimulation (Sarter and Bruno, 1999).

#### *1.5.2.2 HIPPOCAMPAL DEPENDENT MEMORY*

In addition to modulating perception, there is a growing literature supporting that amygdala modulate memory formation in hippocampus (Richter-Levin and Akirav, 2000; McGaugh, 2002; Phelps, 2004). The direct and indirect projections from amygdala to the hippocampus are believed to enhance consolidation (i.e. strengthening the synaptic changes by supporting protein synthesis), in emotional arousing situations (Packard and Cahill, 2001).

Noradrenergic release in the basolateral complex of the amygdala in response to emotional arousing events leads to upregulation of activity in the hippocampal complex (McGaugh, 2004) and also increases synchrony between neuronal firing in these two brain structures at the theta frequency (Pare et al., 2002). The latter is directly linked to the enhanced memory-related plasticity in hippocampus (Pare et al., 2002). In addition, enhanced synchrony of theta activities in the amygdalohippocampal circuitry during memory retrieval enables successful retrieval of emotional memories (Seidenbecher et al., 2003).

In healthy subjects, amygdala activation during encoding predicts subsequent memory for the emotional events (Cahill et al., 1996; Dolcos et al., 2004), and this has not been demonstrated for the equivalent neutral events. In line with this, impaired memory enhancement for emotional events have been observed in patients suffering from amygdala lesions (Richardson et al., 2004) or amygdala atrophy secondary to Alzheimer's disease (Abrisqueta-Gomez et al., 2002).

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### 1.5.3 SOCIAL BEHAVIOR

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To discover the consequences of amygdala impairments, a number of animal studies have investigated alterations in behavior secondary to amygdala lesions. The very early observation from monkeys with bilateral temporal lobectomi lead to the proposal that amygdala is essential for our social behavior. The pioneer work of Kluver and Bucy (Klüver and Bucy, 1937) demonstrated how temporal lobectomi resulted in approaching previous feared objects, hyperorality and hypersexuality. Later, Weiskrantz (Weiskrantz, 1956) raised the hypothesis that amygdala was the structure in the temporal lobe eliciting these behavioral changes, and he named this syndrome The Kluver-Bucy syndrome. As the initial method resulted in large temporal lesions also encompassing hippocampus and enterorhinal cortex, it was difficult to interpretate which of the observed behavioral traits that were due to amygdala lesions. However, newer refined techniques support a role in social cognition for the amygdala, but the initial profound impairments have not been replicated (Amaral, 2002, 2003). In short, monkeys with bilateral amygdala lesions experienced reduced fear inducing potency of predators as well as deficits in complex social interaction (Amaral, 2002), the latter especially if lesions occur in a young age (Amaral, 2003).

This reduced fear is in line with findings in humans with amygdala lesions, as they generally rate people more trustworthy (Adolphs et al., 1998) and exhibit a more approach oriented attitude toward strangers (Kennedy et al., 2009). Moreover, humans with temporal lesions encompassing amygdala, develop no remarkable social deficits (Adolphs, 2003) including an almost normal emotional behavior, perhaps due to cognitive compensation techniques. Only in certain situations, like when facing ambiguous stimuli, do these persons have difficulties

(Bechara et al., 2003). These findings contributed to the evolution of new theories regarding amygdala's functional specialization, which will be discussed next.

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#### 1.5.4 CURRENT THEORIES AND UNCERTAINTIES

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The last decade has seen several new theories evolve regarding amygdala functions. Specifically, the amygdala has been assigned to encoding stimuli relevance (Sander et al., 2003), current value (Salzman et al., 2007) and to modulate vigilance to such biological important events (Whalen, 1998). The pioneering work of Kapp, Gallagher and colleagues who demonstrated that electrical stimulation of the central nucleus increased the arousal and attention of the animal (Kapp et al., 1982), became overshadowed by amygdala's role in fear learning in the 1980's. However, renewed interest for this path came from Whalen and colleagues amongst others in the 1990's. According to their theories, amygdala modulates vigilance at a moment-to-moment basis in response to stimuli that predict biological relevant outcomes (Whalen, 1998; Davis and Whalen, 2001; Whalen, 2007). Upon activation, the amygdala gives rise to a number of central and peripheral responses which promote the processing of such relevant information. The processing of uncertainty and ambiguity is especially potent to recruit the amygdala (Adams et al., 2003; Hsu et al., 2005) and thus modulate vigilance, as there is a need to collect more information in order to disambiguate the stimulus or event. A number of reports have supported this notion by demonstrating how the amygdala responds to ambiguous facial cues (Whalen et al., 2001; Adams et al., 2003) and uncertainty in decision-making (Hsu et al., 2005; Brand et al., 2007). Also, subjects with amygdala lesions tend to perform poorer in gambling involving uncertainty (Bechara et al., 2003). Though much research support that amygdala modulates vigilance, the stimulus dimension which causes amygdala to react is still debated. Though uncertainty is a potent

recruiter of the amygdala, some scientists have questioned if this is the only stimulus quality that amygdala responds to.

Based on electrophysiological recordings in nonhuman primates, it was reasoned that amygdala tracks current value of stimuli (Salzman et al., 2007). By combining electrophysiological recordings in monkeys with appetitive and aversive conditioning schedules, they discovered separate populations of neurons in the amygdala which reflected positive and negative values of both the conditioned stimuli and their reinforcers (Paton et al., 2006). Importantly, the neurons updated their responses rapidly to the conditioned stimuli during the learning (Belova et al., 2008) and they displayed graded responses according to the rewarding or aversive nature of the stimuli (i.e. stronger responses to high rewards than to low rewards) (Paton et al., 2006). However, there are reports linking amygdala to encoding of stimulus's intensity (Anderson et al., 2003; Small et al., 2003) and identity (Kreiman et al., 2000; Gothard et al., 2007), which may be difficult to reconcile with a valence-oriented framework. Also, in a more recent study by Shabel and Janak working with rat amygdala, they reported a group of neurons that responded equally to aversive and appetitive CSs (Shabel and Janak, 2009), thus the function of these neurons are not known.

The theoretical fundament for paper 1-2 in this thesis comes from the proposed role of amygdala in relevance detection first introduced by Sander and colleagues in 2003 (Sander et al., 2003). According to this theory, amygdala contributes to parcel out the relevance of stimuli and events on a moment-to-moment basis. The authors define relevance as: "an event is relevant for an organism if it can significantly influence (positively or negatively) the attainment of his or her goals, the satisfaction of his or her needs, the maintenance of his or her own well-being, and the well-being of his or her species" (Sander et al., 2003). The

notion that amygdala encodes the relevance of stimuli and events was based on a collection of fMRI experiments in humans and neurophysiologic recordings in monkeys indirectly demonstrating that amygdala responds to relevant as opposed to non-relevant events. Importantly, relevant stimuli includes, but also go beyond, emotional ones which is supported by a number of studies showing that amygdala responds to emotional neutral stimuli of social relevance as well (Schwartz et al., 2003; Hsu et al., 2005; Herry et al., 2007). Examples of the latter are bodily movements (Bonda et al., 1996), race groups (Phelps et al., 2000), trustworthiness (Adolphs et al., 1998), raising sound intensities (Bach et al., 2008) and eye gaze (Adams et al., 2003). Also, stimuli that are experienced as arousing or motivating can elicit amygdala responses (Zald, 2003). Consequently, the relevance theory entitles the amygdala with a more general sensory evaluation role than several of the previous theories regarding amygdala function.

Though the relevance hypothesis had gained some indirect support, it was difficult to operationalize it so that the conditions compared only differed according to relevance. Thus, to the best of our knowledge, a direct test of the hypothesis had not been undertaken. The focus of this thesis was to combine emotional neutral stimuli with manipulation of motivational relevancy to test this putative function of the amygdala. Not only would this support the relevance hypothesis, but also demonstrate how motivational significant stimuli without explicit emotional properties engage the amygdala.

## 1.6 AMYGDALA IN DISEASE

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Solitary lesions to the amygdala are rather unusual, but the rare Urbach-Wiethe disorder is an exception. Urbach-Wiethe disorder is an extremely rare genetic disorder with bilateral calcification in the temporal lobes, usually affecting the amygdala and periamygdaloid gyri

bilaterally (Staut and Naidich, 1998). It is caused by a mutation in the chromosome 1 at 1q21 (Hamada et al., 2002), and presents itself with neurological and dermatological symptoms (Siebert et al., 2003; Holme et al., 2005). More common etiologies in humans are severe cases of epilepsy causing medial temporal sclerosis, encephalitis or neurosurgical ablation of the amygdala for medically refractory epilepsy (Adolphs, 2010). The neurodevelopmental disorder Williams syndrome, caused by a deletion on chromosome 7q11.23 has gained great scientific interest, as it provides a model condition for understanding the relation between genetic variation, neural functioning and a well described set of behavioral-cognitive abnormalities (Martens et al., 2008). One striking feature of these patients, are their hypersociability. In general, individuals with Williams syndrome are socially fearless, engaging early in social interaction with others, even strangers (Martens et al., 2008). Simultaneously, they display an undercurrent anxiety to non-social objects, like specific phobias (Meyer-Lindenberg et al., 2006a). Parallel to this, the amygdala exhibited an abnormal reaction pattern to socially relevant and irrelevant stimuli in William syndrome, perhaps due to orbitofrontal cortex deficiency, rendering amygdala-orbitofrontal cortex interactions less useful to guide behavior (Meyer-Lindenberg et al., 2006a). The amygdala has due to its relation to emotion and emotional behavior been linked to a series of neuropsychiatric disorders. Disorders like schizophrenia (Aleman and Kahn, 2005), Alzheimer (Hamann et al., 2002), depression (Phillips et al., 2003b) anxiety (Rauch et al., 2003) and autism (Amaral et al., 2003b) all have functional abnormalities confined to the amygdala.

With the era of functional neuroimaging, a renewed interest in the anxiety disorders has evolved. A general finding among these patients is the subjective experience of excessive fear, and thus the search for the neurological underpinning of these disorders has been closely

intertwined with studies of fear circuits in animal models. Exaggerated responses in the amygdala to fearful stimuli is a general finding (Damsa et al., 2009), and in several of these patients, this is paralleled by functional abnormalities in especially the anterior cingulate cortices and related parts of prefrontal cortex (Rauch et al., 2003). These prefrontal areas are generally considered to regulate the amygdala neural activity (Phillips et al., 2003a), thus a decoupling here may result in the observed amygdala hyperreactivity. Contrary to this, patients suffering from Alzheimer disorder, a neurodegenerative disorder also affecting the amygdala, display amygdala hypoactivity and blunted emotional responses (Hamann et al., 2002). The aforementioned discoveries will hopefully guide new experiments searching for the neural underpinning of these diverse disorders, and in the future possible guide the development of new treatments.

## 1.7 METHODS FOR FUNCTIONAL STUDIES OF THE AMYGDALA IN HUMANS

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Prior to the emergence of functional neuroimaging methods, much of the knowledge regarding human amygdala function was based on observational studies in patients with amygdala impairments as well as the rich animal literature. Notably, as these patients usually had temporal lobe lesions also encompassing other structures like hippocampus and entorhinal cortex, it was difficult to interpretate which of the observed behavioral or cognitive changes that were due to amygdala impairments. However, as functional imaging techniques like fMRI and PET emerged, renewed interest in this brain area evolved. The advances in radiology also inspired clinicians to try selective stimulation of the amygdala under radiological guidance during presurgical evaluation of patients with drug-resistant epilepsy and related conditions. In the present thesis, we used fMRI in all three papers. Therefore, a brief introduction of this method will be given next.



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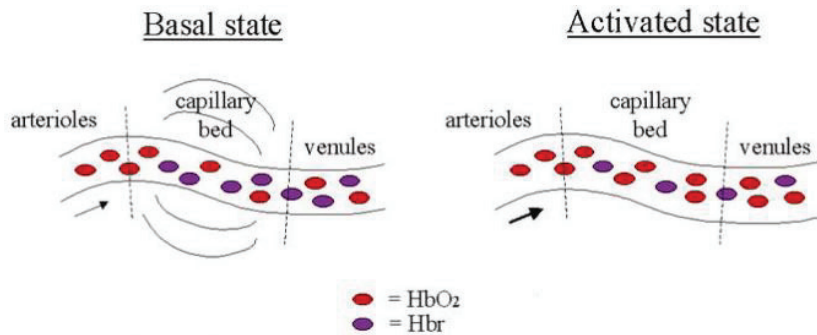
### 1.7.1 BOLD FUNCTIONAL MAGNETIC RESONANCE IMAGING

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Functional MRI is a special type of MRI in which changes in brain hemodynamic responses give a proxy of neuronal activity in the brain. The basis for this method is the Blood-Oxygen-Level-Dependent (BOLD) contrast, which has shown to correspond to neural activity in the brain of humans and animals. Works from the groups of Ogawa, Bandettini and Belliveau in the beginning of the 1990s demonstrated how changes in the magnetic properties of blood could be detected by MRI, which formed the principle of BOLD (Ogawa et al., 1990a; Kwong et al., 1992).

The BOLD contrast depends on the magnetic properties of hemoglobin (Hb), and the relative amount of oxygenated and deoxygenated Hb in an area of the brain. There are two main principles essential for the BOLD effect. First, Hb, the main oxygen transporter in the blood, alters magnetic properties according to its oxygen saturation level. As the Hb loses one of its four oxygen molecules, the resulting deoxygenated Hb gets a significant magnetic moment, with a special influence on the  $T2^*$  magnetic field (Ogawa and Lee, 1990; Ogawa et al., 1990b). When oxygen binds to deoxygenated Hb (resulting in oxyhemoglobin containing four oxygen molecules), the magnetic moment is lost. Secondly, it had previously been demonstrated that neuronal activity is accompanied by changes in local oxygen concentrations and cerebral blood flow (Huettel et al., 2004). In resting state, oxygen dissociates from the Hb within the capillary bed and diffuses into the neural tissue, with the resulting deoxygenated Hb removed by the venous network. However, when activity in neurons raises, their metabolic demand increases, leading to a local hemodynamic response. Thus, local increases in oxygen consumption and cerebral blood flow will occur. Through mechanisms that are not fully known, the local supply of oxygenated blood exceeds the local metabolic consumption, thus resulting in a surplus of oxyhemoglobin in the veins (Buxton

and Frank, 1997). As deoxygenated Hb gives stronger dephasing of protons, a relative decrease in deoxygenated Hb will enhance the T2\* effect, and therefore increase the MRI signal from this brain area. These changes are recorded using a fast recording technique, echo planar imaging (EPI), which is sensitive to changes in T2\* (Huettel et al., 2004).



**Figure 4.** Basal state and activated state of neuronal tissue. In the activated state, the relative amount of oxygenated Hb is increased (fMRI for Newbies, Jody Culham, <http://www.fmri4newbies.com>. Printed with permission). HbO<sub>2</sub>: Oxygenated hemoglobin. Hbr: Deoxygenated hemoglobin.

The data obtained has four dimensions, 1 temporal (time) and 3 spatial dimensions. After collection, the raw data is transformed (Fourier transformation) and amplified to generate the MRI image. The signal difference detected is very small, but usually the stimulus is repeated several times, hence statistical methods can be used to reliably detect areas with significant altered BOLD signal.

The association between the altered neuronal activity and the hemodynamic response is not fully known. One leading proposal is that the energy deficits from active neuronal tissue results in a net increase in blood flow to this area, and a concomitant increased local oxygen consumption of the active neurons (Logothetis and Wandell, 2004). Importantly, the

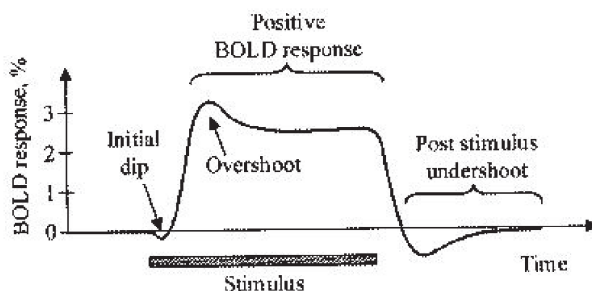
increased local oxygen delivery must exceed local metabolic use to generate the increased oxygenated Hb. However, some researchers have questioned the relations of cerebral blood flow to local metabolic demands, after the discovery that mechanisms not related to lack of energy can control the blood flow to one area. Attwells and colleagues (Attwell and Iadecola, 2002) demonstrated that local blood flow was driven by the presence of fast neurotransmitters like GABA and Glutamate. These neurotransmitters initiate a cascade of secondary transmitter systems when binding to their respective receptors, resulting in vasodilatation and a net increase in local blood flow (Attwell and Iadecola, 2002). Because of the slowness of the hemodynamic response compared to neural activity, the BOLD response comes to represent the local sum of excitatory and inhibitory neuronal activity and not the individual spiking action potentials. Also, as indicated by findings when combining fMRI with electrodes embedded in the neuronal tissue, the BOLD tends to be more associated with presynaptic activity and internal neuronal processes than the output firing of the neurons (Logothetis 2001). Nevertheless, the take home message is that fMRI is an indirect measure of neuronal activity, as it does not directly measure spiking action potentials, thus limiting the conclusions that can be drawn from fMRI data.

#### *1.7.1.1 SPATIOTEMPORAL RESOLUTION*

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The temporal resolution of BOLD fMRI is determined by the temporal characteristic of the BOLD response and by the repetition time (TR), i.e. the time between the beginning of one volume recording and the subsequent one in fMRI. Usually, the TR is between 2-4 seconds. Briefly, the BOLD response starts rising 2-3 seconds after the neuronal activation and progress until it peaks after approximately 4-6 seconds. Then a gradual fall follows terminating in a post-stimulus undershoot before it reaches its baseline. This post stimulus undershoot probably represents a more rapid decrease in blood flow than blood volume

following cessation of the neuronal activity, thus leading to a surplus of deoxygenated Hb and a decrease in  $T_2^*$  (Huettel et al., 2004). Importantly, the amplitude and duration of the BOLD varies according to the brain area involved and also between subjects.



**Figure 5.** The BOLD response (fMRI for Newbies, Jody Culham; <http://www.fmri4newbies.com>. Printed with permission).

The spatial resolution is determined by voxel size, voxels being the three-dimensional small cubes one parts the brain into in neuroimaging. Usually, the voxels are quadratic spanning 2-5 mm in each direction. The total amount of voxels in the brain thus are quite large, often in the range of 40 000-300 000. Recent technical advancements have increased the spatial resolution due to the use of higher magnetic fields and multichannel radio frequency coils. This has been used to study finer scaled networks in the visual cortex.

#### 1.7.1.2 EXPERIMENTAL DESIGN

There are principally two different designs used in fMRI, the block design and the event-related design. In block design, multiple similar trials are grouped together in blocks lasting from ~15 seconds to several minutes. The basic idea is that within one block, trials are similar according to type of stimuli presented or specific cognitive process evoked, hence BOLD signal for each trial will add up linearly. In order to make statistical inferences, similar blocks are grouped together and signal averaged across the blocks (i.e. experimental blocks), and

these are further compared to another set of averaged blocks which differ only in the effect of interest (i.e. control blocks) (Huettel et al., 2004).

In the last years, researchers have increasingly used the event-related designs. Basically, one tries to model the BOLD signal changes associated with each trial, as opposed to the block designs where trials are grouped. As the hemodynamic response is slow, the BOLD response associated with each trial will overlap, but this can be explicitly modeled if the underlying design is good. The event-related designs have several advantages, and for some experimental questions this is the only design available, like when the goal is to analyze responses to infrequent presented targets in a series of stimuli. This design allows one to examine the BOLD response to individual trials and if trial presentations are mixed, prevent anticipation and habituation effects (Huettel et al., 2004).

## 1.8 THE NEUROBIOLOGY OF INDIVIDUAL VARIATION IN AMYGDALA RESPONSE

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There is a growing interest in elucidating biological and environmental factors which cause individual differences in brain functions, and specifically amygdala functioning. Importantly, such individual differences in amygdala function have been linked to both individual variation in behavioral traits (Hariri, 2009) and even psychopathology (Rauch et al., 2003). So far, the focus on genes altering monoaminergic neurotransmission in amygdala have received much attention, as these transmitters are thought to have a regular function on amygdala neuronal excitement (LeDoux, 2007). A number of candidate genes regulating central dopaminergic, serotonergic and noradrenergic signaling (i.e. the monoamines) have been related to individual differences in amygdala activity and morphology. For instance, individuals carrying low expression alleles of the monoaminergic Catechol-*O*-

methyltransferase (COMT) enzyme (Smolka et al., 2005) and the Monoamine oxidase A (MAOA) enzyme (Meyer-Lindenberg et al., 2006b), implicated in the catabolism of monoamines, exhibit increased central levels of monoamines and hyperreactive amygdala to emotional stimuli. Further, individuals carrying a low expression variant located in the promoter region of the serotonin transporter gene (the 5-HTTLPR S allele), analogous to the low expression carriers of MAOA and COMT, demonstrate the same pattern (Hariri et al., 2002). These findings illustrate how functional genetic variants in monoaminergic signaling pathways affect amygdala reactivity to relevant stimuli, contributing to the observed interindividual variations in amygdala activity and perhaps vulnerability to neuropsychiatric disorders.

The effect of each gene variant is probably modest, and thus there is an ongoing search for new genetic mechanisms affecting amygdala reactivity. The recent advantages in genetics, with the emergence of whole-genome data, allows for a new hypothesis-free investigations regarding molecular pathways affecting amygdala activity. Thus, in paper 3 we used this new and exciting approach to search for genes causing individual variations in amygdala neural activity.

## AIMS

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The main aim of the thesis was to increase the knowledge regarding the functional specialization of the human amygdala and to discover new gene variants contributing to the individual variation in amygdala activity, by performing functional MRI studies and combining these with genome-wide association (GWA) data. Based on accumulating data demonstrating amygdala responsivity to a large scale of motivating stimuli and events, a more general functional specialization for the amygdala was proposed.

Specific aims:

*1: determine if the human amygdala BOLD response reflects the instructed relevance of a series of emotional neutral visual stimuli*

*2: determine if the human amygdala BOLD response varies according to the goal relevancy of a sensorimotor task*

*3: explore if the use of GWA data combined with functional imaging phenotypes of the amygdala will lead to the discovery of novel gene variants associated with individual variation in amygdala hemodynamic activity*

## MATERIAL AND METHODS

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### 2.1 FACILITIES

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The papers of this thesis are based on functional Magnetic Resonance Imaging (fMRI) examinations performed at the Section of Radiology and Nuclear Medicine, Oslo University Hospital. For paper 1 and 3 a 1.5 T Siemens Magnetom Sonata scanner supplied with a standard head coil was used (Siemens Medical Solutions, Erlangen, Germany), while in paper 2, images were acquired on a 3 T GE Sigma HDx scanner (General Electric Company; Milwaukee, WI, USA). E-prime software controlled stimuli presentation, and the stimuli were presented using Visual System (Nordic NeuroLab, Bergen, Norway). Finger responses were collected using Response Grips (Nordic NeuroLab, Bergen, Norway).

### 2.2 PARTICIPANTS

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The subjects took part in the Thematically Organized Psychosis (TOP) study, a collaborative study involving the University of Oslo and Oslo University Hospital, funded by the University, Regional Health Authorities and the Research Council of Norway. The subjects in paper 1,2 and the healthy control subjects in paper 3 were randomly selected from the Norwegian citizen registration of people living in Oslo and around the Oslo area, and invited by letter. Before participating, subjects were screened to exclude somatic and psychiatric illness, substance abuse, MRI-incompatibility or serious head trauma. All subjects gave written informed consent before participation. They received an honorarium.

In paper 3, the Norwegian sample consisted of 127 patients with a psychiatric diagnosis in addition to 94 healthy control subjects. The patients were recruited from psychiatric units at Oslo University Hospital. Clinical assessment was carried out by trained psychiatrists and



clinical psychologists. Diagnoses were based on the Structural Clinical Interview for DSM-IV Axis I disorders (SCID-I) administered by a MD or a clinically trained psychologist. In the North American replication sample, 14 subjects had a psychiatric diagnosis. In the Norwegian sample, all subjects were Caucasians (subject ethnicity determined during the clinical interviews). However, for the North American sample, a greater diversity was present.

Exclusion criteria for all groups in the Norwegian samples were: hospitalized head injury, neurological disorder, IQ below 70 and age outside the range of 18-60 years. The Norwegian healthy control sample was screened with the Primary Care Evaluation of Mental Disorders (PRIME-MD) or the MINI, and subjects were excluded if they or any of their close relatives had a life time history of a severe psychiatric disorder (schizophrenia, bipolar disorder and major depression), if they had a medical condition known to interfere with brain function (including hypothyroidism, uncontrolled hypertension and diabetes), or substance abuse in the last three months.

### 2.3 IMAGING PROTOCOLS

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In paper 1, full-brain coverage fMRI data were acquired in the axial plane (30 contiguous axial slices, each slice spanning 4 mm aligned with the anterior commissure-posterior commissure (AC-PC) plane) using a 1.5 T scanner. An echo planar imaging (EPI) BOLD sequence (repetition time (TR) = 2400 ms, echo time (TE) = 40 ms, field of view (FOV) = 200 x 200 mm, flip angle = 90°, matrix size 64 x 64) was used for generation of the volumes. Equivalently, a series of 24 interleaved axial slices (4 mm thick with 1 mm gap) aligned with AC-PC plane were acquired in paper 3 using the same, but slightly adjusted, BOLD EPI sequence (TR = 2040 ms, TE = 50ms, flip angle = 90°, matrix 64 x 64, FOV 192 x 192 mm).

The first seven volumes in each study were discarded as dummies to ensure homologous tissue magnetization. Prior to the BOLD fMRI scanning in paper 1 and 3, sagittal T1-weighted 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) images were collected (TR= 2000 ms, TE=3.9 ms, flip angle =7°, matrix 128 x 128, FOV 256 x 256 mm) for better localization of functional data. In paper 2, the functional MRI scans were acquired by a 3 T scanner (General Electric Company; Milwaukee, WI, USA) supplied with a standard eight-channel head coil. Using a T2\*-weighted EPI sequence sensitive to the BOLD contrast (TR= 2000 ms, TE= 25 ms, Flip angle 90°, FOV 260 x 260 mm , 64 x 64 matrix), a total of 192 volumes were collected for each session. The first 3 volumes were discarded. Each volume consisted of 36 slices covering the whole brain parallel to the AC—PC plane (sequential acquisition; 3.5 mm thick with a 0.5 mm gap). For localization purposes of the functional data, FSPGR T1-weighted anatomical images (TR=7.7 ms, TE=3.0 ms, Flip angle 12°) were acquired.

## 2.4 FMRI TASKS

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### GO-NOGO TASK

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In paper 1, the experimental task was a modification of the classical Go-NoGo task. To ensure no emotional interference, only emotionally neutral stimuli were used. Purple letters or numbers were presented serially against a black background for 1 s, separated by a jittered inter-trial interval (ITI) lasting  $3.5 \pm 1$  s. During the ITI a fixation cross appeared on the screen. The subject was instructed to give a specific index finger response for all colored letter stimuli that appeared on a screen (50 % of all the trials, including random purple letters and a green “r”, each presented in 25 % of the trials), except for the letter “t”, for which an index finger response with the other hand was requested. Letter “t” (25 % of trials) was

instructed as particularly important compared to the other stimuli, due to its change of response. The use of hands was counterbalanced. Equivalently, numbers (25 % of trials) interspersed in the sequence requiring a response stop also represented a response shift from the main response. Thus, by instructions one response was highlighted as main response occurring in 50 % of the trials, while the behavioral changes of letter “t” and numbers were instructed as particularly relevant for overall performance.

A fourth condition was added to the paradigm, to test the effect of salience per se. This salience condition was a randomly occurring green “r”, with the accompanying instruction to respond similar as for the other letters. We chose to add this condition for two reasons. First, letter “r” was perceptually salient compared to the other stimuli, and by using this in the contrast, we aimed at eliminating the salience factor that by instructions were given to letter “t” and numbers. Secondly, the change in color for letter “r” was behaviorally irrelevant (same response as for other letters); hence we could compare behaviorally relevant information (response shifts for letter “t” and numbers) with behaviorally irrelevant information (the color change of “r”). We conducted a total of 80 trials (20 letter “t”, 20 other letters, 20 letter “r” and 20 numbers) in a randomized event-related design. The total duration of the experiment was 5 min and 48 s.

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#### 4-CHOICE STIMULUS-RESPONSE TASK

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To replicate and extend the findings from paper 1, a new experiment was designed for paper 2. We investigated human amygdala responses to emotionally neutral stimuli in a 4-choice stimulus-response task, while manipulating relevance. The experiment consisted of two 6 min and 42 s runs. Each run consisted of 28 trials, half of which belonged to a Low relevance condition and the other half to a High relevance condition. The conditions were randomly

presented. Each trial consisted of two tasks: a relevance task followed by a reward receipt task. Trials were separated by a jittered ITI lasting  $5 \pm 2$  s. The relevance task consisted of four white boxes presented against a black background. Four sequentially presented colored circles, each lasting 800 ms, appeared in the boxes, in a randomized order. Only one circle appeared at a time. The color of the circles varied according to the two conditions, so that the circle appeared in black in Low relevance trials and in purple in High relevance trials, respectively. The task was to press the key corresponding to the box in which the circle appeared. The relevance task was followed by reward receipt task, where a number, corresponding to the amount the subject could win in NOK (i.e. 0 NOK or 5 NOK), appeared in one of the four boxes for 2.0 s. A response terminated the task. Reward was received when the participant correctly indicated in which box the number appeared. A jittered inter-stimulus interval lasting  $3.5 + 1.5$  s separated the relevance task and the reward receipt task.

The subjects were given verbal instructions prior to the scanning. They were told about the two possible colors of the circles, and the consequences of a wrong response for the black and purple circles, respectively. In the High relevance condition, successful indication of all four stimuli was necessary for a reward opportunity (i.e. 5 NOK) in the reward receipt task. If not, 0 NOK appeared in one of the boxes. Contrary, in the Low relevance condition, the opportunity to respond for reward was independent of performance in the relevance task and was instead presented in 80 % of the trials. This 80% distribution was chosen based on accuracy in each condition in a pilot version of the task. Consequently, the only difference between the High and Low relevance condition was whether a certain response accuracy was necessary for a subsequent chance to respond for a reward.

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## FACES MATCHING TASK

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In paper 3, a widely used and validated paradigm was employed to elicit amygdala reactivity (Hariri et al., 2002; Carre et al., 2010). Emotional faces from the NimStim series appeared on the screen against a black background. The task was to decide which of two images at the bottom of the screen that matched the target on the top of the screen. In the experimental task these pictures were faces expressing either fear or anger (face matching task), whereas in the sensorimotor control task, geometrical figures were displayed. We used a block design with four blocks of the faces matching task, where each block consisted of 6 emotion-specific face trios. Interleaved between these blocks, participants completed 5 blocks of the sensorimotor control task. Each trial (faces or figures) was presented for 5.4 s with no inter-stimulus interval, for a total block length of 32.6 s. The total paradigm lasted 5 min and 12 s.

## 2.5 HANDLING OF DATA

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All of the DICOM images were converted to analyze (paper 1 and 3) or nifti (paper 2) format using the nICE software. Subsequent preprocessing and statistical analysis were then performed in SPM2 (paper 1 and 3) or SPM8 (paper 2) (<http://www.fil.ion.ucl.ac.uk/spm>). Before any analysis, the images were visually inspected for signal dropout in the amygdala, as this region is prone to magnetic susceptibility. In paper 1, one of the subjects had to be excluded due to signal dropout. All behavioral data were analyzed in SPSS (Statistical Package for Social Sciences 16.0. SPSS Inc., Chicago, USA).

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### 2.5.1 PREPROCESSING AND QUALITY CONTROL

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Individual subject data were realigned to the first volume of the time series in order to correct for head motion, and then the mean functional image and the anatomical image was coregistered to ensure that they were aligned. Subjects that moved more than 3 mm during

the scan were excluded. None of the subjects had to be excluded due to excessive movement. For the individual subject data acquired on the 3T scanner, additional warping was applied due to the greater signal distortion in data acquired at such high field strengths. Next, the images were spatially normalized into the standard stereotactic space of the Montreal Neurological Institute template using the 12-parameter affine model offered by SPM, and then resampled at 2 x 2 x 2 (paper 1 and 3) or 3 x 3 x 3 (paper 2) mm voxels. Subsequently, to reduce additional noise and residual differences in individual anatomy, data were smoothed using a 6 (paper 1 and 3) or 8 (paper 2) mm full width half maximum (FWHM) isotropic kernel. Subsequently, data were high pass filtered using a cut-off value of 128 s and then an AR1 function was applied.

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### 2.5.2 STATISTICAL ANALYSIS

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The general linear model (GLM) of SPM2 (paper 1 and 3) or SPM8 (paper 2) was used for analysis in all papers. Further details will be given for each paper separately. As we had apriori hypotheses regarding the amygdala, small volume correction based on anatomically defined bilateral amygdala using the SPM Anatomy toolbox (paper 1) or SPM WFU Pickatlas toolbox (version 2.3, <http://fmri.wfubmc.edu/cms/software#PickAtlas>; Wake Forest University School of Medicine) (paper 2 and 3) and false discovery rate- (FDR) (paper 1 and 3) or family-wise error- (FWE) (paper 2) corrected p-values were applied. In addition, exploratory whole-brain analyses were performed in paper 1 and 2 using the same methods to correct for multiple comparisons as for the concomitant small volume analysis.

### *PAPER 1*

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The model was built by convolving stick functions for the onsets of four different event-types with the canonical hemodynamic response function (HRF) in SPM2. The four event types

were letter “t”, “r”, other letters and numbers. Parameter estimates of the HRF for each of the four event types were then calculated by SPM using least square fit of the model to the time series of the data. The individual contrast images of these parameter estimates were then entered into a second-level random-effects model for group analysis. Separate one sample t-tests were used to test the effect of relevant vs. less relevant stimuli; letter “t” > letter “r”, numbers > letter “r” and letter “t” > other letters. Also, the effects of perceptual salience was tested with the contrast letter “r” > other letters.

## *PAPER 2*

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In this paper, the model consisted of short boxcar functions representing the onsets of each 4-choice stimulus-response task, i.e. purple (i.e. High relevance condition) and black (i.e. Low relevance condition) circle stimuli, convolved with the canonical HRF in SPM8. The duration of each boxcar was 3.2 s. Additional events were modeled as regressors of no interest. This included the reward receipt task in the High and Low relevance condition and wrong response trials of the relevance task (i.e. trials where one or more wrong responses were given in the 4-choice stimulus-response task). Individual contrast images were entered into a second-level random-effects model for group analysis. The contrast of interest was the High relevance condition > Low relevance condition, which was tested in a one sample t-test as implemented in SPM8.

Based on the results from the whole-brain GLM analysis, a supplementary psychophysiological interaction (PPI) was performed to investigate differences in functional connectivity between amygdala and the rest of the brain for the two levels of relevance. The goal of a PPI analysis is to test if correlation in neural activity between two brain areas varies according to variation in a psychological variable (i.e. High or Low relevance). In this paper,

anatomically defined (WFU pickatlas) right amygdala was defined as seed region, and High vs. Low relevance as the psychological variable. Right amygdala was chosen based on the proposed predominance of the right hemisphere in detection of behavioral relevant stimuli (Mormann et al., 2011). In summary, we generated a GLM in which the explanatory variable was the interaction term, and the main effects of time-course, the task regressors and the motion regressors were included as covariates of no interest. The individual t-contrast images of the interaction gained from the PPI were then entered into a random effects one-sample t-test. As we had an apriori hypothesis regarding ventral striatum, we applied small volume corrections using anatomically defined ventral striatum (Fox and Lancaster, 1994; Nielsen et al., 2004).

### *PAPER 3*

Data for all subjects were first analyzed using a single-subject fixed-effect model built by convolving boxcar functions for the onsets of the two different conditions (faces and figures) with a canonical HRF. Individual contrast images were created by subtracting “figures” from “faces”. To delimit activated voxels within the anatomically defined bilateral amygdala for each individual, the automatic anatomical labels (aal) amygdala mask in the WFU PickAtlas toolbox provided in the SPM2 was used. Each subject’s contrast values for everyone of these voxels were then exported from SPM2. Next, a t-test was applied to every voxel in the software package “R”, and the voxel with most evidence of differential activation across the individuals were chosen as the peak voxel for that hemisphere. The activation of these two voxels was carried forward as the phenotypes for the genetic association analysis. A two-sample t-test within SPM2 was performed to study differences in amygdala BOLD-activation for the allelic variants of the significant gene variant (i.e. CT/TT > CC).



# RESULTS

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## PAPER 1

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Classically, amygdala has been regarded as a critical component of the neural circuitry mediating conditioned fear responses, and hence important for fear learning and expression. This is based on electrophysiological and lesion studies in rodents and cats (Ursin, 1960; LeDoux, 2000) and later on imaging studies in humans (LaBar et al., 1998). During the last years, this hypothesis has been challenged by findings relating amygdala to other emotions (Zald, 2003) in addition to nonemotional social relevant events (Schwartz et al., 2003; Hsu et al., 2005; Herry et al., 2007). The aim of the first paper was to investigate if the human amygdala activity reflected the behavioral relevance of a set of neutral visual stimuli as hypothesized by Sander and colleagues (Sander et al., 2003), using fMRI. In summary, we modified a Go-NoGo task so that it comprised of behaviorally relevant and irrelevant letter and number stimuli. The subject was instructed to give a specific index finger response for all colored letter stimuli that appeared on a screen (25 % consisted of random purple letters and 25 % of a green “r”), except for the purple letter “t” (25 % of trials), for which an index finger response with the other hand was requested. In addition, for 25 % of the trials, a purple random number appeared on the screen, which required a behavioral stop.

We hypothesized that letter “t” which was instructed as particularly behaviorally relevant due to its change of response hand, would cause significantly larger amygdala responses than letter “r”, for which the color change was of no behavioral importance and thus this letter requested no change from the main response. To replicate that behaviorally relevant stimuli yielded significantly greater amygdala responses than less relevant stimuli, we contrasted

numbers > letter “r”. A region of interest analysis yielded significant bilateral amygdala activations for the contrast letter “t” > letter “r”, and a trend in left amygdala for the second contrast (numbers > letter “r”). To test if amygdala responded to salient stimuli per se, we contrasted letter “r” vs. other letters. The results revealed no significant amygdala responses. To fully explore the data, we also contrasted letter “t” > other letters. We found a trend in right amygdala, though this fell short of significance. The accompanying behavioral data indicated that the subjects responded fastest to letter “r” slowest for other letters with a response time for letter “t” in between. The results support a more general role for the human amygdala in detection of behaviorally relevant stimuli.

## PAPER 2

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While relevance was manipulated based on instructions in paper 1, a more sophisticated design was used in paper 2, linking the event’s relevancy to the reception of a monetary reward. Using fMRI, we investigated human amygdala responses to emotionally neutral stimuli in a 4-choice stimulus-response task. Four white boxes were presented on a black screen. Within these boxes, four sequentially presented colored circles appeared, in a randomized order. The participant had to press the key corresponding to the box in which the circle appeared. The task was operationalized as highly relevant if a subsequent opportunity to respond for a reward depended on response accuracy of the task, and less relevant if the reward opportunity was independent of task performance. The color of the circles indicated the High and Low relevance condition. A region of interest analysis revealed bilateral significant amygdala activation in response to the High relevance condition > Low relevance condition of the task. An exploratory whole-brain analysis yielded robust activations in the bilateral ventral striatum for the same contrast. A subsequent functional connectivity analysis

demonstrated increased connectivity between right amygdala and right ventral striatum for the highly relevant events compared to the less relevant events.

In order to explore how the “coupling” differed between amygdala and ventral striatum for the two conditions, we extracted the individual beta values using the group level peak voxel for right ventral striatum and the individual peak voxel within the anatomically defined right amygdala region of interest. We found a trend positive correlation between right amygdala and right ventral striatum activity in the High relevance condition, while in the Low relevance condition no correlation appeared. As to the behavioral data, no significant differences in response time or accuracy between the two conditions were obtained.

In summary, the data replicated and extended the findings from paper 1, supporting that amygdala encodes or calculates relevance. By using reward to manipulate relevance and not only instructions, the central importance of the highly relevant events’ became more explicit in the second experiment. Secondly, based on the previously demonstrated unidirectional connections from amygdala to the ventral striatum in primates (Fudge et al., 2002) in addition to temporal recordings indicating that amygdala activation precedes that of ventral striatum (Ambroggi et al., 2008), the present results may indicate that amygdala transfer information regarding relevance to the ventral striatum. Thus, these structures interaction probably goes beyond what has been observed for stimuli-reward associations to encompass a broader range of relevant information.

Based on neuroimaging studies illustrating the predictive links between amygdala activation, personality traits and behaviors, an important next step has been to identify biological and environmental factors driving variability in amygdala function (Hariri, 2009). A large corpus of candidate gene studies indicate that individual differences in amygdala activity may be caused by genetic variants within monoaminergic signaling pathways, such as dopamine, serotonin and noradrenalin (Hariri et al., 2002; Smolka et al., 2005; Meyer-Lindenberg et al., 2006b). However, to our knowledge, the use of genome-wide data to discover genetic variants underlying variation in adult amygdala activity is novel. We combined genome-wide data with functional imaging phenotypes to discover genetic variants which affect amygdala activation to emotional stimuli. The functional imaging phenotypes were created by extracting the amygdala fMRI activation (using an anatomical region of interest approach) from each individual while the subjects underwent an emotional faces matching task. In this task, subjects had to select which of two emotional faces that matched a third target face. The faces depicted either fear or anger. The most significant signal was associated with rs10014254; this had a p value of  $4.16 \times 10^{-8}$ . Adjusted for multiple testing across both phenotypes (peak voxel in left and right hemispheres) and all SNPs using the Bonferroni correction gave a  $p=0.045$ . Supplementary SPM analysis revealed significantly enhanced bilateral amygdala responsivity for the heterozygote and the homozygote T-allele carriers of rs10014254. This SNP lies in a regulatory region upstream of the Paired-like homeobox 2b (PHOX2B) gene, and it is thus possible that variants in this region could affect this gene's expression. An attempt to replicate the findings in a new sample was unfortunately unsuccessful. However, the analysis was conducted in a hypothesis free framework; no a priori assumptions were made as to likely candidate gene regions. Therefore, we believe an

undirected finding within such a plausible genetic region, adding further support to the importance of monoaminergic signaling pathways in regulating amygdala activity, is of importance.

## DISCUSSION

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### 4.1 SUMMARY OF RESULTS

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In paper 1 and 2 we provide data supporting that encoding or computation of relevance is an important amygdala function. In paper 1, amygdala responses reflected instructed behavioral relevance of a set of neutral letter and number stimuli. We found increased amygdala responses to behavioral relevant letter and number stimuli as compared to less relevant letter stimuli. Equivalently, in paper 2, amygdala responses to a neutral stimulus – response task varied according to the goal relevancy of the task. Thus, significant greater amygdala responses was observed in high relevance conditions where reception of a monetary reward was contingent upon subject’s task performance compared to low relevance conditions where the reward was received unrelated to this task. The data support that amygdala is engaged by a broad range of motivating stimuli, including both emotional and nonemotional ones, perhaps computing the stimulus’ relevance (Adolphs, 2010; Cunningham and Brosch, 2012) or importance (Weierich et al., 2010). Further, the functional connectivity between amygdala and one of its targets, i.e. the ventral striatum, varies according to the event’s relevancy. This may indicate that amygdala transfer information regarding relevance to some of its target areas, subsequently modulating cognition (Phelps and LeDoux, 2005; Schaefer et al., 2006) and motor action (Sagaspé et al., 2011). In addition, the findings of paper 3 contribute to our current understanding of biological mechanisms causing individual differences in amygdala reactivity. Our data indicate that individual variance in a gene variant possibly influencing monoaminergic signaling is associated with individual differences in amygdala activity to emotional stimuli.

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#### 4.1.1 AMYGDALA FUNCTION

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Previous findings linking amygdala to reward-related learning (Baxter and Murray, 2002; Murray, 2007), decision making (Bechara et al., 2003) and establishment of stimuli's current value (Paton et al., 2006; Salzman et al., 2007) were not easily reconciled with the theory of a functional specialization solely for fear and fear-related learning. Consequently, alternative theories on amygdala's functional specialization evolved, one of them relating amygdala to encoding of stimulus' relevance. The "relevance detector theory" proposes that through evolution, the amygdala has become less functionally specialized to currently reflect the event's importance for the organism and its well-being (Sander et al., 2003). Notably, the relevance hypothesis entitle the amygdala with a more general evaluation function than several of the previous theories, and thus integrates many of the diverse findings from animal and human studies which has been difficult to reconcile with existing hypothesis.

Importantly, relevant events include both emotional and non-emotional ones, with the amygdala activity reflecting their relevancy, regardless of their emotional valence (Santos et al., 2011). The work of this theses demonstrates significant amygdala BOLD responses in relation to both emotional (i.e. paper 3) and neutral (paper 1 and 2) relevant stimuli, supporting this putative function of the amygdala.

The emotional faces used in paper 3 are naturally more relevant than geometrical shapes, as they may signal important environmental information with the potential to influence one's well-being or goal achievement. In paper 1, we manipulated the behavioral relevance of stimuli to investigate if this was reflected in amygdala hemodynamic responses. Half of the trials requested a consistent response (for instance right index finger), and this response was considered as the main response during instructions. However, for letter "t" and numbers, each stimulus occurring in 25 % of the trials, a change from the main response pattern was

required. Responding correctly to the two latter stimuli was highlighted as especially important in order to perform optimal. Thus, by instructions, some stimuli were highlighted as more relevant than others. However, though the response to “t” and numbers were different than the main response, and occurred less often, there were no consequences related to either a correct or a wrong response. Therefore, one could argue that the results of paper 1 did not reflect manipulation of relevance, as we suggested in the paper. Still, in the main contrast of interest (i.e. “t” > “r”), except for the change of hands, the task was otherwise similar for the two conditions. Therefore, it is less likely that variations in motor demands did cause the observed effect. In addition, supported by the trend for “t” > other letters, there were no differences in visual features that could have caused the effect. Thus, we would argue that the effects are due to a difference in relevance or motivational significance.

Further, to replicate and extend the findings in paper 1, a new experiment was designed and tested in paper 2. By using reward to manipulate relevance and not only instructions, the central importance of the highly relevant events’ became more explicit in the second experiment. In this experiment, we compared two types of trials that were equivalent in reward value, but differed in the relationship between accuracy in the task and the obtainment of the reward. The subjects performed a simple stimulus-response task where the color of the stimuli (circles) indicated the accuracy necessary for a subsequent reward opportunity. Thus, in purple circle trials, all stimuli had to be correctly indicated in order to get a reward opportunity, while in black circle trials; performance did not influence later chances of receiving a reward. Notably, the reception of reward was separated from the task of interest and the task conditions were carefully matched according to reward value, reward occurrence rate, visual appearance and motor responses. By such, we aimed at minimizing the influence of reward and related emotions on amygdala responses. We believe the findings of paper 2



reflect the greater relevance of the purple circle condition compared to the black circle condition. According to one of the definitions of relevance, relevant events have the potential to “significantly influence the attainment of his or her goals” (Sander et al., 2003). In the present experiment, the goal was represented by the reward, and the purple circle condition was more relevant than the black circle condition as performance significantly influenced the later obtainment of the goal in the first condition.

That amygdala parcel out the relevance of stimuli and events is supported by studies demonstrating how the amygdala response is significantly enhanced to targets with a specific behavioral request embedded in a stream of baseline stimuli with no related responses. For instance, data from the widely used and validated auditory oddball paradigm where an infrequent target tone (Kiehl et al., 2001b; Kiehl et al., 2001a) or visual stimulus (Kiehl et al., 2001a) is interspersed in a series of frequently presented baseline tones or visual stimuli, support this notion. Similar results were obtained to visual and auditory Go-stimuli requiring a motor response compared to baseline NoGo stimuli in a Go/NoGo task (Laurens et al., 2005), and to a target face among non-target faces in a visual search task (Santos et al., 2011). Supposing the subjects’ aim of optimal performance of the task, the targets represent events that can significantly influence overall performance and thus are more relevant during the experiment than the baseline. Parallel to this, two recent studies explicitly manipulating task relevancy (or salience) in addition to emotional valence, demonstrating that task relevancy activated the amygdala independently (Santos et al., 2011) or additively (Hindi Attar et al., 2010) to the emotional content. The last two studies support that relevance more than identifying emotional significance is what engages the amygdala, in line with other neuroimaging studies (Fitzgerald et al., 2006; Wright and Liu, 2006). A limitation of several of these studies was that stimuli requiring a motor response were compared to stimuli with no

behavioral demands. Thus the effect of a motor response per se could not be ruled out from the results. Hence, in paper 1 and 2, contrasting stimuli with the same motor demands sought to overcome this limitation.

The results of paper 1 and 2 are also consistent with an emerging literature demonstrating how the amygdala response may be context dependent and sensitive to current goals. This is in line with the concept of relevance, which stresses how the context and current goals may shape the relevance of a stimulus (Sander et al., 2003). For example, making judgments about faces can strongly modulate the amygdala response to those faces (Hariri et al., 2000; Cunningham et al., 2008). Further, personal traits like anxiety (Etkin et al., 2004) or extraversion (Canli et al., 2002) modulate the amygdala response to fearful and happy faces, respectively. The role of expectations in modulating amygdala response has also been documented in experiments involving electrophysiological recordings in monkeys (Belova et al., 2007). These findings are in line with the findings of paper 1 and 2. Though the task was (almost) similar for the two conditions, the letter “t” or purple colored circles signaled a context in which performance determined goal achievement (successful performance in paper 1 or a monetary reward in paper 2), and this change of context by itself elicited a boost in amygdala neural responding.

It is essential to distinguish between stimuli and actions which under normal conditions are supported by the amygdala on one hand, and stimuli and actions that require amygdala involvement on the other. It is possible that encoding of stimulus relevance requires the cooperation of a network of brain areas, in which amygdala is one of the candidates. Also, the encoding of relevance could rely on upstream brain areas and then subsequently passed on to the amygdala. If amygdala is not required for relevance encoding, than subjects with lesions

to the amygdala should have no difficulties in judging relevance. Indeed, this is supported by some recent studies (Tsuchiya et al., 2009; Bach et al., 2011). However, it is possible that the patients of these studies sorted stimuli based on other categories than relevance, i.e. they had developed cognitive compensational mechanisms. Indeed, the lesions in several of these patients occurred at a relatively young age, supporting that development of alternative neurocircuits had occurred. Contrary, the amygdala may be essential to relevance encoding, with a resultant impairment in judging or comparing the significance of events secondary to depleted amygdala function.

An alternative possibility would be that the results obtained in study 1 and 2 in fact are due to emotional components added to the stimuli and not relevance. Classically, emotional experiences are divided according to valence and arousal in which valence span from positive to negative and arousal from calm to excited (Lang and Davis, 2006). Previous reports relate the amygdala to both valence (Anders et al., 2004; Anders et al., 2008) and arousal (Anderson et al., 2003; Small et al., 2003). The subjects' may have found the letter "t" or the purple circle more exciting in this experimental setting, as they aimed at perfect responding. This state of increased arousal or "hypervigilance" potentially mediated by the amygdala (Whalen, 1998) gives rise to numerous central and peripheral responses that may improve attention (Vuilleumier, 2005) and motor performance (Schaefer et al., 2006). However, the subjects were not aware of the paradigm rationale, and did not report that the most relevant stimuli caused any change in arousal. Also, amygdala responses induced by emotional stimuli often habituates over time. This was explicitly tested in paper 1 in which we did a parametric modulation with time as a parameter to test for linear habituation during the "t" or "r" condition. The analysis revealed no linear habituation in the amygdala for either of the two conditions; however this does not exclude other habituation patterns. We argue that the

significant responses obtained in paper 2 are not attributable to differences in reward or predictive value. The design of the task aimed at separating brain responses to reception of reward from the relevance task. In addition, the reward value after correct responses to the targets in both conditions was identical, and the conditions were matched according to reward occurrence rate. We have no reason to believe that the change of color between the High and Low relevance condition in paper 2 triggered elevated attention and amygdala activity, based on the findings of paper 1 (Ousdal et al., 2008). In addition, there were no significant behavioral differences between the two conditions in paper 2, which would have been indicative of attentional differences (Lim et al., 2009).

Still there are scientists who argue that amygdala may be better viewed as tied to fear-related functions (Ohman and Mineka, 2001). In favor of such a theory is the notion that amygdala is part of the phylogenetical old and “primitive” brain, and thus would be an effective fear detector conserved across evolution (Amaral, 2003). In primates, the basolateral amygdala occupies more of the amygdala complex than in rodents, parallel to the evolution of neocortex (Stephan et al., 1987). Also, their amygdala-cortex connectivity is greatly enhanced (Pessoa, 2010). Thus, across evolution, the functional profile of the amygdala may have become less specialized, in order to cope with new environmental challenges (Sander et al., 2003). Indeed, new direct connections between amygdala and lateral PFC have been demonstrated in primates (Ghashghaei et al., 2007), and indirectly, the primate amygdala is able to reach most of the prefrontal cortex after just one single connection within prefrontal areas (Averbeck and Seo, 2008). Thus, new and altered connections may be ways amygdala has expanded its repertoire of functions across evolution.

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#### 4.1.2 INDIVIDUAL DIFFERENCES

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Though recent studies have established that BOLD fMRI measures represent reliable intrasubject measures of brain function (Manuck et al., 2007), there is great variability between subjects in observed BOLD responses. To investigate such differences, neuroscientists have turned to the field of genetics to find individual variations in biological pathways subsequently shaping differences in brain function. Hence, imaging genetics seeks to establish connections between common genetic polymorphisms, variation in neurotransmitter function and individual differences in brain activation patterns (Hariri, 2009). Obviously, the BOLD function is just an indirect measure of the underlying neuronal activity, and does not reflect a specific transmitter system. Still, its tendency to correlate better with presynaptic than postsynaptic activities (Logothetis and Wandell, 2004), may favor a relation between BOLD responses and neurotransmission. In paper 3, the combination of genome-wide data and functional imaging phenotypes yielded a significant association between a genetic variant possibly affecting monoaminergic signaling and the amygdala.

In the amygdala, monoamines like norepinephrine and dopamine influence how the different inhibitory and excitatory neurons interact (LeDoux, 2007). The monoamines are released widely from their nerve terminals, and thus have more diffuse effects than for instance GABA and Glutamate in the amygdala. Their specificity comes from the distribution of the receptors for the various amines within each amygdala nuclei (LeDoux, 2007). Animal studies using pharmacological inactivation (Chung et al., 1999) and analogous studies in humans (Takahashi et al., 2005) support that manipulating amygdala concentrations of monoamines results in altered amygdala neuronal activity. Interestingly, manipulation of these transmitters does not only affect the responses in amygdala neural circuits, but also peripheral stress responses and subjective emotional experiences (Burghardt et al., 2004). The

Catechol O-methyltransferase (COMT) gene and the X-linked monoamine oxidase (MAOA) gene both code for enzymes necessary for the degradation of monoamines, and thereby effect the concentration of dopamine and noradrenalin within amygdala (Smolka et al., 2005; Meyer-Lindenberg et al., 2006b). Low expression alleles of these enzymes are associated with less catabolization of monoamines with resultant increased cerebral concentrations and a heightened amygdala BOLD- responsiveness to emotional stimuli (Smolka et al., 2005; Meyer-Lindenberg et al., 2006b). The current results are in line with these earlier candidate gene studies, as they demonstrate how genetic variation in a regulatory step of monoaminergic signaling possibly affecting central monoaminergic tone subsequently relates to amygdala neuronal signaling. As GWA data has no biases regarding which pathways to be linked to amygdala neural activity, the resultant findings of a gene variant related to monoaminergic signaling, offer unique support for these transmitters effect. To the best of our knowledge, this is the first study combining GWA data and functional neuroimaging phenotypes of the adult human amygdala, though a similar approach has been used in one study in adolescents (Liu et al., 2010).

We attempted to replicate the findings in paper 3 in an independent sample from North America, but did not succeed. This brings up a general issue in neuroimaging genetic studies. A number of reported findings so far have been difficult to replicate, thus questioning their validity. One obvious possible explanation for this is the small sample sizes in the early neuroimaging genetic studies (de Zubicaray et al., 2008). Looking at the classical genetic studies, with thousands of subjects, neuroimaging studies have often made inferences based on less than hundred individuals. Thus, while the original neuroimaging study may have “winner’s curse”, replication establish the true effect size (Zollner and Pritchard, 2007) which may be more modest and hence difficult to replicate in small replication samples. Also, the

issue of population stratification, i.e. that the sample has subpopulations with a systematic difference in allele frequency (for instance secondary to different ancestry) may influence the observed associations (Hao et al., 2010). In the present study, this was less likely to occur, due to a homogenous Norwegian sample. Further, the reliability of some of the methods used to define amygdala in neuroimaging studies may be questioned, as an anatomical region of interest approach combined with normalization, may still not be sufficient to account for individual differences in anatomical organizations and hence it results in variation across studies (de Zubicaray et al., 2008). To overcome this, amygdala may have to be defined individually using manual methods and anatomical landmarks.

The problem of multiple comparisons, which occurs in imaging genetic studies as thousands of gene variants are tested against thousands of voxels in the brain, may cause false positive associations. In the present study, this was accounted for by using Bonferroni correction, which is the gold standard to counteract the problem of multiple testing. However, if less stringent methods are used, false positive associations may be reported. At last, other factors influencing the populations studied may affect the ability to replicate. As highlighted by Chanock and colleagues, the samples under study should match as much as possible to maximize chances of replication (Chanock et al., 2007). Both subtle differences in study design (i.e. different stimulus sets) and age range (all students in the North American sample and a greater diversity in the Norwegian sample) between the Norwegian and North American samples, may have caused our failure to replicate.

#### 4.2 IMPLICATION OF THE FINDINGS

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The focus of this thesis has been twofold. In the first two papers, we provide data supporting a broader functional specialization of the amygdala beyond its emotional function (Sander et

al., 2003; Adolphs, 2010; Cunningham and Brosch, 2012). Secondly, we searched for biological mechanisms contributing to variations in amygdala function, by combined neuroimaging phenotypes with individual GWA data. This led to the discovery of a new gene variant possibly affecting individual amygdala responsivity to emotional stimuli.

The last decades have brought new insight into types of stimuli that engage the amygdala and the consequences of its engagement. In common for several of these stimuli are their motivating natures, either based on their emotional properties (Zald, 2003) or self-relevance (Adolphs, 2008). That manipulation of stimulus' relevance is reflected in amygdala activity was supported by paper 1 and 2 of this thesis. In addition, we also found a close relation between amygdala and ventral striatum responses, and have suggested that amygdala may gate ventral striatum responses to motivational relevant stimuli. Previously, the well-known projections from amygdala to ventral striatum have been implicated in modulating reward-related responses in ventral striatum (Ambroggi et al., 2008), but the current work supports interactions beyond reward-related processes to encompass a broader range of relevant stimuli. Alternatively, their activities are both gated by common dopaminergic inputs, which is an important monoaminergic transmitter, released from the ventral tegmental area and substantia nigra in the brainstem (Bjorklund and Dunnett, 2007). The notion that monoamines regulate amygdala neural activity was supported by our third paper. Thus amygdala firing is not all dependent on external and internal stimuli, but also the available monoamines in amygdala perhaps regulating its responsivity. At the time of stimulation, the current level of monoamines within amygdala may decide how it reacts to the stimulus, with its downstream effect on other neurocircuits and ultimately behavior. It is possible to speculate that such individual variations in amygdala responsivity (perhaps to the stimulus' relevance) contribute



to differences in complex behavioral traits and at a later stage vulnerability for several neuropsychiatric disorders (Hariri, 2009).

#### 4.3 LIMITATIONS OF THE METHODS

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Although fMRI currently is viewed as one of our most powerful methods to study the human brain (Logothetis, 2008), there are some limitations that need consideration. Perhaps the most important hereof, is that the BOLD signal is an indirect measure of neuronal activity. The measured hemodynamic response ultimately yielding the BOLD effect is related to neuronal activity, but despite numerous of investigations, the exact neuronal events measured by the BOLD and the neurovascular coupling is still debated. As demonstrated by Logothetis and colleagues, the BOLD tends to be more associated with presynaptic activity and internal neuronal processes than the output firing of the neurons (Logothetis et al., 2001). Thus BOLD was suggested to reflect the release of both fast and modulatory neurotransmitters into one region (Attwell and Iadecola, 2002). The binding of fast neurotransmitters to their receptors, initiate a cascade of events ultimately yielding vasodilatation and subsequently increased local blood flow (Rossi, 2006). Another theory relates BOLD to the energy consumptions of active neurons (Logothetis and Wandell, 2004). As the metabolic activity increases, the local blood flow parallels, to compensate the local decrease in oxygen and glucose.

The resolution of fMRI merits consideration. The spatial resolution in fMRI is determined by the voxel size, and it is usually about 2-3 mm (but can be even 1 mm). However, these voxels contain approximately 5.5 million neurons, billions of synapses and a fine-grained vascular network (Logothetis and Wandell, 2004). Consequently, each voxel contains both excitatory and inhibitory neurons, and thus the vascular changes observed are the net sum of excitatory

and inhibitory activity within a voxel. Further, as the BOLD signal results from the effects of deoxygenated hemoglobin, which is mainly located in capillaries and veins, it is possible that the observed effects come to represent draining veins (Keilholz et al., 2006). This was especially a problem for low field magnets (i.e. 1.5 T), but seem to be less a problem with the advance of field strength (i.e. 3 T). The temporal resolution of BOLD also needs to be considered. The BOLD response peaks after 4-6 seconds, and last approximately 16 seconds. Therefore, it is difficult to distinguish the BOLD response to different events which occurs in close temporal proximity. To overcome some of these limitations, careful considerations of timing in the experimental design has to be done, or fMRI could be combined with other techniques which have a greater temporal resolution (i.e. electroencephalography (EEG) or magnetoencephalography (MEG)).

Functional imaging of the amygdala faces some difficulties. First, the amygdala is a rather small brain structure, with an altogether diameter of around 15 mm (Zald, 2003). Secondly, the animal literature indicates that the different nuclei of the amygdala complex possess distinct functions and connections (Balleine and Killcross, 2006). However, the spatial resolution of fMRI does not permit examination of these different nuclei in humans. Third, the amygdala is located in close proximity to the sphenoid sinus. Consequently, inhomogeneity in magnetic susceptibility with distortions of the field occurs, which in worst case may cause signal loss (Zald, 2003). The emergence of more focused high-resolution fMRI may help overcome some of these shortcomings.

The present experimental designs may have some important limitations. The difficulties associated with GWA studies have already been discussed. There is a possibility that the design of paper 1 did not test relevance as proposed. The relevance manipulation was based

on instructions, and there is a risk that the subjects did not find the high relevance stimuli more relevant than the low relevance stimuli. However, the replication in paper 2 using a more sophisticated design supports the findings of paper 1. Also, there is an ongoing discussion regarding proper sample sizes in fMRI. The present sample sizes fulfilled the recommendation of Desmond and Glover (Desmond and Glover, 2002), however, other sample sizes may be more ideal to get a well-powered study.

#### 4.4 CONCLUSIONS

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- The present data supports a more general role for the human amygdala in parceling out the relevance of stimuli and events.
- Amygdala may supply relevance information to ventral striatum and other target areas, as supported by greater functional connectivity between amygdala-ventral striatum for highly relevant than less relevant events.
- Individual variations in amygdala functional activity may rely on genetic variations within monoaminergic signaling pathways.

#### 4.5 FUTURE RESEARCH

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In the extension of this thesis, the relevance - theory should be tested using animal models and pharmacological manipulation in humans. Further, it would be interesting to compare amygdala responses in healthy controls with patients suffering from for example Alzheimer disease (Hamann et al., 2002), schizophrenia (Aleman and Kahn, 2005) and autism disorder (Amaral et al., 2003b) to manipulation of stimulus' relevance, as all of these disorders have structural and functional abnormalities within the amygdala. Our speculation regarding amygdala – ventral striatum functional interaction in response to relevant stimuli merits

further investigation. Both of these brain areas are central components of the neural workspace implicated in emotion, reward and motivation (Haber and Knutson, 2010) thus their interaction should be further investigated. If amygdala is involved in relevance detection, this might have great implications in the clinic. Several of the patients with Alzheimer, schizophrenia, anxiety and autism have social impairments and it is possible to relate these to subtle impairments in relevance detection. Thus, the unproportionally increased relevance given to phobic objects in phobic anxiety disorder or decreased ability to correctly label the relevance of environmental stimuli in schizophrenia may come from abnormal amygdala responsiveness. Hence, one could speculate that pharmacological agents, which normalize amygdala function, may also help treating these symptoms.

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**Associations between variants near a monoaminergic pathways gene (PHOX2B) and amygdala reactivity: A genome-wide functional imaging study.**

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**Running title: Amygdala genome-wide functional imaging study**

## **Abstract**

As the amygdala is part of the phylogenetic old brain and its anatomical and functional properties are conserved across species, it is reasonable to assume genetic influence on its activity. A large corpus of candidate gene studies indicate that individual differences in amygdala activity may be caused by genetic variants within monoaminergic signaling pathways, such as dopamine, serotonin and norepinephrine. However, to our knowledge, the use of genome-wide data to discover genetic variants underlying individual differences in adult amygdala activity is novel. In the present study, the combination of genome-wide data and functional imaging phenotypes from an emotional faces task yielded a significant association between rs10014254 and the amygdala using a region of interest approach. This SNP is located in a regulatory region upstream of the Paired-like homeobox 2b (PHOX2B) gene; therefore it could affect the expression of this gene. PHOX2B regulates the expression of enzymes necessary for the synthesis of several monoamines and is essential for the development of the autonomic nervous system. However, an attempt to replicate the finding in an independent sample from North America did not succeed. The synthesis of functional magnetic resonance imaging (fMRI) and genome-wide data takes a hypothesis-free approach as to which genetic variants are of interest. Therefore we believe that an undirected finding within such a plausible region is of interest, and that our results add further support to the hypothesis that monoaminergic signaling pathways play a central role in regulating amygdala activity.

## **Introduction**

The amygdala is a complex brain structure, central to a wide range of mental processes and behavioral functions. While the amygdala historically has been implicated in fear and fear-related learning (Amaral, 2003; LeDoux, 2003), more recent work suggests that it support more subtle functions such as distributing the stimulus emotional significance, current value or relevance (Morrison & Salzman, 2010; Ousdal et al., 2008; Sander et al., 2003; Zald, 2003). Upon activation, the amygdala gives rise to a number of central and peripheral responses to facilitate information processing and appropriate behavioral responses (Davis & Whalen, 2001). Emotional stimuli are particularly strong instigators of amygdala activity (Sergeier et al., 2008), and individual variation in amygdala reactivity to negative emotional stimuli is associated with both complex behavioral traits (Etkin et al., 2004; Hariri, 2009) and anxiety-related disorders (Shin et al., 2004; Stein et al., 2002). As such, sources of individual variability in amygdala reactivity may point to mechanisms involved in the pathophysiology of these disorders.

The notion that variation in functional and structural indices of the amygdala has a genetic basis is supported from a number of studies. Twin studies of humans revealed that structural measurements of the amygdala have high heritability; in particular focal grey matter density has a heritability estimated at over 80% (Hulshoff Pol et al., 2006; Peper et al., 2009). Other work has implicated common functional genetic variants in monoaminergic pathways genes as affecting amygdala reactivity to emotional stimuli and behavioral traits in humans (Hariri, 2009). The monoamines, such as dopamine, serotonin and norepinephrine, are important modulators of amygdala activity (LeDoux, 2007). Findings from both animal and human pharmacological neuroimaging studies indicate that increasing levels of dopamine, serotonin

and norepinephrine within amygdala potentiates its functioning, subsequently affecting downstream neurocircuits and related behaviors (Buffalari & Grace, 2007; Burghardt et al., 2007; Takahashi et al., 2005; van Stegeren et al., 2005).

Blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI) has proven an effective and reliable tool for investigating individual differences in amygdala neural functioning (Manuck et al., 2007). The combination of fMRI and single gene data has yielded associations between amygdala reactivity and genetic variants affecting the expression of the serotonin transporter (SLC6A4 gene; the serotonin transporter gene) (Hariri et al., 2002), the Catechol-*O*-methyltransferase enzyme (COMT gene) (Smolka et al., 2005) and the Monoamine oxidase A enzyme (MAOA gene) (Meyer-Lindenberg et al., 2006). All three gene variants affect synaptic clearance of monoamines, thus regulating monoaminergic neurotransmission. The low-expression variant of the MAOA gene results in increased levels of monoamines within the amygdala, and has been associated with diminished amygdala volume and hyperresponsive amygdala to emotional stimuli (Meyer-Lindenberg, et al., 2006). Similarly, a low expression variant located in the promoter region of the serotonin transporter gene (the 5-HTTLPR S allele) has been consistently associated with heightened amygdala reactivity to emotional stimuli (Hariri et al., 2005), increased serotonergic signaling and reduced grey matter volume in the amygdala (Pezawas et al., 2005). Together, these findings support an at least partially genetic explanation for variation in information processing within the amygdala. However, it is likely that the effect of single variants on amygdala reactivity is small (Hariri, 2009) and few variants have been discovered so far.

As mentioned, individual differences in amygdala neural activity have been linked to behavioral traits and even psychopathology. Therefore, the discovery of genetic variants

contributing to individual variation in amygdala neural activity may subsequently point to molecular pathways involved in the pathophysiology of these disorders, and is thus important. Genome-wide association studies offer the possibility of interrogating the whole genome for variants affecting a given phenotype, uncovering novel candidate loci that affect amygdala function through pathways and molecular mechanisms currently unknown. To the best of our knowledge, this is the first genome-wide association study of amygdala activation in adults, however a similar approach has been used in one study in adolescents (Liu et al., 2010). We combined genome-wide microarray genotype data with data from an emotional fMRI task and discovered a genomic region significantly associated with amygdala activation.

## **Materials and Methods**

### **The TOP study**

#### ***Participants***

Participants were recruited from the Thematically Organized Psychosis (TOP) study, an ongoing collaborative study involving the University of Oslo and Oslo University Hospital in Norway. There were 224 individuals (109 women) for whom both fMRI and genotype data were successfully collected (Table I). Participants were healthy individuals or patients with diagnoses of schizophrenia spectrum disorder, bipolar disorder or psychosis not otherwise specified. Patients were recruited from the psychiatric unit of Oslo University Hospital and underwent the Structural Clinical Interview for DSM-IV Axis I disorders (SCID-I) administered by a MD or a clinically trained psychologist, to assess the presence of AXIS I disorders. Diagnostic reliability was satisfactory ( $\kappa = 0.77$ , 95% CI: 0.60-0.94) (Ringen et al., 2008). Healthy control subjects were randomly selected from the Norwegian citizen registration of people living in the same catchment area and invited to participate by letter.

Before participation, control subjects were screened to exclude serious somatic and psychiatric illness, substance abuse or MRI-incompatibility. All subjects gave written informed consent before participation. The study was conducted at Oslo University Hospital, Norway, and approved by the Norwegian Data inspectorate and the Regional Committee for Medical Research Ethics.

### ***fMRI Amygdala Reactivity Task in the TOP study***

A widely used and validated paradigm was employed to elicit amygdala reactivity (Carre et al., 2010; Hariri, et al., 2002). In this task participants select which of two stimuli (displayed at the bottom of the screen) matches a target stimulus (displayed at the top). The images displayed were either human faces expressing anger or fear (face matching task) or geometrical shapes (the sensorimotor control task). Participants completed 4 blocks of the faces matching task, where each block consisted of 6 emotion-specific face trios derived from a standard set of facial affect pictures (Tottenham et al., 2009). Interleaved between these blocks, participants completed 5 blocks of the sensorimotor control task. Each trial (faces or shapes) was presented for 5.4 seconds with no inter-stimulus interval, for a total block length of 32.6 seconds. The total paradigm lasted 310 seconds. E-prime software (version 1.0 Psychology Software Tools, Inc, Pittsburgh, PA, USA) controlled the presentations of the stimuli using VisualSystem (NordicNeuroLab, Bergen, Norway). Responses were recorded through MR-compatible ResponseGrips (NordicNeuroLab, Bergen, Norway).

### ***BOLD fMRI data acquisition in the TOP study***

MRI scans were acquired on a 1.5 T Siemens Magnetom Sonata scanner (Siemens Medical Solutions, Erlangen, Germany) supplied with a standard head coil. Volumes (n = 152, 24 axial slices, 4 mm thick with 1 mm gap) covering the whole brain were acquired in the axial



plane, using a BOLD EPI sequence (TR=2040 ms, TE=50ms, flip angle=90°, matrix 64 x 64, FOV 192 x 192 mm). The first seven volumes were discarded. Prior to BOLD fMRI scanning, a sagittal T1-weighted 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) scan (TR= 2000 ms, TE=3.9 ms, flip angle =7°, matrix 128 x 128, FOV 256 x 256 mm) was collected for better localization of functional data.

### *fMRI Data Analysis in the TOP study*

SPM2 (<http://www.fil.ion.ucl.ac.uk/spm>) was used for preprocessing of data and subsequent single-subject fixed-effect analysis. Before analysis, images were visually inspected for signal dropout in the amygdala, as this region is prone to magnetic susceptibility. None of the subjects had to be excluded due to signal dropout. All of the functional images were realigned to the first image in the time series to correct for head motion (Friston et al., 1995). None of the subjects moved more than 3 mm in any direction during the scan. Subsequently, the mean functional image and the anatomical image were coregistered to ensure that they were aligned. The images were spatially normalized to the stereotactical Montreal Neurological Institute (MNI) template (Friston, et al., 1995), and resampled at 2x2x2 mm voxels. Thereafter, all images were smoothed using a 6 mm full width-half maximum (FWHM) isotropic kernel. Subsequently, data were high pass filtered using a cut-off value of 128 s and then an AR1 function was applied. Data for all subjects were first analyzed using a single-subject fixed-effect model built by convolving boxcar functions for the onsets of the two different conditions (faces and figures) with a canonical hemodynamic response function (HRF). Individual contrast images were created by subtracting “figures” from “faces”. To delimit activated voxels within the anatomically defined bilateral amygdala for each individual, the automatic anatomical labels (aal) amygdala mask in the WFU PickAtlas toolbox provided in the SPM was used (version 2.3,

<http://fmri.wfubmc.edu/cms/software#PickAtlas>; Wake Forest University School of Medicine) (Maldjian et al., 2004; Maldjian et al., 2003). This gave us a total of 319 voxels, 161 voxels in the left and 158 voxels in the right hemisphere. Each subject's contrast values for everyone of these voxels were then exported from SPM2. Next, a t-test was applied to every voxel in R software (Ihaka & Gentleman, 1996), and the voxel with most evidence of differential activation across the individuals was chosen as the peak voxel for that hemisphere (left amygdala peak voxel;  $t=20.54$ ,  $p = 4.43 \times 10^{-60}$ , right amygdala peak voxel;  $t=20.12$ ,  $p = 1.84 \times 10^{-58}$ ). The activations of these two voxels were carried forward as the phenotypes for the genetic association analysis.

#### ***Genotyping and quality control in the TOP study***

The TOP sample was genotyped at Expression Analysis Inc (Durham, NC, USA) using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix Inc, Santa Clara, CA, USA). Individuals with discrepancies between reported and genotyped sex were removed; to control for population stratification individuals with a calculated ancestry different from the majority of the TOP sample were removed. These were identified by inspecting plots of the first two multi-dimensional scaling components of the Identity by State score, as calculated by PLINK (Purcell et al., 2007), of the individuals in the TOP study and in the HapMap study ("The International HapMap Project," 2003). Individuals who clustered towards different ethnic groups in HapMap were excluded. All SNPs located in mitochondrial DNA, on the sex chromosomes, or in unknown locations were removed. After this, information on 244 individuals and 872,242 SNPs was available. Quality control was implemented by removing individuals or SNPs that had call rates below the following percentile cut-offs: first individuals < 90 % (leaving 242 individuals); second, SNPs < 95% (750,574 SNPs remaining); third, remaining individuals < 97 % (226 individuals); fourth, remaining SNPs <

97% (708,351 SNPs). Next, SNPs with a minor allele frequency less than 5% were removed (546,381 SNPs). Finally, individuals with outlying (greater than three standard deviations from the mean) levels of heterozygosity were removed (n=2). After QC, information on genotype was available for 546,381 SNPs and 224 individuals.

### ***Statistical Analysis of TOP data***

The individual contrast values for the right and left amygdala peak voxels were tested for association with each SNP separately. An additive model of genetic effect was used, controlling for diagnosis using three indicator variables that coded for schizophrenia, bipolar disorder and other psychosis. Gender and age variables were not included as previous analyses had suggested no significant effect for these variables. Multiple testing over SNPs and phenotypes was controlled for using the Bonferroni correction. Subsequently, a random-effects two sample t-test (CC vs. CT/TT) SPM analysis was performed with the top candidate SNP to explore the difference in amygdala BOLD response as a function of genotype profile. As the amygdala was the region of interest, small volume correction based on anatomically defined bilateral amygdala and false discovery rate (FDR) corrected p-values were used to correct for multiple comparisons. For the SPM analysis, there was no correction across SNPs, only across voxels. The anatomically defined regions of interest (ROIs) were created using the aal mask in the SPM WFU Pickatlas toolbox (Maldjian, et al., 2004; Maldjian, et al., 2003).

### ***Pathway analysis of TOP data***

Each of SNPs entered into the GWA analysis was annotated to the closest gene using Affymetrix annotations. A list of these annotated genes was produced, ranked by p value of the most significant SNP associated with that particular gene. This ranked list was submitted

to the Gene Set Enrichment Algorithm (GSEA) (Subramanian et al., 2005), with weights corresponding to the  $-\log_{10} p$  values of the corresponding SNPs, to look for overrepresentation of Gene Ontology (GO) categories.

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Insert Table I about here

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## **The DNS study**

### ***Participants***

A total of 100 participants for whom both fMRI and genetic data were available were included from the ongoing Duke Neurogenetics Study (DNS). The Duke Neurogenetics Study recruits participants from surrounding colleges. All participants provided written informed consent in accordance with Duke University guidelines and received \$100 for participating. One participant was excluded from analyses due to poor BOLD fMRI signal in amygdala regions of interest (see below) leaving a final sample of 99 individuals (Table II).

All participants were free of the following DNS exclusion criteria: 1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms, 2) use of psychotropic, glucocorticoid, or hypolipidemic medication, and 3) conditions affecting cerebral blood flow and metabolism (e.g., hypertension). Diagnosis of any current DSM-IV Axis I disorder or select Axis II disorders (i.e., Antisocial Personality Disorder, Borderline Personality Disorder), was assessed with the electronic Mini International Interview (Sheehan et al., 1998) and Structured Clinical Interview for the DSM-IV Axis II Personality Disorders (SCID) (First, 1997). The presence of an Axis I or Axis II disorder is not an exclusion criterion for DNS

participation because the DNS seeks to establish broad variability in multiple behavioral phenotypes related to psychopathology (Table II).

### ***fMRI Amygdala Reactivity Task in the DNS study***

The DNS task was similar to the task used in the TOP study with 4 face matching and 5 interleaved sensorimotor control blocks, but there were some minor differences. This version consisted of one block each of fearful, angry, surprised and neutral facial expressions presented in a pseudorandom order across participants, and used a different set of standard facial affect pictures (Ekman P., 1976). To be consistent with the TOP study only blocks containing angry and fearful expressions were included in analyses reported here. Within faces matching blocks, 6 face trios were presented for 4 seconds with a variable inter-stimulus interval of 2-6 seconds, for a total block length of 48 seconds. Each sensorimotor control block consisted of 6 different shape trios each presented for 4 seconds with a fixed inter-stimulus interval of 2 seconds, for a total block length of 36 seconds. The total paradigm length was 390 seconds. Reaction times and accuracy were recorded through an MR-compatible button-box.

### ***fMRI data acquisition in the DNS study***

DNS participants were scanned using a research-dedicated GE MR750 3T scanner equipped with high-power high-duty-cycle 50-mT/m gradients at 200 T/m/s slew rate, and an eight-channel head coil for parallel imaging at high bandwidth up to 1MHz at the Duke-UNC Brain Imaging and Analysis Center. A semi-automated high-order shimming program was used to ensure global field homogeneity. A series of 34 interleaved axial functional slices aligned with the anterior commissure-posterior commissure (AC-PC) plane were acquired for full-brain coverage using an inverse-spiral pulse sequence to reduce susceptibility artifact

(TR/TE/flip angle=2000 ms/30 ms/60; FOV=240 mm; 3.75×3.75×4 mm voxels; interslice skip=0). Four initial RF excitations were performed (and discarded) to achieve steady-state equilibrium. To allow for spatial registration of each participant's data to a standard coordinate system, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR/TE/flip angle=7.7 s/3.0 ms/12; voxel size=0.9×0.9×4 mm; FOV=240 mm, interslice skip=0).

### ***BOLD fMRI Data Analysis in the DNS study***

The general linear model of SPM8 (<http://www.fil.ion.ucl.ac.uk/spm>) was used for whole-brain image analysis. Individual subject data were realigned to the first volume in the time series to correct for head motion before being spatially normalized into the standard stereotactic space of the MNI template using a 12-parameter affine model. Next, data were smoothed to minimize noise and residual differences in individual anatomy with a 6 mm FWHM Gaussian filter. Subsequently, data were high pass (cut-off 128 s) filtered. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean. Next, the ARTifact detection Tool (ART) (Whitfield-Gabrieli, 2009) was used to account for additional noise in the images. Specifically, individual whole-brain BOLD fMRI volumes meeting at least one of two criteria were assigned a lower weight in determination of task-specific effects: 1) significant mean-volume signal intensity variation (i.e., within volume mean signal greater or less than 4 standard deviations of mean signal of all volumes in time series), and 2) individual volumes where scan-to-scan movement exceeded 2 mm translation or 2° rotation in any direction. To ensure that an adequate signal from the amygdala was obtained, an amygdala ROI mask (aal from the WFU Pickatlas) (Maldjian, et al., 2004; Maldjian, et al., 2003) was used to exclude all participants with less than 90% coverage of the amygdala ROI (n = 1).

After these preprocessing steps, linear contrasts using canonical HRFs estimated an angry and fearful faces > shapes contrast image for each individual. These contrast images were entered into a second-level random effects model (one sample t-test) to determine mean task-related responses within the anatomically defined right and left amygdala (WFU Pickatlas). To correct for multiple comparisons, FWE-correction across the amygdala ROIs with a combined voxel-level threshold of  $p < 0.05$  and cluster threshold of  $\geq 10$  contiguous voxels, was applied. Subsequently, BOLD contrast estimates were extracted for the group peak voxels within right and left amygdala for each participant. Extracted values were then entered into regression models outside of SPM. Importantly, extracting BOLD parameter estimates from peak voxels activated by the paradigm rather than voxels specifically correlated with the independent variables of interest, will preclude the possibility of any regression coefficient inflation that may result from capitalizing on the same data twice (Viviani, 2010).

#### ***Genotyping and quality control in the DNS study***

Genomic DNA was isolated from buccal cells derived from Oragene DNA self-collection kits (DNA Genotek, Inc., Kanata, Ontario, Canada). Samples were genotyped using the Illumina Omni Express chip and a custom array containing an additional 330,000 SNPs by 23andme ([www.23andme.com](http://www.23andme.com); Mountain View, CA, USA). Because rs10014254 was not available in the DNS genotyping array, SNAP (Johnson et al., 2008) was used to identify a SNP that could function as a proxy based on linkage disequilibrium. SNAP showed that rs10014254 was in complete linkage disequilibrium with rs17529323 ( $r^2 = 1.0$ ) within the CEPH population of 1000 genomes. Hence, rs17529323 (A/C) was used for DNS analyses.

#### ***Statistical Analysis of DNS data***

A series of multiple regressions including gender and the number of rs17529323 C alleles were conducted in PASW (v. 18, SPSS Inc., Chicago, IL, USA) to predict amygdala reactivity to emotional stimuli extracted from the peak voxels in the right and the left amygdala. These analyses were conducted in the sample including Caucasians only and in the entire sample with the addition of self-reported ancestry as covariates (i.e., dummy coded African American, Asian, or Other). Analyses were repeated within the entire sample with individuals with a psychiatric diagnosis excluded.

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Insert Table II about here

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## Results

As in prior studies, the task robustly recruited amygdala in both studies. In the Norwegian sample, there were significant associations between activation of the amygdala peak voxel in the left hemisphere and three SNPs in high LD: rs10014254, rs11722038 and rs17529323. The most significant signal was with rs10014254, this had a  $p = 4.16 \times 10^{-8}$ ,  $p = 0.045$  after adjustment for multiple testing across both phenotypes and all SNPs using Bonferroni correction (Figure 1 and Table III). The SNP is located upstream of the Paired-like homeobox 2b (PHOX2B) gene. The effect of this SNP on both left and right hemisphere activations is shown in Figure 2. Inspection of activation against genotype shows one individual to have outlying activation, after exclusion of this individual the p value remains highly significant but not at a genome-wide level ( $p = 5.52 \times 10^{-5}$ ). An estimation of genomic inflation using all SNPs found little evidence of such an effect ( $\lambda = 1.00$ ; Figure 3). Table III shows estimates of effect for the combined sample and for separate analyses of each subsample (these separate analyses are displayed in Figure 4); here we see consistent estimates of effect in all groups



except “other psychosis” where only one individual carried the minor allele. A statistical test for interactions between SNP and diagnosis also found no evidence ( $p = 0.28$ ). The results from the SPM random-effects two sample t-test analysis revealed significantly increased activation in right ( $x = 16, y = -8, z = -16, Z = 2.87, \text{cluster-size} = 34 \text{ voxels}, p_{(\text{SVC})} < 0.05$ ) and left ( $x = -26, y = -4, z = -14, Z = 3.60, \text{cluster-size} = 73 \text{ voxels}, p_{(\text{SVC})} < 0.05$ ) amygdala for the T-allele carriers of the rs10014254 (i.e. combining heterozygote, CT ( $n = 21$ ), and minor allele homozygote, TT ( $n = 1$ )) relative to major allele homozygotes, CC ( $n = 199$ ), though this analysis ignores multiple testing across SNPs. The SPM activations results are displayed in Figure 5.

Analysis on a proxy SNP, rs17529323, in the North American study failed to produce significant effects in the entire sample (right amygdala peak voxel;  $x = 28, y = -4, z = -20, t \text{ statistic} = 0.24, p = 0.81$ , left amygdala peak voxel;  $x = -24, y = -6, z = -18, t \text{ statistic} = 0.05, p = 0.96$ ) or in the Caucasian only sample (right amygdala;  $t \text{ statistic} = 1.04, p = 0.31$ , left amygdala,  $t \text{ statistic} = 0.70, p = 0.49$ ).

Adopting a strict Bonferroni multiple testing correction, ignoring LD within the genome, risks discarding potentially interesting findings. For this reason a list of all regions containing an association signal with p value less than  $10^{-5}$  in the Norwegian GWAS, along with corresponding p values from the North American study, is included in Table IV. We see that for the most significant region after the PHOX2B region, represented by rs5767645 and with  $p = 6 \times 10^{-7}$  for association with right hemisphere activation, there is also a nominally significant association with right hemisphere activation in the North American study ( $p = 0.024$ ) though this would not survive multiple testing correction.

We looked for further evidence of the importance of monoaminergic signaling pathways using the TOP data; both by inspecting well known candidate loci and by using the tools of pathway analysis. For two of the previously linked genes, MAOA and COMT, genotype information was available either at the previously identified locus (rs4680 for MAOA) or a locus in perfect LD (rs3027401 is in perfect LD with rs6323, previously linked with COMT expression). Applying the same model as used in the GWA analysis, there was a nominally significant association between the COMT loci and left hemisphere peak activation ( $p=0.034$ ) but no association with right hemisphere peak activation ( $p=0.33$ ). There was no evidence of association for the MAOA SNPs (left hemisphere,  $p=0.71$ , right hemisphere,  $p=0.081$ ). No SNPs were available which were in LD with rs25532, the variant linked to differential expression of SLC6A4.

A pathway analysis of the GWAS results was performed to look for overrepresentation of SNPs annotated to particular GO categories amongst the more significant GWAS associations. The GO categories of interest were those which contained the monoaminergic signaling genes linked to amygdala activity (i.e. COMT, MAOA, and SLC6A4). The GSEA tool (<http://www.broadinstitute.org/gsea/index.jsp>) was used to search for such overrepresentation. None of these particular GO categories were significant with  $FDR < 0.25$ . There could be a number of explanations for this: pathway analysis suffers from reduced power because of factors such as the incompleteness of GO categories, and random noise in the ranking can swamp genuine signals. In addition, when annotating SNPs to genes many links between SNPs and monoaminergic genes may have been missed; this would further reduce power.

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Insert Figure 1-5 and Table III-IV about here

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## Discussion

In this study, the combination of genome-wide data and fMRI phenotypes in a Norwegian sample suggested an association between a common gene variant in a regulatory region upstream of PHOX2B and neural function of the amygdala. However, analyses in a secondary North American dataset failed to replicate this association.

It is possible that the gene variant rs10014254 regulates the expression of PHOX2B *in cis*. ChIP-Seq data, collected by the ENCODE consortium (Birney et al., 2007) and displayed in the UCSC genome browser (<http://genome.ucsc.edu/>), found three transcription factors binding to this locus. However, confirmation of a regulatory effect of rs10014254 on expression of this gene would require further experiments. PHOX2B codes for a transcription factor exclusively expressed in the nervous system, including the amygdala (Lein et al., 2007). Of its main functions, PHOX2B is necessary for the development of the autonomic nervous system, and has a primary role in the generation and survival of adrenergic neurons (Pattyn et al., 1999). Further, it regulates the expression of enzymes necessary for the biosynthesis of dopamine and norepinephrine (Brunet & Pattyn, 2002). It is also involved in the serotonergic neurogenesis (Jacob et al., 2007). Interestingly, a mutation in PHOX2B causes Congenital Central Hypoventilation Syndrome (CCHS) with autonomic nervous system dysfunction such as reduced drive to breathe, abnormal heart rate variability, exaggerated sweating, poor temperature control and abnormal pupillary-dilatation (Antic et al., 2006; Patwari et al., 2010). Further, some CCHS patients show subtle cognitive and affective impairments such as problems with working memory functions and elevated levels of anxiety (Ruof et al., 2008; Vanderlaan et al., 2004). The CCHS findings are consistent

with animal and human studies which demonstrate that the amygdala is involved in mediating autonomic reactivity and the allocation of attentional resources in response to significant environmental stimuli (LeDoux, 2007). The amygdala receives cardiopulmonary information and projects directly to autonomic control regions such as the hypothalamus. Further, stimulating the central amygdala leads to alterations in blood pressure, heart rate and respiration (Davis, 1992). Because of these findings, the amygdala is considered as part of the neural circuit which mediates descending control over the autonomic nervous system (Kapp et al., 1982), likely integrating autonomic responses with emotional or relevance factors. Thus, changes in PHOX2B expression may alter autonomic functions both directly and indirectly, the second by modulating amygdala neuronal firing, with resultant changes in subjects' physiological and cognitive responses to relevant environmental stimuli.

An association has also been suggested between a PHOX2B polymorphism and schizophrenia, in particular for subgroups with ocular misalignment (Toyota et al., 2004). An emergent feature among schizophrenia patients is impairments in emotion recognition and abnormal amygdala responses to emotional salient information; this implicates the amygdala as part of the neural substrate of this disorder (Morris et al., 2009). However, as no interaction with diagnosis was found in the current study, and the direction of effect was the same for the control group, those with schizophrenia and those with bipolar disorder (little can be said about the other psychosis group, as it contained only one minor allele carrier), the present results suggest this variant acts independently of any neuropsychiatric disorder. Similar findings have been obtained with other genes controlling important brain phenotypes (Rimol et al., 2010).

As argued previously, at least some of the observed variation in amygdala activity appears to rely on differences in monoaminergic signaling pathways. For instance, both impaired amygdala structure and increased amygdala activation have been linked to genetic variants in important monoaminergic pathways, in particular the serotonin transporter gene (Hariri, et al., 2005; Hariri, et al., 2002) and the MAOA enzyme gene (Meyer-Lindenberg, et al., 2006). Interestingly, low-expression variant in MAOA is associated with impulsive violence (Caspi et al., 2002) and the corresponding variant of the serotonin transporter gene (the 5-HTTLPR S allele) with anxiety (Gross & Hen, 2004), linking genetics variants to complex behavioral traits. The current result is in line with these earlier candidate gene studies, as it demonstrates how genetic variation in a regulatory step of monoaminergic signaling which affects cerebral monoaminergic tone subsequently relates to amygdala neuronal signaling. Moreover, volumetric effects of variants linked to the MAOA gene and the serotonin transporter gene on amygdala structures are consistent with emerging evidence which indicates some structural damage in amygdala and interconnected limbic structures in CCHS patients (Kumar et al., 2006; Kumar et al., 2005). These results indicate that genetic variation in monoaminergic signaling pathways affects not only amygdala neural activity, but also structural integrity. It would be interesting to investigate in a future study if variants of the rs10014254 affect amygdala structure. The synthesis of fMRI and genome-wide data is a hypothesis-free approach, with no preconceived notion as to which genetic pathways affect a trait. As the gene variants tested for associations are not limited to a few candidates, but hundreds of thousands of SNPs, it allows the discovery of unexpected genetic variants and novel mechanisms. Hence, this unguided discovery of a genetic variant within a monoaminergic signaling pathway constitutes further evidence for the unique role these transmitters play in regulating amygdala activity.

The effect of the rs10014254 on amygdala reactivity was not replicated in a North American sample. Such a replication in an independent dataset would greatly strengthen the credibility of the variant we propose (Chanock et al., 2007). While all science progresses by the independent validation of experimental results, with GWA studies replication reduces the probability that the result is related to subtle population stratification (though our study is conducted in a homogeneous population, well suited for GWA studies) or the issue of multiple comparisons (which we control for using the gold standard, and many argue overly conservative, Bonferroni correction). Replication could also give truer estimate of effect size, which initially could be inflated by “Winner’s curse” (Zollner & Pritchard, 2007). Winner’s curse has been posited as a reason GWAS results fail to replicate, because of the initial overestimation of effect size, subsequent replication experiments are underpowered to confirm their findings. The reason for the current failure is not clear. It is possible to speculate that both task- and genotype related factors influence these relations. For instance, winner’s curse would be exacerbated by the smaller size of the replication sample. Chanock et al suggest as far as possible study design and phenotype should match to maximize the chance of replication (Chanock, et al., 2007).

Other factors including the samples under study and task-related differences may have contributed to the lack of replication. While the Norwegian task used pictures of faces expressing fear or anger from the NimStim set, the North American study used faces from the Ekman set. As the amygdala is a heterogeneous structure composed of several nuclei, parts of the observed discrepancy may be ascribed to activation of different nuclear groups within the amygdala. These nuclear groups have different connectivity profiles and are even thought to operate independent of each other in some neural processes (Balleine & Killcross, 2006).

Unfortunately, the resolution of whole brain fMRI does not allow one to discriminate subgroups of nuclei within the amygdala, and therefore this remains speculative.

In summary, we report here an association between amygdala reactivity and genetic variants upstream of PHOX2B, which controls pathways related to monoaminergic biosynthesis.

Although we failed to replicate this association within a second sample, we believe that the centrality of such pathways to amygdala neural activity, as well as the links between this gene and phenotypes related to amygdala function, mean that this result merits further investigation.

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**Table I.** Demographic Variables from Norwegian TOP sample by rs10014254 Genotype

Group	CC (n = 199)	CT (n = 21)	TT (n = 1)	Missing genotypes	Total (n = 221)
<b>Gender</b>	101	8	0	2	109
<b>(women)</b>					
<b>Age</b>	32.5±9.1	28.9±8.5	28.0	40.7±4.7	32.1 ±9.1
<b>Controls</b>	85	9	0	3	94
<b>Schizophrenia</b>	47	4	0	0	51
<b>Diagnosis</b>					
<b>Bipolar</b>	56	7	1	0	64
<b>Diagnosis</b>					
<b>Other</b>	11	1	0	0	12
<b>psychosis</b>					

Note: Age is presented as mean ± standard deviation.

**Table II.** Demographic Variables from North American DNS sample by rs17529323

Genotype Group

	All Ethnicities		Caucasians Only		Total
	AA (n = 89)	AC (n = 10)	AA (n = 45)	AC (n = 5)	(n = 99)
<b>Gender</b>	48	5	23	1	53
<b>(women)</b>					
<b>Age</b>	19.7±1.3	19.0±1.2	19.7±1.4	19.4±1.5	19.6±1.3
<b>Psychiatric</b>	11	3	6	2	14
<b>Diagnosis</b>					
<b>Caucasians</b>	45	5	45	5	50
<b>African</b>	10	3	0	0	13
<b>Americans</b>					
<b>Asians</b>	27	2	0	0	29
<b>Other</b>	7	0	0	0	7

Note: Psychiatric disorders were as follows: Generalized Anxiety Disorder = 1; Major Depressive Disorder and Alcohol Abuse = 1; Alcohol Dependence = 4; Alcohol Abuse = 5; Alcohol Abuse and Cannabis Dependence = 1; Alcohol Abuse and Cannabis Abuse = 1; Cannabis Abuse = 1. Age is presented as mean ± standard deviation.

**Table III.** Effect of significantly associated SNPs on activation of the left amygdala in the entire Norwegian sample and in the subgroups.

<b>Population</b>	<b>Variant</b>	<b>Major (Minor) Allele</b>	<b>Beta (SE)</b>	<b>P value</b>
	rs10014254	C(T)	0.387 (0.0681)	$4.16 \times 10^{-8}$
<b>Combined</b>	rs11722038	A(G)	0.387 (0.0681)	$4.20 \times 10^{-8}$
	rs17529323	A(C)	0.386 (0.0682)	$4.66 \times 10^{-8}$
	rs10014254	C(T)	0.476 (0.100)	$7.55 \times 10^{-6}$
<b>Controls</b>	rs11722038	A(G)	0.474 (0.101)	$8.30 \times 10^{-6}$
	rs17529323	A(C)	0.474 (0.101)	$8.30 \times 10^{-6}$
	rs10014254	C(T)	0.339 (0.184)	0.0712
<b>Schizophrenia</b>	rs11722038	A(G)	0.339 (0.184)	0.0712
	rs17529323	A(C)	0.334 (0.185)	0.0774
	rs10014254	C(T)	0.388 (0.109)	$7.41 \times 10^{-4}$
<b>Bipolar</b>	rs11722038	A(G)	0.388 (0.109)	$7.41 \times 10^{-4}$
	rs17529323	A(C)	0.388 (0.109)	$7.41 \times 10^{-4}$
	rs10014254	C(T)	-0.212 (0.375)	0.584
<b>Other psychosis</b>	rs11722038	A(G)	-0.212 (0.375)	0.584
	rs17529323	A(C)	-0.212 (0.375)	0.584

Note: We report effect size, standard error and p value using an additive model and, in the combined analysis, controlling for diagnosis.

**Table IV.** Other associated regions.

Hemisphere	SNP	Nearest Gene	Beta (SE)	P value	DNS p
Left	rs1121554	FUNDC2P2 (upstream)	0.16 (0.035)	$9.1 \times 10^{-6}$	0.087
Left	rs10178425	UNC80 (intron)	0.26 (0.058)	$9.8 \times 10^{-6}$	-
Left	rs10212227	CNTN6 (upstream)	0.35 (0.073)	$2.2 \times 10^{-6}$	-
Left	rs10014254	PHOX2B (downstream)	0.39 (0.068)	$4.2 \times 10^{-8}$	0.96
Left	rs911008	SLC25A21 (intron)	-0.17 (0.036)	$3.0 \times 10^{-6}$	0.57
Right	rs2170561	FAM5C (downstream)	0.25 (0.052)	$3.4 \times 10^{-6}$	0.97
Right	rs4746818	VPS26A (intron)	0.24 (0.052)	$5.3 \times 10^{-6}$	0.064
Right	rs433782	FAM155A (downstream)	0.16 (0.034)	$4.8 \times 10^{-6}$	0.64
Right	rs1035540	BCAR1 (intron)	-0.22 (0.043)	$6.2 \times 10^{-7}$	0.22
Right	rs150757	CYYR1 (downstream)	-0.23 (0.048)	$3.4 \times 10^{-6}$	0.052
Right	rs5767645	TBC1D22A (downstream)	-0.18 (0.035)	$6.0 \times 10^{-7}$	0.024

Note: A list of all regions associated with one of the peak voxel phenotypes at a significance threshold of  $10^{-5}$  in the Norwegian sample. Most regions contain groups of associated SNPs in high LD, we report a single SNP from each region (the SNP with the most significant p value). We report effect size, standard error and p value for the SNP assuming an additive model and correcting for diagnosis. The nearest gene is based on Affymetrix annotations. The final column gives the p value for testing the peak voxel in the same hemisphere in the Duke Neurogenetics Study (DNS) using an additive model, in the sample where people of different ancestry have been removed. The DNS analysis was based on the same SNPs where available, otherwise the following proxies were used (R<sup>2</sup>=1 unless otherwise stated): rs1121554 tagged with rs1429381, rs10014254 with rs17529323, rs2170561 with rs6656158, rs4746818 with rs4746817, rs1035540 with rs2870471 (R<sup>2</sup>=0.83), rs150757 tagged with rs219655 (R<sup>2</sup>=0.925). Rs10178425 and rs10212227 had no proxies in the DNS experiment, and thus were not included.

**Figure 1. Manhattan plot of genome-wide p values from the Norwegian sample**

-Log<sub>10</sub> p values for association between SNP and activation of the peak voxel in the amygdala in the left and right hemispheres, plotted against genomic location. The red line corresponds to genome-wide significance accounting for multiple testing across SNPs and phenotypes.

**Figure 2. Peak voxel activation by genotype in the Norwegian sample.** Activation of the peak voxel in the left and right hemispheres, adjusted for diagnosis, plotted against the genotype of rs10014254.

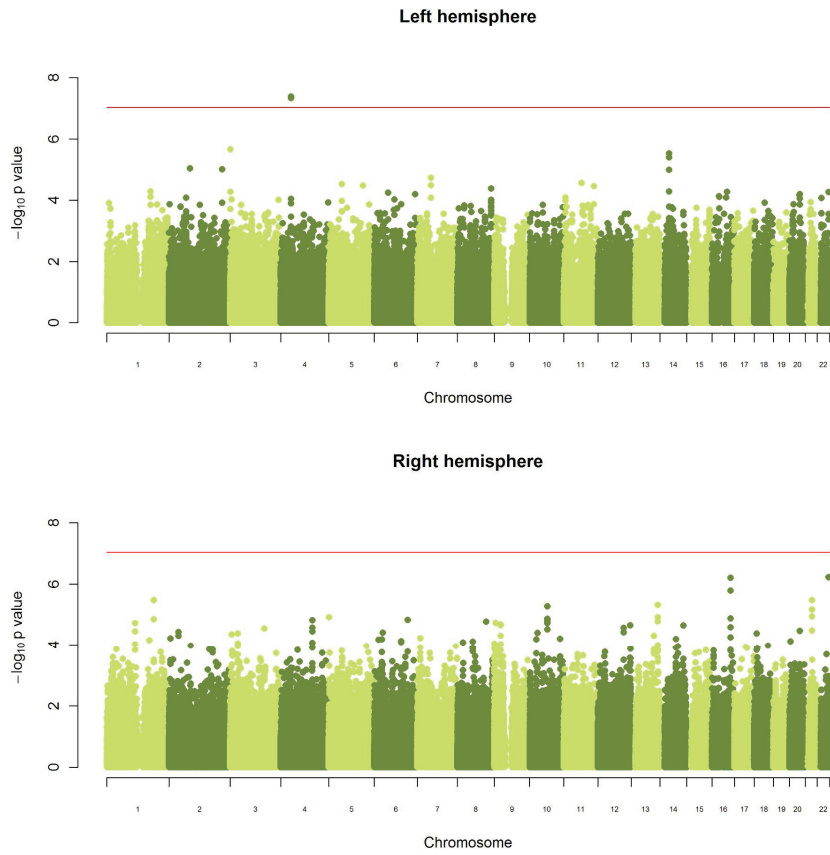
**Figure 3: Q-Q plot of the Norwegian sample**

This Q-Q plot of the expected against observed  $-\log_{10}$  p values for association with the left and right hemisphere phenotypes across all SNPs shows little evidence of genomic inflation.

**Figure 4. Peak voxel activation by genotype and diagnosis.** Activation of the peak voxel in the left and right hemispheres is plotted against the genotype of rs10014254 for each of the diagnostic groups separately. We see consistent effect sizes of genotype on left hemisphere on left hemisphere activations across all groups except other psychosis; this group includes only one minor allele carrier.

**Figure 5. Effect of genotype.** (A) The association between the rs10014254 and amygdala activation in the Norwegian TOP sample. Participants carrying the T-allele (i.e. CT or TT) exhibited significantly heightened activation in bilateral amygdala (right amygdala peak voxel;  $x = 16, y = -8, z = -16, Z = 2.87, p_{(SVC)} < 0.05$ , left amygdala peak voxel;  $x = -26, y = -4, z = -14, Z = 3.60, p_{(SVC)} < 0.05$ ) in comparisons to those homozygous for the C-allele. The

results are corrected for multiple comparisons across voxels using small volume correction, but not for the multiple comparisons across SNPs inherent in the GWAS analysis. As such, this plot is principally included to present the areas of greatest SNP effect. The colors refer to t-values as coded in the bar at the right of the figure. (B) and (C) Contrast estimates for the peak voxel in left and right amygdala, respectively, for the same contrast.



**Figure 1**



Figure 2

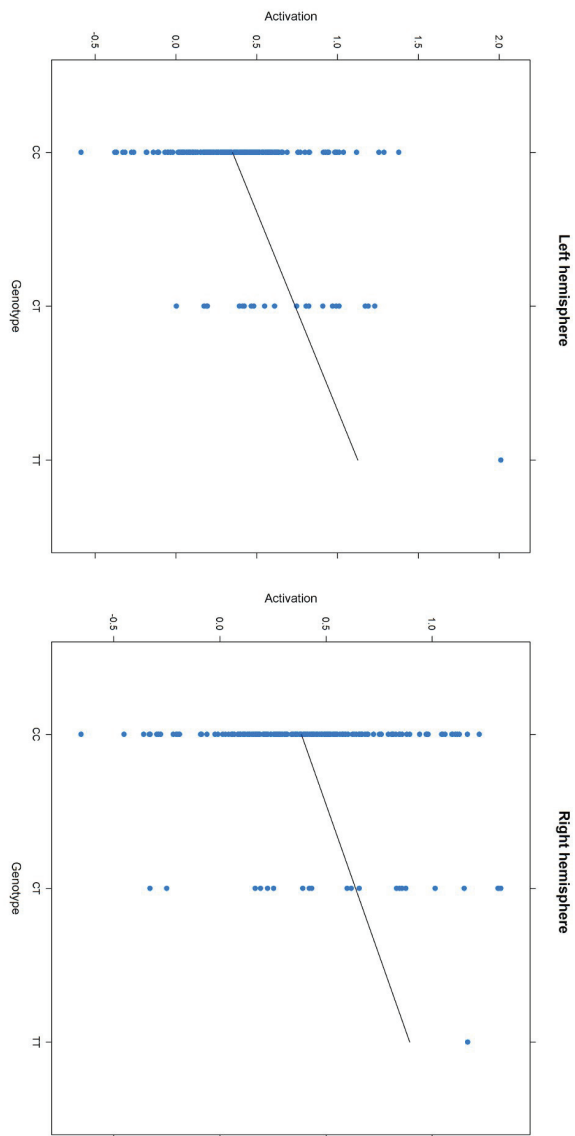


Figure 3

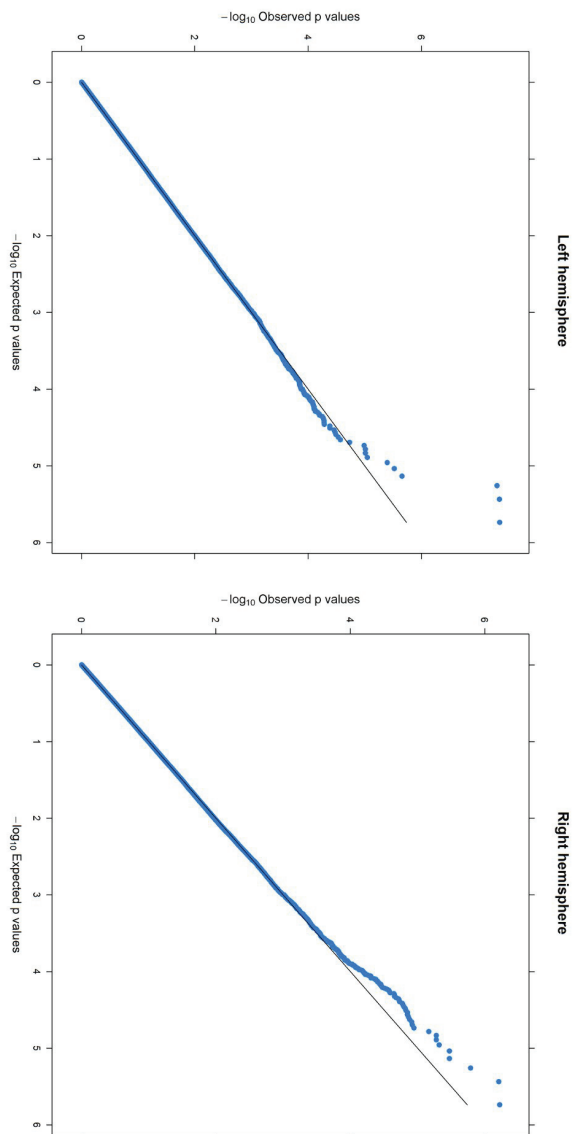
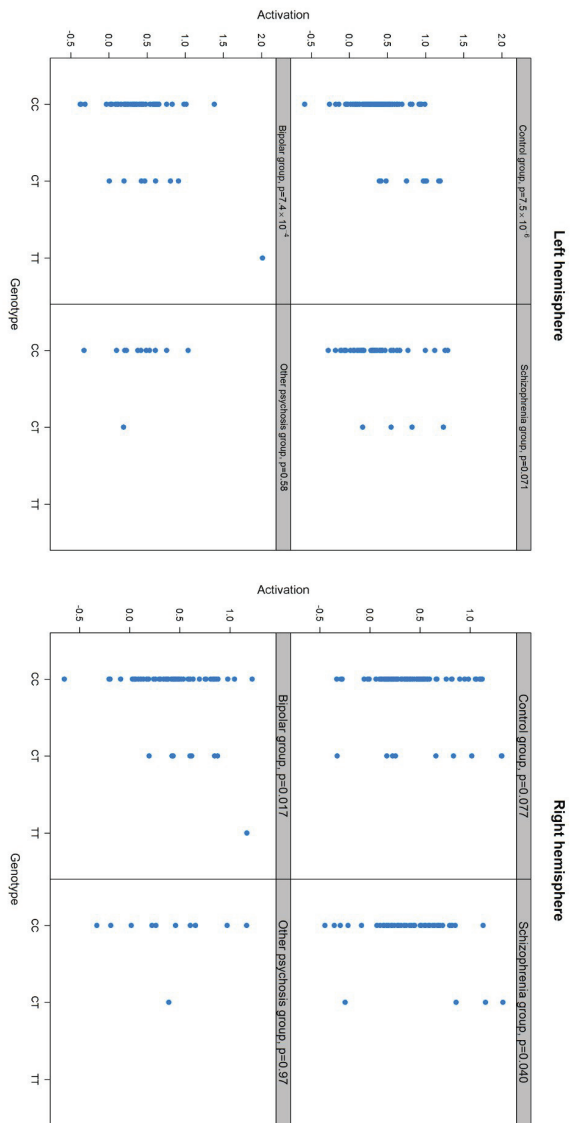


Figure 4



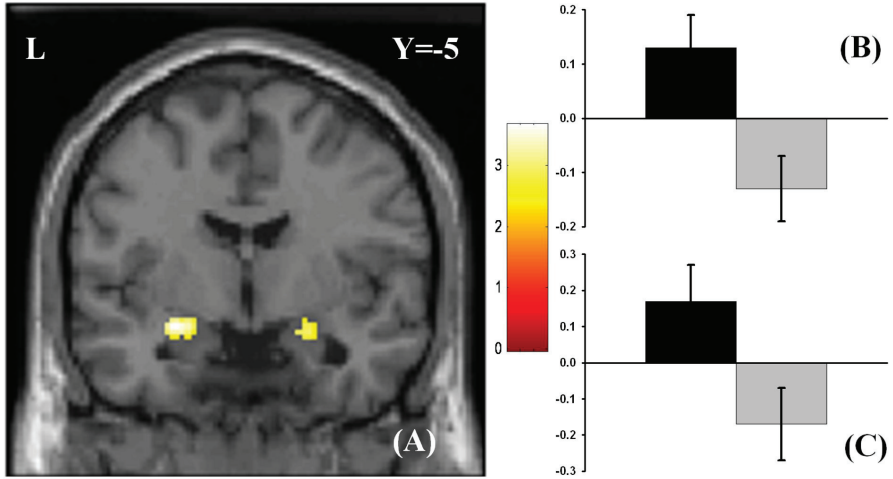


Figure 5