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Master's Thesis

The Involvement of the Anterior Cingulate Cortex in Safety and Danger Coding and in Cognitive Control

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Abstract

Imagine yourself on a walk in the park. At every moment of time you get bombarded with information; children laughing, dogs barking, birds singing, wind stroking your skin, the smell of grass and green leafs, not to mention all the visual information. Still, you do not focus on all this information all the time. Several regions of your brain work together in deciding which information to use and which to suppress depending on your behavioral goals (Gold and Shadlen, 2007, Walton et al., 2007). The anterior cingulate cortex (ACC) plays a major role in this processing and discrimination of novel information, is particularly active in selective attention when attention is focused on a goal of interest (Isomura et al., 2003, Casey et al., 2000) and in flexible shifting of behavioral states if the goal of interest changes (Kolling et al., 2016, Miller, 2000). Moreover, the ACC is implicated in both cognitive and affective aspects of this information processing (Bush et al., 2000). The neurons of the mouse ACC have also been shown to code for spatial information as certain neurons called annulus cells fire when the mouse explores the perimeter of an open field, while others called bull's-eye cells fire when the center is explored (Weible et al., 2012). Because rodents have a natural aversion of open spaces (Montgomery, 1955) we hypothesized that the annulus cells could be a neural correlate of safety and the bull's-eye cells a neural correlate of danger. By use of behavioral tasks and electrophysiological recordings the current study investigated the role of the mouse ACC in selective attention and safety and danger coding. For the selective attention task the mice had to learn a set of behavioral tasks as described by Kolata et al. (2007). We failed to replicate their behavioral results and could therefore not record neural correlates of selective attention. From the safety and danger coding tasks we found that many ACC neurons have shifting firing patterns over several recording sessions. This suggests that the same type of information can be coded in different cells each time it enters the ACC, and that the ACC can be viewed as an integrating structure that fires in response to inputs from other cortical structures. However, some annulus and bull's-eye cells had stable firing patterns over several sessions, which indicate that the ACC also may have specific functions as safety and danger coding. Thus, this study strengthens the hypothesis that the ACC may be involved in safety and danger coding.

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Contents

Abbreviations

ACC	**A**nterior **C**ingulate **C**ortex
ACd	**D**orsal **A**nterior **C**ingulate Cortex
ACv	**V**entral **A**nterior **C**ingulate Cortex
CCT	**C**ognitive **C**ontrol **T**est
CL	**C**hance **L**evel
EF	**E**levated **F**ield
EPM	**E**levated **P**lus **M**aze
IL	**I**nfra-**L**imbic Cortex
ITI	**I**nter **T**rial **I**nterval
KI/CNC	**K**avli **I**nstitute for Systems Neuroscience/**C**entre for **N**eural **C**omputation
MWU	**M**ann-**W**hitney **U**
NS	**N**on-**S**pecific
ODL	**O**lfactory **D**iscrimination **L**earning
OF	**O**pen **F**ield
PL	**P**re-**L**imbic Cortex
RS	**R**etrosplenial **C**ortex
SEM	**S**tandard **E**rror of the **M**ean
SLR	**S**imple **L**inear **R**egression
SOP	**S**tandard **O**perating **P**rocedure
SPSS	**S**tatistical **P**ackage for the **S**ocial **S**ciences
VDL	**V**isual **D**iscrimination **L**earning
WSR	**W**ilcoxon **S**igned **R**ank

Chapter 1

Introduction

When animals perform an action they generally do so because they believe the benefits of the action will compensate for the costs. Consequently they evaluate whether the action is worth carrying out or if they should explore alternatives (Kolling et al., 2016). Successful behavioral adaptions are dependent on the ability to flexibly and rapidly shift between behavioral actions. Such cognitive or higher order processes depend on large dynamic networks of interconnected brain structures (Miller, 2000, Bressler, 1995). Behavioral studies of humans with brain lesions, together with imaging studies of healthy individuals, have revealed many of the brain areas and interconnected networks that are involved in cognitive processing (Bressler, 1995). Cognitive functions can therefore not be solely attributed to one brain region but also to connections between brain areas (Delbeuck et al., 2003). However, one brain region that has been shown to be involved in cognitive functions as evaluating actions and directing attention is the cingulate cortex (Kolling et al., 2016, McGovern and Sheth, 2016, Miller, 2000). The cingulate cortex is one of the major parts of Broca's great limbic lobe; an assembly of medial forebrain structures found in all mammals that have been implicated in a large variety of processing, from cognitive to emotional (Roxo et al., 2011). Advances in understanding the neurobiological basis of these structures will improve the ability to refine mental illness treatments for cognitive dysfunction, e.g. in mood and anxiety disorders (Kandel et al., 2013). A part of the cingulate cortex that has been shown to be involved in both affective and cognitive processing of novel information (e.g. Weible et al., 2012, Chang and Sanfey, 2009, Bontempi et al., 1999) and flexible switching of behavior (Miller, 2000) is the ACC. However, the overarching function of the ACC remains unclear. This study therefore investigates the role of the ACC in novel information processing, moreover its involvement in affective danger and safety coding and cognitive processing of selective attention.

1.1 Overview of the anterior cingulate cortex

The ACC is the frontal part of the cingulate cortex, which is located in the medial cerebral cortex superior to the corpus callosum (Fig. 1.1). The cingulate cortex comprises the ACC, posterior cingulate cortex (retrosplenial cortex; RS), prelimbic (PL) and infralimbic (IL) cortices (Jones and Witter, 2007). The subdivisions are separated from each other both in

terms of connections (see paragraph 1.4) and functions. The PL and IL and are involved in fear responses and fear memory consolidation (Stern et al., 2014, Laurent and Westbrook, 2009), while the RS and ACC are involved in evaluative functions (e.g. spatial memory and navigation) and executive functions (e.g. working memory and attention), respectively (Isomura et al., 2003, Cooper et al., 2001, Vogt et al., 1992). However, studies have shown that these subdivisions also cooperate, for example in spatial learning (Warburton et al., 1998) and avoidance learning (Freeman et al., 1996).

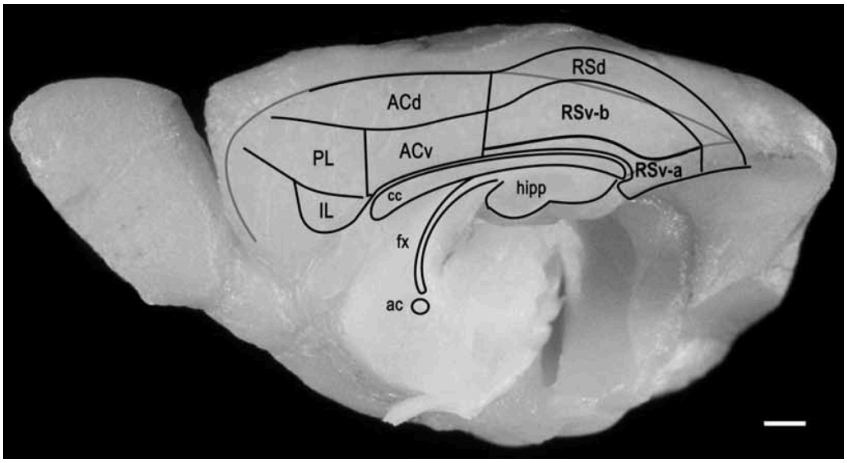

Figure 1.1 Overview of the cingulate cortex of the rat. The reconstruction is made on the right brain hemisphere where the cerebellum and brain stem has been removed. ac, anterior commissure; ACd, dorsal anterior cingulate cortex; ACv, ventral anterior cingulate cortex; cc, corpus callosum; fx, fornix; hipp, hippocampus; IL, infralimbic cortex; PL, prelimbic cortex; RSd, dorsal retrosplenial cortex; RSv-a, ventral retrosplenial cortex ventral part; RSv-b, ventral retrosplenial cortex dorsal part. Scale bar = 1 mm (adapted from Jones et al., 2005).

The ACC in mammalian species can be divided into a dorsal part (ACd) related to cognition, and a ventral part (ACv) related to emotion (Bush et al., 2000). Furthermore, Heidbreder and Groenewegen (2003) have proposed that the ACd is involved in temporal shifting of behavior, while the ACv is involved in shifting of behavioral strategies according to spatial information. These different functions arise from the different connections of the subdivisions (see paragraph 1.4). In short, ACv is densely connected to the RS and subcortical structures involved in affective coding such as the amygdala, nucleus accumbens, insula and the hypothalamus. The ACd is densely connected to the RS, PL and structures involved in cognitive control such as the prefrontal and parietal cortex (Ebitz and Platt, 2015, Rolls, 2015, Jones et al., 2005). Despite of the different connections and functions, the borders between the cingulate areas are not abrupt but show a gradual change in both the rostral-caudal and ventral-dorsal direction. However, an objective representation of the border between the ACC

and RS coincides with the appearance of the rostral tip of the hippocampus in coronal sections of the rat brain (Jones et al., 2005).

As previously mentioned, the ACC is involved in executive brain processes, which implies that it takes part in a vide variety of brain functions. Examples include anticipatory and motivational responses to pain-related events, coding for consequences of pain affect (Wang et al., 2003), evaluation, expression and generation of emotions (Rolls, 2015, Etkin et al., 2011), novel location and novel object recognition and long-term object memory (Weible et al., 2012, Weible et al., 2009) and cognitive control (Botvinick et al., 2004, Bush et al., 2000). In addition, it has been shown that certain cells in the ACC have firing patterns that correspond to safe and dangerous locations of an open field (Weible et al., 2012, Montgomery, 1955). The danger and safety coding properties and cognitive control will be described further in paragraphs 1.2 and 1.3.

1.2 Danger and safety coding properties of the anterior cingulate cortex

In addition to contributing in cognitive processes, which often involve several cortical structures, the neurons in the ACC have also been found to have specific functions. The ACC neurons exhibit a variety of different firing correlates depending on the task of interest. For example, rodent ACC neurons have been shown to code for novel object recognition and localization of objects, in addition to long time object memory and errors. This was based on studies by Weible et al. (2012) and Weible et al. (2009) who showed that some ACC neurons had a significantly higher firing rate around the location of familiarized objects, which continued to be persistent even when the objects were removed. This is a property that is consistent with memory coding. In addition, some neurons were silent when the objects were present, but started to fire when the objects were removed, which is consistent with both memory and error coding. The firing rates also increased with the amount of familiarization to the objects. Weible et al. (2012) also investigated the firing properties of mouse ACC neurons in open fields (without objects present). It was found that some neurons called bull's-eye cells had significantly higher firing rates in the center of the field compared to the perimeter, while others called annulus cells had significantly higher firing around the perimeter compared to the center. During open field sessions with objects present the bull's-eye cells also showed a decrease in firing around the objects, while the annulus cells showed increased firing around the objects (Fig. 1.2). Because rodents have a natural aversion of open fields (Montgomery,

1955) the bull's-eye cells could be a neural correlate of dangerous locations, while the annulus cells could be a neural correlate of safe locations.

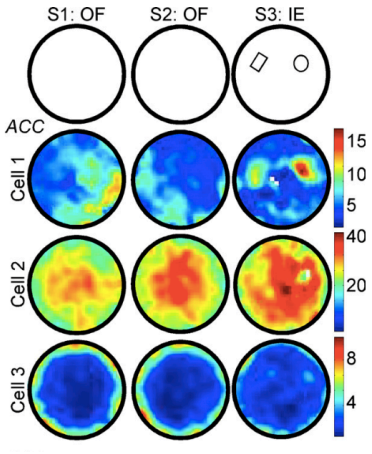

Figure 1.2 Firing properties of ACC cells in an open field with and without objects. In the open field, most neurons did not have a persistent activity pattern over several sessions but showed increased firing around objects (cell 1). Bull's-eye cells had a significantly higher firing in the center of the field compared to the perimeter (cell 2) and showed decreased firing around objects (S3). Annulus cells had a significantly higher firing around the perimeter compared to the center (cell 3) and showed increased firing around objects (S3). IE: initial exposure; OF, open field; S, session; Scale bar, firing frequency in hertz (adapted from Weible et al., 2012).

This study uses the elevated plus maze (EPM) to investigate whether the ACC is involved in safety and danger coding. This maze is based on the elevated Y-maze described by Montgomery (1955) who showed that rodents fear open and elevated locations. Because the EPM has both elevated/open and closed regions it has clearly defined dangerous and safe zones. The EPM was first proposed by Handley and Mithani (1984) as a model to investigate fear-motivated behavior in rats and was further validated by Pellow et al. (1985) who looked at arm entries as a measure of anxiety in the rat. It has also become a standard test of anxiety in mice because it exploits the conflict between the mice's natural desire to explore novel locations and their fear of open and elevated places (Lister, 1987). Investigations of where the bull's-eye and annulus cells fire on the elevated plus maze might reveal if these cells are involved in danger and safety coding. If the bull's-eye cells fire on the open (dangerous) arms they might code for danger, and conversely if the annulus cells fire on the closed (safe) arms they might code for safety.

1.3 The anterior cingulate cortex and cognitive control

According to Miller (2000) cognitive control can be described as executive brain functions that adapt information processing from one moment to another, such that the corresponding

behavior matches the animal's current goals. Studies have shown that the ACC, and especially the ACd (Bush et al., 2000), is involved in cognitive information processing. For example, ACC lesions have been shown to result in impaired ability to learn the value of actions and to invest high effort in effort-based decision-making tasks. Hence, the ACC play an important role in cost-benefit tasks and reward-guided behavior, which are important aspects of cognitive control (Walton et al., 2009, Kennerley et al., 2006, Rudebeck et al., 2006). In addition, imaging studies have shown that the ACC is involved in cognitive motor functions, i.e. directing attention to specific features of a stimulus and selecting a response corresponding to the stimulus ("attention for action"), motor preparation and execution of the response and error recognition if the response did not yield the expected result (Isomura et al., 2003, Paus, 2001). Neural activity in the ACC is particularly increased in selective attention tasks with conflicting stimuli as in the Stroop-task and Flanker-task. Selective attention or attention for action is a feature of cognitive control where information is filtered such that irrelevant information (depending on setting or task) can be ignored (Milham and Banich, 2005, Casey et al., 2000, Botvinick et al., 1999).

In the Stroop-task, test-subjects are asked to name the color of the font of a word spelling another color (e.g. BLUE) or conversely, spell the word and ignore the color (Stroop, 1935). In this task the subjects are therefore presented with conflicting information and have to suppress their reaction to either say the word or the color (Fig. 1.3 A top). In a study by Milham and Banich (2005) functional magnetic resonance imaging was used to investigate the activity in the ACC when presenting test-subjects with both incongruent information (e.g. BLUE) and congruent information (e.g. BLUE). They found increased activity throughout the ACC during presentation of conflicting stimuli, which is consistent with several other studies (e.g. Banich et al., 2000, Botvinick et al., 1999). But Milham and Banich (2005) also found that the anterior part of the rostral ACC was especially active during presentation of incongruent information, while the posterior part of the rostral ACC was active during presentation of both incongruent and congruent information. Meaning that the anterior part of rostral ACC was active during presentation of conflicting stimuli, while the posterior part of rostral ACC was active during presentation of task irrelevant information regardless of conflict (i.e. color).

The Stroop-task has been used frequently in studies of selective attention, and has also been redesigned to work for animals. In a study by Michelet et al. (2016) they use the Stroop-like

task where monkeys learn to associate a fruit shape with a color (banana-yellow; apple-red; pear-green). The monkeys then had to pick the correct color belonging to the fruit when the fruit shape was presented with a wrongful color. Hence, they had to ignore the conflicting color presented on the shape (Fig. 1.3 A bottom). In this study they found an increased activity in ACd in response to incongruent stimuli, which is consistent with the findings of Milham and Banich (2005). In the mouse Stroop-task (Sauce et al., 2014, Matzel and Kolata, 2010, Kolata et al., 2007) mice learn to discriminate between three scents in one environment ("context 1") and three visual cues in another environment ("context 2"), of which one of each type of cue is paired with a food reward. In the selective attention task all cues are presented together in either context 1 or 2. To find the food reward in this task the mice had to ignore one set of cues based on which environment they were placed in, i.e. use scents (ignore visual cues) in context 1 and use visual cues (ignore scents) in context 2 (Fig. 1.3 B). These studies were used to assess general learning ability and intelligence in mice and were therefore not directly related to ACC and cognitive control. This study, however, uses the mouse Stroop-task to investigate the involvement of the ACC in cognitive control. When mice have learned to discriminate between two distinct environments and the cues within the environments, recordings from the ACC will be performed while they are performing the selective attention task.

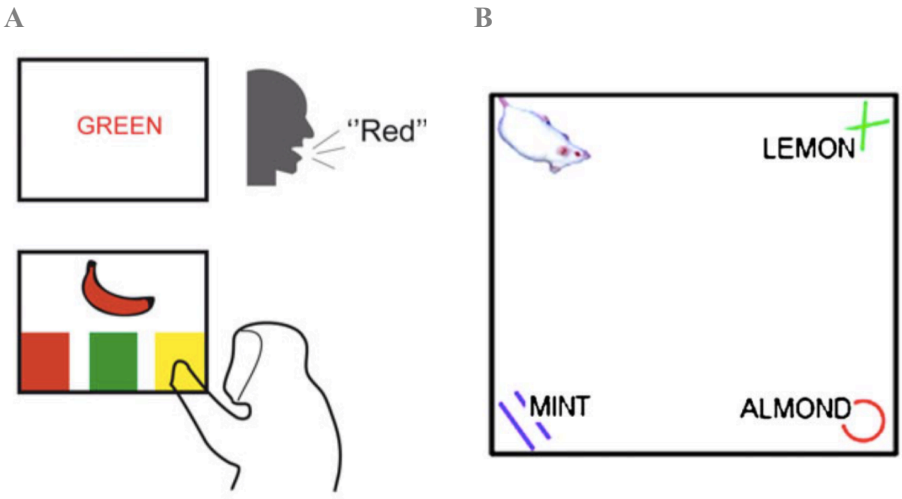

Figure 1.3 Human, monkey and mouse Stroop-tasks. A top) Stroop-task for humans. Test-subjects are presented with conflicting information, i.e. word spelling a color presented in different colored ink, and have to name the color of the ink and therefore ignore reading the word. **A bottom)** Stroop-task for monkeys. The monkeys have learned to associate fruit shapes with colors (banana-yellow; apple-red; pear-green). In the Stroop-task they are presented with a fruit shape with a wrongful color and have to pick the learned corresponding color and therefore ignore the color presented on the fruit. **B)** Stroop-task for mice. *Continued on the following page.*

Figure 1.3 continued. The mice have learned to use the mint scent to find a reward in one environment ("context 1") and the green X to find a reward in another environment ("context 2"). In the Stroop-task both types of cues are presented in either context 1 or 2. To find the reward, the mice have to ignore one set of cues depending on which context the cues are presented in: context 1 = mint, context 2 = green X (A adapted from Michelet et al., 2016, B adapted from Matzel and Kolata, 2010).

There is, however, increasing recognition that cognitive processes not only depend on a single structure such as the ACC but on dynamic networks of interconnected brain structures as described by Bressler (1995). The projections of the ACC will therefore be described in the following paragraphs.

1.4 Architecture and projections of the anterior cingulate cortex

1.4.1 Cytoarchitecture of the anterior cingulate

It has been shown that the ACC contains most of the same neurons as neocortical areas, including pyramidal, multipolar, bitufted and bipolar cells. In the ACC, layer I and II comprises small multipolar cells, while layer III and V comprises medium and large multipolar cells. Bitufted cells are also frequent in layers II and III, while bipolar cells are found in layers II, III and V. The pyramidal cells occur in layers II and III (Vogt and Peters, 1981). There is also a rostral-caudal gradation in cytoarchitectural features in the ACC. Neurons in all layers of rostral ACC are smaller than those in the caudal ACC. Rostral parts also have dense neural networks in layers I, III and Vb, which are minimized in caudal parts. In addition, the neurons in layers II-V are larger than the neurons in layers I and VI, where layer V has the largest neurons and the less dense packing (Vogt and Paxinos, 2014).

1.4.2 Intrinsic projections of the cingulate cortex

In the cingulate cortex, the IL is mainly connected with the PL, while the rostral 1/3 of ACd is connected to the PL, and the caudal 2/3 of ACd and ACv is connected with the RS. In general, the connections between the ACC and RS are topographically organized such that the rostral ACC has reciprocal connections with the caudal RS, and caudal ACC has reciprocal connections with rostral RS. More specifically, the caudal ACd has reciprocal connections with the dorsal RS and dorsal part of the ventral RS. The caudal ACv also has reciprocal connections with the dorsal part of the ventral RS, and receives projections from the dorsal RS. The rostral ACv project to the ventral part of the ventral RS and receives projections from all parts of the RS in addition to some from the PL. In addition, the rostral and caudal ACd are interconnected and project to the caudal ACv, while rostral and caudal ACv project to

rostral ACd (Jones et al., 2005). Figure 1.4 summarizes the intrinsic connections of the cingulate cortex.

Within the ACC there is also a ventral-dorsal topography with a trend for superficial layers to project to superficial layers and deep layers to deep layers, with the exception of ACC layer I that also receive input from deep layers. The RSd projections to the ACC also terminate in layers I and III, while ACC projections to RS terminate in layers I, III and V (Jones et al., 2005, Van Groen and Wyss, 2003, Fisk and Wyss, 1999).

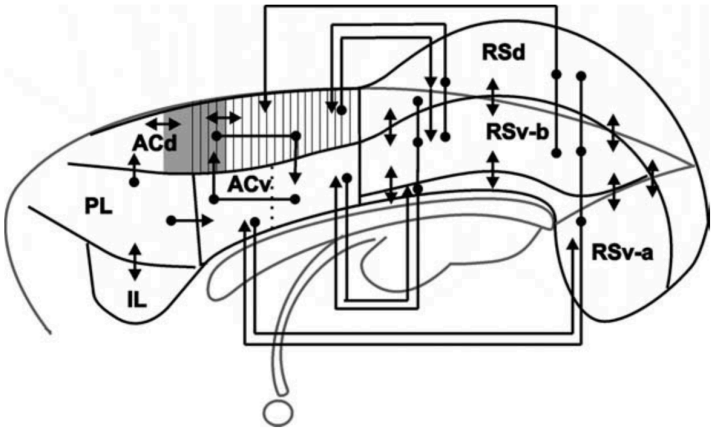

Figure 1.4 Intrinsic connections of the rodent anterior cingulate cortex. In general there are dense connections between the rostral ACd and PL; rostral ACC and caudal RS; caudal ACC and rostral RS. ACd, dorsal anterior cingulate cortex; ACv, ventral anterior cingulate cortex; IL, infralimbic cortex; PL, prelimbic cortex; RSd, dorsal retrosplenial cortex; RSv-a, ventral retrosplenial cortex ventral part; RSv-b, ventral retrosplenial cortex dorsal part. Arrows indicate projections and dots indicate origin of projection (adapted from Jones et al., 2005).

The intrinsic connectivity of the cingulate suggests that specific cingulate functions depend on a number of interconnected cingulate subregions that form functionally separated networks (Jones et al., 2005). For example, RS is involved in spatial memory and navigation, while ACC is involved in working memory and attention (Isomura et al., 2003, Cooper et al., 2001, Vogt et al., 1992). Furthermore, ACd is involved in cognitive tasks and ACv is involved in emotional tasks (Bush et al., 2000). However, these functions are not exclusively located in the cingulate cortex but rather depend on dynamic networks of interconnected brain areas (Bressler, 1995). In addition to being interconnected with the cingulate cortex the ACC is connected to a variety of other different areas, which are described in the subsequent paragraph.

1.4.3 Cortical and subcortical projections of the anterior cingulate

The distinctive sets of projections to and from the ACC contribute in specializing its functions. The ACC receives and sends projections to widespread areas of the brain representing all sensory modalities (directly and indirectly), which it integrates and uses in cognitive and affective processes (Hoover and Vertes, 2007). Afferent projections to the ACC arise in many cortical and subcortical structures; medial prefrontal, posterior parietal, primary and secondary visual, perirhinal and entorhinal cortices, hippocampus, hypothalamus, claustrum, amygdala, substantia nigra, ventral tegmental area, periaqueductal gray and monoaminergic nuclei of the brainstem. In addition, ACC receives projections from a number of thalamic nuclei of which the densest projections arise in anteromedial, interanteromedial, mediodorsal, paracentral, central lateral, reuniens and rhomboid nucleus (Hoover and Vertes, 2007, Jones and Witter, 2007). There is also a rostral-caudal differentiation in where thalamic nuclei project to the ACC. Rostral ACC receives primarily anteromedial, interanteromedial, reuniens and paratenial input, while caudal ACC receives primarily anteromedial and anterodorsal input (Shibata, 1993, Horikawa et al., 1988). In addition, there are several less dense afferent projections to the ACC, both from cortical and subcortical structures (Hoover and Vertes, 2007). In order to avoid an even more extensive listing of brain areas these structures are shown in figure 1.5, which summarizes the projections to the ACC.

The ACC projects back to many of the areas it receives information from, meaning that many connections are reciprocal. For example, there are efferent projections from ACC to the medial prefrontal cortex (Hoover and Vertes, 2007), posterior parietal cortex (Kolb and Walkey, 1987) and to the parahippocampal region but, however, not to the hippocampal formation. The rostral 1/3 of ACd mainly project to the perirhinal and lateral entorhinal cortex, while projections from the remaining ACC target postrhinal and medial entorhinal cortices in addition to presubiculum and parasubiculum (Jones and Witter, 2007). The projections between certain thalamic nuclei and the ACC are also reciprocal; ACC projects to the anteromedial, interanteromedial, mediodorsal, centromedial, paratenial, paraventricular nucleus and the nucleus reuniens (Vertes, 2002). The ACd has also been shown to project to dorsal lateral hypothalamus (Reppucci and Petrovich, 2015). In addition, Heidbreder and Groenewegen (2003) have shown that the ACd is most connected to sensorimotor and association neocortical areas, while the ACv is most connected with amygdala and temporal, limbic association cortices, septum, medial preoptic and hypothalamic areas. They also found that projections to monoaminergic cell groups were stronger from ACv than ACd.

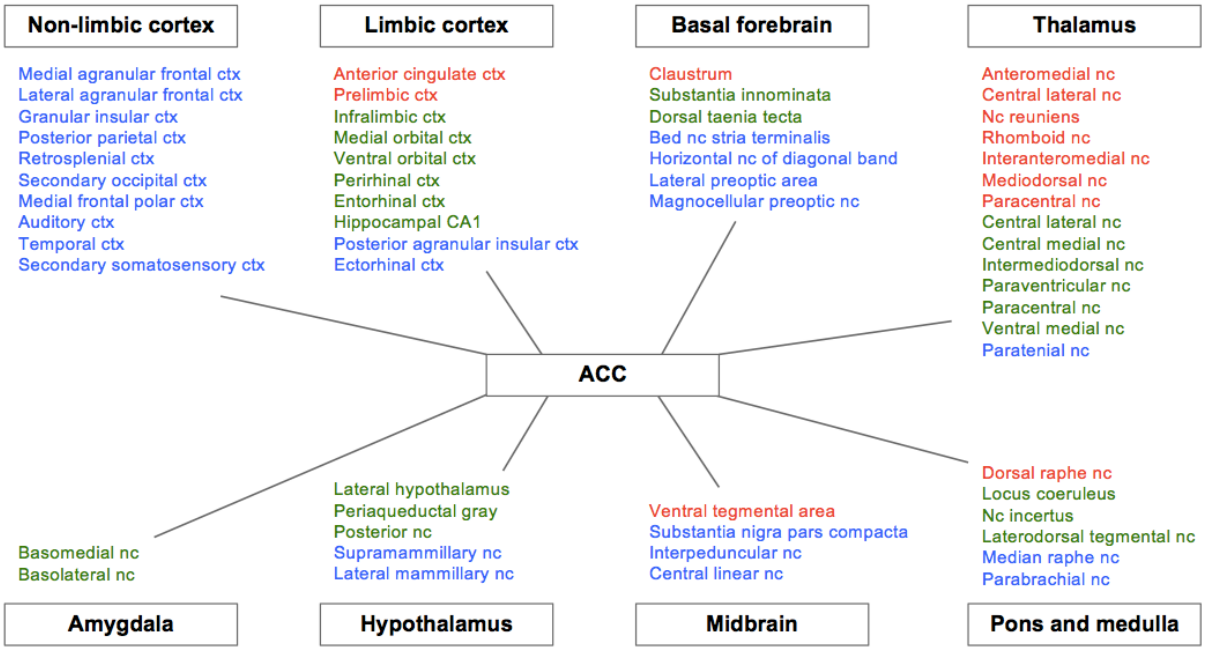

Figure 1.5 Overview of the projections to the anterior cingulate cortex. Red, green and blue colors represent high, moderate and light projections, respectively. Non-limbic cortices represent motor, somatosensory and associational regions of the cortex, while limbic cortices represent the remaining parts of the cortex, including orbital cortices and the hippocampal formation. The projections were found by use of retrograde tracing techniques. Ctx, cortex; nc, nucleus (replicated from Hoover and Vertes, 2007 fig. 11 B).

Pathways by which the ACC could contribute in cognitive and affective coding are via connections with many of the mentioned brain structures, which mediate different aspects of these processes. For example, cholinergic projections from the basal forebrain have been shown to serve important roles in behavioral arousal and attention mechanisms by enhancing processing of thalamic inputs in sensory areas (Sarter et al., 2005), while projections from the amygdala have been shown to convey information on affective properties of sensory stimuli involved in cognitive functions (Vertes, 2006). In addition, the medial prefrontal cortex, posterior parietal cortex, dorsolateral striatum and lateral posterior thalamic nuclei are parts of a large thalamo-cortical-basal ganglia network that serve central roles in directing spatial attention (Reep and Corwin, 2009). Furthermore, the projections from hippocampus to ACC and between the parahippocampal region and ACC have been suggested to be parts of networks important for learning and memory processes. The ACC may provide flexible attention and a discriminative approach towards novel information, which induce effective learning in the (para-) hippocampus (Jones and Witter, 2007).

1.5 The use of mice in behavioral and electrophysiological studies

1.5.1 The C57BL/6 mouse

Mice offer many advantages for investigating brain functions. For example, they can be used in behavioral, electrophysiological, histological and lesion studies. In addition transgenic mice can be used in knockout studies. In this study the C57BL/6 mouse is used. This is a strain of inbred mice often used in behavioral and physiological experiments. C57BL/6 show high levels of locomotion and low levels of anxiety in open fields and have the ability to learn complex tasks as for example Morris water maze, eight-way radial arm maze, conditional spatial alternation tasks and contextual fear conditioning (Crawley et al., 1997). More specifically, the female C57BL/6 have shown better performance in the rotarod task and more exploratory behavior in the open field compared to the males. However, as the mice familiarize with an environment the exploration decrease and a habitual activity pattern is observed. In the open field this means that they spend more time around the perimeter and less in the center, that the speed of exploring increases, and that the animal will spend more time resting (Deacon, 2006, Bothe et al., 2005).

1.5.2 Electrophysiological recordings

Methods for recording single-unit (single-neuron) activity in large populations of neurons have opened the opportunity for analyzing the relationship between neuronal activity and animal behavior. A number of recording techniques have been developed (e.g. micro electrodes and stereotrodes; McNaughton et al., 1983) but the tetrode has been shown to increase the reliability of isolating the single-unit from the remaining neuronal population (Gray et al., 1995, Wilson and McNaughton, 1993). The tetrode (four electrodes twisted together, implanted into the extracellular matrix) relies on the fact that the action potential amplitude is a declining function of the distance between the neuron and the electrodes. Therefore, the action potential amplitude from one neuron will change in the same way on all channels/electrodes. This relationship can be used as a criterion to separate units. By providing six pairs of amplitude ratios the tetrode reduces classification errors commonly encountered in single-unit recordings, e.g. incomplete separation of clusters in 2-D projections, incomplete separation of different neurons with similar waveform or classifying one neuron with different waveforms as two neurons. Because the amplitudes change in the same way on all channels the clusters that overlap in one projection can often be separated in another projection, the amplitude ratios will be different from neurons with similar wave

shapes depending on the neurons position relative to the electrodes, and the amplitudes from a neuron with different wave shapes will change in a coherent way on the channels (Gray et al., 1995).

1.6 Aim of study

The previously mentioned studies have provided evidence that the ACC may be involved in coding for dangerous and safe locations and in cognitive processing. However, the ACC remains poorly understood at the functional level due to its complex associative nature, and few studies directly examining the firing correlates of its neurons by means of recording experiments. This study therefore aims to investigate the firing characteristics of ACC neurons in two different behavioral tasks, the elevated plus maze and a cognitive control task based on the mouse Stroop-task, to better understand how the information processing in the ACC relates to behavior. More specifically, the first aim of this study is to investigate the role of the ACC in safety and danger coding by examining how the firing of bull's-eye and annulus cells correlates with dangerous and safe locations in an environment. The second aim of this study is to investigate how electrophysiological correlates of selective attention unfolds in the ACC.

Chapter 2

Materials and methods

2.1 Animals

In the following experiments a total of 15 male and 10 female C57BL/6 mice were used. The mice were housed individually in environmentally enriched clear shoebox cages in a temperature (20-24 °C) and humidity (45-65 %) controlled room on a reversed 12-hour light/dark cycle.

2.2 Animal handling

All animals were handled according to the regulations of the Norwegian Food Safety Authority (Regulation 761: The Use of Animals in Research 2015 §§1-30). In order to minimize the effect of stress responses to handling, all animals were handled extensively prior to the start of the behavioral training and testing. Procedures from two nature protocols were used to habituate the mice to handling (Deacon, 2006, Deacon and Rawlins, 2006). The habituation process started with accustoming the mice to the researchers hand. This was done by slowly placing the hand in the cage of one mouse and letting it rest there for 2-3 minutes, followed by slowly tracing the mouse and pulling the hand back. Treats (cream filling from Pepita crackers [Sætre, Orkla Confectionery & Snacks Norge] or a crumb of chocolate flavored cereal [Weetos Choco, Weetabix Food Company]) were also put onto the hand to induce approaching behavior of the mice. The treats gave the mice a positive association to the hand and often led to the mice walking towards the hand and jumping onto it. Hence, they ceased being anxious when the hand was present. At this point, habituation to lifting was started. The lifting was carried out by gripping the proximal part of their tail and lifting them onto the hand, lifting one of the toys they were sitting on, or moving two hands towards them such that they jumped onto one of the hands. The habituation process lasted for 5-10 minutes per mouse per day, for a minimum of ten days and at the end of the handling period all mice walked freely onto the hand of the researcher. The mice were also habituated to the room where the experiments would take place by bringing them there from day four of the handling period.

2.3 Experimental procedures

The experiments were performed at the KI/CNC, NTNU. Prior to the experimental sessions, 4-6 mice were brought to the testing room on a trolley. They were kept in their home cages throughout the time in the testing room, except during experiments and in the inter-trial intervals (ITI) in the olfactory- and visual discrimination learning, and cognitive control test. Before the experiments started the exercise wheels was removed from each cage in order to reduce noise in the room. All test apparatus was cleaned with ethanol (70 %) and dried before all trials.

2.3.1 Danger and safety coding neurons of the anterior cingulate cortex

The elevated plus maze

The design of the EPM used in the following experiment was based on several other EPM designs for mice (Walf and Frye, 2007, Rodgers and Cole, 1993, Lister, 1987). The EPM (Fig. 2.1) was built of plywood boards (1 piece 65 cm long, 5 cm wide, 1 cm thick; 2 pieces 30 cm × 5 cm × 1 cm) fastened together such that they formed a plus sign with 30 cm long arms and a central platform measuring 5 cm × 5 cm. The maze was elevated 50 cm above the floor by use of steel feet. The 15 cm high walls for the two closed arms were made of clear polycarbonate plastic (Lexan®) covered with matte black adhesive paper and were fastened to the arms by use of clamps. A piece of the inner top part of the walls (3 cm × 12 cm) was removed so the drives of implanted mice would not collide with the walls when they entered the arms. In order to increase the visibility of the mice, and to increase the mice's foothold on the maze, the arms and central platform was covered with grey duct tape. The EPM was then placed under a video camera in a circular recording area surrounded by long dark blue curtains. Three different setups of the EPM were used. Setup 1: EPM with clear walls enclosing two of the arms, placed in a test area with subdued lights; setup 2: EPM with black walls, placed in a test area with subdued lights; setup 3: EPM with black walls, placed in a test area with bright lights.

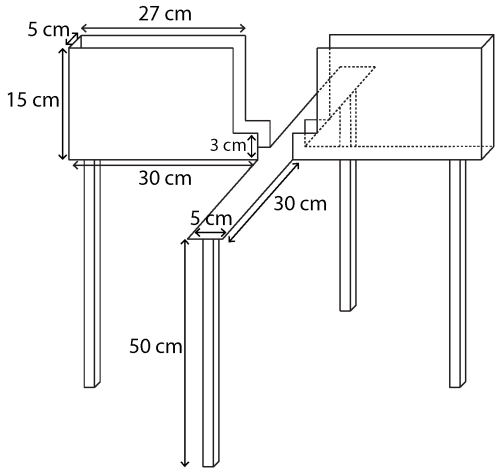

Figure 2.1 Illustration of the elevated plus maze used in this study. The elevated plus maze was placed under a video camera in a circular recording area surrounded by long dark blue curtains, where experiments were performed both with subdued and bright lights. The clear Lexan® walls were covered with matte black adhesive paper in some of the experiments.

Behavioral and electrophysiological procedure

The behavioral procedure for the EPM was based on a Nature protocol by Walf and Frye (2007). This maze is used to investigate fear and anxiety. Therefore the mice did not get habituated to the EPM before the test session. There was also only one five-minute test session per mouse to make sure that the first innate anxiety responses were recorded. These responses were quantified by recording the number of entries to the open and closed arms and time spent on the open and closed arms. An entry onto one of the arms was counted when all four paws of the mouse were on the arm. The session started with placing a mouse on the central platform facing away from the experimenter and a stopwatch set for five minutes was started while the experimenter left the test area. All sessions were recorded by the video camera placed in the ceiling above the maze. If a mouse fell of one of the open arms during the experiment it was picked up and placed back onto the arm in order to give all mice the same exposure on the maze. However, the entry and latency scores from this mouse were excluded from the total results. At the end of the five minutes the mouse was picked up and brought back to its home cage.

After making sure that the setup of the EPM worked, i.e. the mice spent more time and had more entries onto the closed than the open arms, recordings from ACC neurons were made while mice explored the maze. The recordings were carried out by Cornelis Herman Zuiderveen Borgesius, researcher at the KI/CNC. The same procedure as the behavioral testing was used with one exception: the mice were allowed to explore the maze for up to 20

minutes, to ensure recordings from neurons both on the open and closed arms. In order to investigate if the firing patterns on the EPM belonged to neurons that could code for safe and/or dangerous locations, i.e. annulus and bull's-eye cells, the mice were recorded in a circular open field (OF; diameter: 65 cm; walls: 45 cm) for 30 minutes before being recorded on the EPM. Some mice were also recorded on a circular elevated field without walls (EF; diameter: 68 cm; drop: 46 cm) for 30 minutes after being recorded on the OF.

2.3.2 The anterior cingulate cortex and cognitive control

Olfactory- and visual discrimination learning

The design of this experimental setup was based on a design for a selective attention task as described by Kolata et al. (2007). Here the object is for the mice to learn to discriminate between three scents and three visual cues in two distinct chambers. The black and white chambers (50 cm x 50 cm wide, 30 cm tall walls) used in the following experiments (Fig. 2.2) were made of Plexiglas®; the black box had a grey inlay covering the floor to increase the visibility of the mice. In three corners of the chambers a metal food cup (diameter: 5 cm) was placed, while in the fourth corner a removable wall (15 cm x 15 cm wide, 30 cm tall) formed the start point of the tasks. All food cups were loaded with 1/8 chocolate cereal ring and in two of the cups the crumbles got covered with wire mesh (on the very first trial an extra crumble of cereal was placed on the edge of the target food cup). In the black olfactory chamber different scented cotton swabs were fastened to the cups (scents: 30 µl of vanilla, raspberry or lemon food flavoring [Mors Hjemmebakte, Idun Industri AS]). The accessible crumble (not covered with wire mesh) was always associated with the vanilla scent, while raspberry and lemon were always associated with the inaccessible crumbles independent of the positioning of the cups. In the white visual chamber different symbols (silver colored X, Δ or □ cardboard figures covered with clear adhesive paper) was fastened above each cup. The X was always associated with the accessible crumble, while Δ and □ were associated with the inaccessible crumbles. Prior to the start of the experimental trials the chambers were placed under a video camera in a circular recording area surrounded by long dark blue curtains. Experiments were performed with subdued lights.

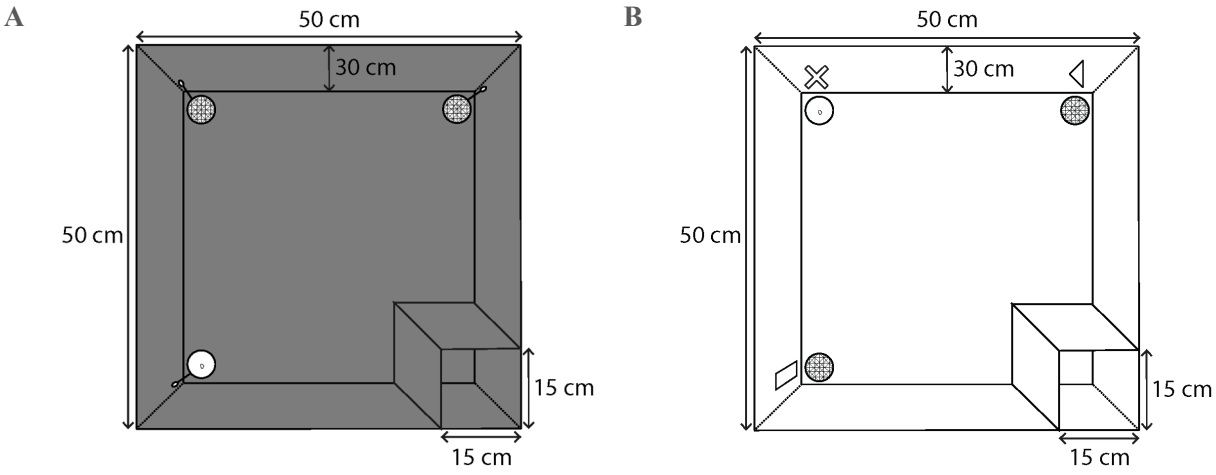

Figure 2.2 Test chambers for the olfactory (A) and visual (B) discrimination learning. The olfactory and visual chambers were made of black and white Plexiglas®**,** respectively. Independent of the positioning of the cups, the accessible crumble was always associated with the vanilla scent in the olfactory chamber and with the symbol X in the visual chamber. The removable 15 cm x 15 cm walls formed the start point of the tasks.

Learning procedure

Prior to the start of the olfactory discrimination learning (ODL) and visual discrimination learning (VDL) the mice were food restricted according to the regulations of the Norwegian Food Safety Authority (for standard operating procedure (SOP) at the KI/CNC see appendix A). This was done to encourage foraging behavior in the tasks. Each mouse also had one day of acclimatization to the olfactory chamber. Here they explored the chamber, containing only empty food cups, for 20 minutes. At the end of the acclimatization the mice were brought back to their home cages where they received half a chocolate cereal ring used as the reward (accessible crumble) in the training. Two different strategies were used in the ODL and VDL, henceforth denoted as ODL1, ODL2, VDL1 and VDL2. All trials in both tasks were recorded by the video camera in the ceiling above the cambers.

The procedures for the ODL1 and VDL1 were based on designs by Kolata et al. (2007). In the ODL1 the mice received four trials of training per day, for three days. Each trial started with placing a mouse in the start point of the black chamber. After 20 seconds the start point walls were removed and the mouse was allowed to explore the chamber until the reward was found in the target cup (scented with vanilla). Latency and number of errors before finding the reward was counted on all trials. An error was defined as trying to retrieve a non-target crumble or making contact with a non-target cup (scented with raspberry or lemon). Revisiting a non-target cup was not counted as an error, so a maximum of two errors was counted on each trial. After the reward was found the mouse was allowed to consume the

reward for 15 seconds before it was picked up and placed in a holding chamber for 6 minutes. In the ITI the olfactory chamber was cleaned and a new reward was placed in the target cup before the cups was rotated randomly. The scented cotton swabs were replaced between each mouse. To investigate if the mice had learned to associate the vanilla scent with the reward, a control experiment was carried out on day four. Here the mice received four trials where the crumble in the vanilla scented cup was made inaccessible, while the crumble in the raspberry cup was made accessible. Latency and number of errors before finding the reward was counted again. After completing the ODL1 the mice proceeded on to the VDL1. The procedure for the VDL1 was the same as for the ODL1 except all trials were performed in the white chamber and the scents were replaced with the visual cues. Hence, the target cup was now marked with X and the non-target cups were marked with Δ and □. The number of training days was also increased from three to four.

The ODL2 was based on an experiment by Muzzio et al. (2009), where mice learn to associate a scent with a reward by digging in scented wood chips. In the following experiment the mice received ten days of training, each with four trials (6 minutes ITI). In the first four trials (day 1) the procedure was exactly the same as the ODL1, but from trial five (day 2) the food cups got filled with wood chips (clean unscented standard bedding used in the animal facility) such that it covered the cereal crumbles and wire meshes. The wood chips were changed in each ITI, but beyond that the procedure was the same as the ODL1. In the ODL2 an error was defined as digging in a non-target cup. Revisiting or digging in a previously opened non-target cup was not counted; hence a maximum of two errors could be counted on each trial. A control experiment, to investigate if the mice had learned to associate the reward with the vanilla scent, was performed on day 11. This was the same test as in the ODL1 but with the addition of wood chips in the cups. After completing the ODL2 the mice proceeded on to the VDL2. The procedure for the VDL2 was the same as for the VDL1 except the food cups were filled with wood chips on all trials and the number of training days was increased from four to ten. The mice were also presented with four control trials on day 11 to investigate if they had learned to associate the X with the reward. In these trials the X was placed above a cup containing an inaccessible crumble, while Δ was placed above the cup containing the accessible crumble.

Cognitive control test

Some of the mice that had completed both discrimination tasks were tested in a complex discrimination task based on the mouse Stroop-task (Matzel and Kolata, 2010, Kolata et al., 2007). In this cognitive control test (CCT) both scents and visual cues were presented together on the cups in either the black or the white discrimination chamber. The visual cues would act as distractors when presented in the black olfactory chamber, and the scents would act as distractors when presented in the white visual chamber. The two target cues (vanilla and X) would never be placed on the same food cup. That is, vanilla was presented together with Δ or □ in the olfactory chamber and X was presented together with raspberry or lemon in the visual chamber. Hence, the mice had to remember which cues belonged to which discrimination chamber in order to find the reward without making errors. The task consisted of one three trial session in both chambers (6 minutes ITI) and both sessions occurred on the same day. Depending on if the mice had completed the ODL/VDL 1 or 2, they performed the CCT1 without wood chips or CCT2 with wood chips, respectively. Latency and number of errors was recorded on all trials. In the CCT1 an error was defined as trying to retrieve a non-target crumble or making contact with a non-target cup, while in the CCT2 an error was defined as digging in a non-target cup. Revisiting a non-target cup was not counted as an error.

2.3.3 Summary of the discrimination learning and cognitive control test

Figure 2.3 on the following page shows a schematic comparison of the ODL1, VDL1 and CCT1 versus ODL2, VDL2 and CCT2.

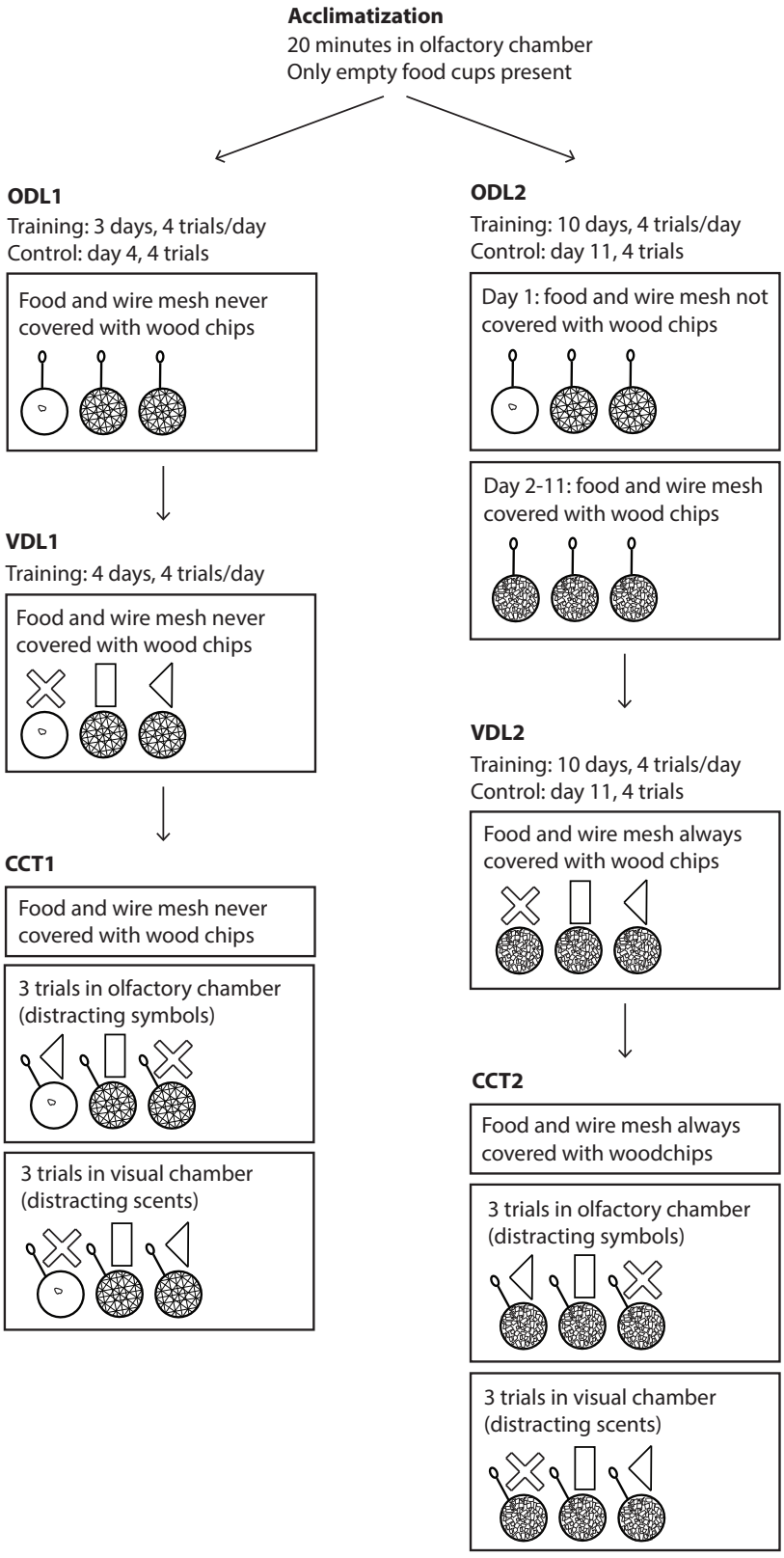

Figure 2.3 Schematic comparison of the two strategies used in the discrimination learning and cognitive control test. The food cups were placed in three corners of the black olfactory or white visual chamber and were rotated randomly between each trial. The accessible crumble was always associated with the vanilla scent and symbol X in the olfactory and visual chamber, respectively except in the control trials. In the CCT the two target cues (vanilla and X) never occurred on the same cup.

2.3.4 Electrophysiological recordings during olfactory discrimination

Recordings in the olfactory task were conducted in collaboration with Cornelis Herman Zuiderveen Borgesius. These recordings were part of a pilot study investigating whether neurons in the ACC could code for specific scents. Two mice were retrained in the olfactory task after having completed the ODL, VDL and CCT 1. The retraining lasted for 10 days and followed the procedure from the ODL2 day 2-10. After the retraining, recordings were made on four trials of the olfactory discrimination task. The procedure on these trials were also the same as ODL2 day 2-10, except the mice explored the box for 10 minutes per trial to ensure recordings on all locations in the chamber.

2.4 Surgical procedure

Cornelis Herman Zuiderveen Borgesius performed all the surgeries needed to implant electrodes to record activity from ACC neurons. The mice were anesthetized with isoflurane (5 %) in an induction chamber. When anaesthetized they were placed on a stereotaxic frame (Kopf ®) were anesthesia was continued from a tube mask (isoflurane: start concentration 3 %, lowered to 1 % during surgery). The surgical field was then shaved and eye ointment was put on the cornea. After ensuring that a mouse was fully anaesthetized, by checking for reflexes by pinching the skin between the toes, the head was fixed in the stereotaxic frame. When the correct position was achieved, the scalp was disinfected with iodide and a small anterior-posterior incision was made on the scalp to reveal the scull bone. Lambda and bregma were then used as the reference frame to cut away a small piece of bone to reveal the cerebral cortex (Paxinos and Franklin, 2001). A custom made microdrive (Axona Ltd.) holding eight separately moveable tetrodes (each: four 17 μm platinum/iridium wires twisted together) was implanted into the ACC (anterior-posterior between 1 and 0 mm from bregma; medial-lateral 0.2 to 0.3 mm from midline; dorsal-ventral 1.0 mm from skull level). The wound was closed and the head stage connected to the tetrodes was fastened to the scull with dental cement. Small screws were placed into the skull to provide extra grip and stability to the drive and dental cement. All mice were given post-surgical analgesia and were let to recover for two weeks before recording experiments started. For complete surgical/medical SOP at the KI/CNC see appendix B.

2.5 Statistics and data analysis

2.5.1 Chance level of the discrimination learning

For the discrimination learning experiments a chance level of errors was calculated (appendix C) to compare with the actual number of errors made by the mice. Random choices of cups were calculated to result in two attempts, and therefore one error, to find the reward per trial. Hence, the chance level equaled one. If the mice performed significantly better (made fewer errors) than the chance level this would show that they had learned to use the cues. However, the chance level was only valid for mice that never returned to a cup they had visited before on the same trial. Therefore, to be able to compare the chance level with the errors made by the experimental group, returning to an already visited non-target cup was not counted as an error.

2.5.2 Statistical data analysis in SPSS

The statistical analysis program SPSS (Statistical Package for the Social Sciences, IBM Analytics) was used to check whether the experimental data were normally distributed and to generate hypothesis tests. These tests were used to investigate whether data in two groups were statistically significant different. Depending on if the data were normally or non-normally distributed the parametric paired and unpaired t-tests or the non-parametric Wilcoxon signed-rank (WSR) and Mann-Whitney U (MWU) tests were used, respectively. A simple linear regression (SLR) analysis was also generated in order to check whether there was a statistically significant linear relationship between number of days and errors/latency to find the reward in the discrimination learning. In all experiments the significance level was set to 5 % ($\alpha = 0.05$).

2.5.3 Cluster cutting and data analysis in MATLAB

The program Spike Sort 3D (Neuralynx, Inc.) was used to cut spike clusters from the recordings. This was done by drawing borders formed like Gaussian ellipses around clusters of spikes with similar orientation, in a way that minimized overlap between neighboring clusters and noise. The clusters were then given a subjective hallmark based on Isolation Distance and L_{ratio} (Schmitzer-Torbert et al., 2005), wave shape and in some cases the amount of the cluster that was cut away by the recording threshold. The hallmarks varied from high quality 1 to low quality 4; only clusters with hallmarks 1-3 were used in further analysis. Timestamp files containing information about when the ACC neurons spiked were then generated from the clusters, also by use of Spike Sort 3D. These timestamp files were

analyzed in MATLAB (The MathWorks, Inc.) by use of a custom MATLAB code and smoothed rate maps that showed firing frequency of different neurons on different locations on the EPM, circular fields or olfactory chamber were created. Unpaired t-tests on unsmoothed rate maps from the OF and EF were also generated in MATLAB. These t-tests were used to investigate if the neurons had a significant change ($p < 0.05$) in firing rate between the center and perimeter within one session of the OF or EF.

Chapter 3

Results

3.1 Danger and safety coding neurons of the anterior cingulate cortex

3.1.1 Establishment of the functional elevated plus maze

Bull's-eye and annulus cells of the ACC fire in the center and around the perimeter of open fields, respectively (Weible et al., 2012). Based on rodent's natural aversion of open spaces (Montgomery, 1955), these cells could code for dangerous and safe locations. In order to test this hypothesis we recorded from the ACC while mice were exploring the EPM, which has clearly defined open (dangerous) and closed (safe) zones. If the bull's-eye cells fired on the open arms and annulus cells fired on the closed arms they could code for danger and safety, respectively. However, we first had to make sure that the EPM was working. That is, the mice had a higher preference for the safe than the dangerous zones. A total of 16 mice were used in this behavioral EPM experiment. Each mouse explored one of three different setups of the EPM for a single five-minute session (for reference see paragraph 2.3.1). The preference of each mouse for the different divisions of the EPM was indicated by the amount of time spent and number of entries into that location. More time and entries indicated a higher preference than less time and entries.

A Shapiro-Wilks test ($p > 0.05$) and visual inspection of the normality plots showed that the differences in time and differences in entries between the open and closed arms were approximately normally distributed in all setups. Paired sample t-tests were therefore used to investigate whether the difference in time and entries between open and closed arms, within one setup, was statistically significant ($p < 0.05$). In setup 1 (Fig. 3.1 A, $n = 4$ males) the EPM had transparent walls and was placed in a dimly lit recording area. Here, the mice had no significant difference in time spent on the open and closed arms (time 117 ± 18 s and 97 ± 18 s, mean ± SEM for open and closed arms respectively, $p = 0.63$), or in entries to the open and closed arms (entries 11 ± 1.9 and 8 ± 1.9, mean ± SEM for open and closed arms respectively, $p = 0.25$), which indicated that setup 1 was not working. Therefore the EPM walls were made black (setup 2) to increase the safety-danger difference between the arms. This was based on mice's preference for darker than brighter locations (Deacon, 2006, Wax and Goodrick, 1975). There was also no significant difference in time spent on the open and closed arms (time 107 ± 15 s and 113 ± 16 s, mean ± SEM for open and closed arms respectively,

p = 0.85) or in entries to the open and closed arms (entries 17 ± 2.7 and 17 ± 1.7, mean ± SEM for open and closed arms respectively, p = 0.96) in setup 2 (Fig. 3.1 B, n = 5 males). Hence, this setup was not working either. Therefore the EPM (with black walls) was placed in a brightly lit recording area (setup 3), to further increase the safety-danger difference between the arms. In setup 3 (Fig. 3.1 C, n = 1 male, n = 6 females) the mice spent significantly more time on the closed than the open arms (time 42 ± 4 s and 180 ± 5 s, mean ± SEM for open and closed arms respectively, $p < 0.01$), and they made significantly more entries onto the closed than the open arms (entries 6 ± 0.6 and 16 ± 1.7, mean ± SEM for open and closed arms respectively, $p < 0.01$). This indicated that setup 3 worked.

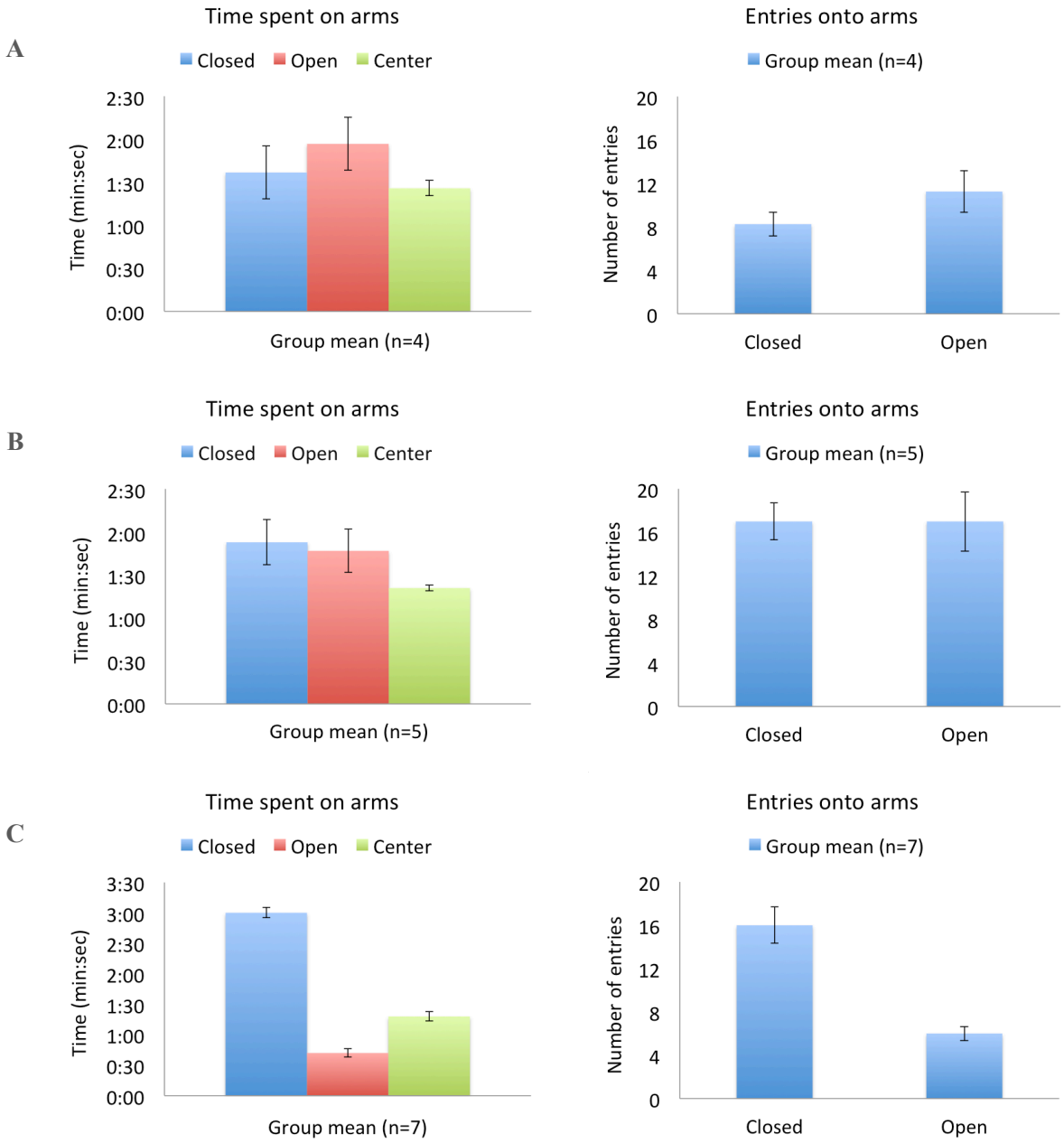

Figure 3.1 Time and entries (± SEM) on the open and closed arms on all elevated plus maze setups. **A)** Setup 1 (dimmed light; transparent walls): the mice spent on average more time and had a higher number of entries on the open arms (time 117 ± 18 s, entries 11 ± 1.9) compared to the closed arms (time 97 ± 18 s, entries 8 ± 1.9), but the differences were not statistically significant ($p > 0.05$). This indicated that the group had no clear preference for either place on the plus maze. **B)** Setup 2 (dimmed light; black walls): these mice had approximately equal distribution of time (closed 113 ± 16 s, open 107 ± 15 s) and number of entries (closed 17 ± 1.7, open 17 ± 2.7). Therefore there was not a significant difference in time or entries between the open and closed arms, which indicated that the group did not have a preference for either type of arm. **C)** Setup 3 (bright light; black walls): the mice had a significantly ($p < 0.05$) higher amount of time spent and entries made on the closed arms (time 180 ± 5 s, entries 16 ± 1.7) than the open arms (time 42 ± 4 s, entries 6 ± 0.6), which indicated that they had a preference for the closed arms.

However, non-normality is often hard to detect in small sample sizes so a nonparametric equivalent of the paired t-test was also performed. For all setups the WSR test did show the same as the paired t-test: no significant difference in time spent or in entries made in setup 1 (time $p = 0.72$; entries $p = 0.20$) and 2 (time $p = 0.89$; entries $p = 1.00$), significant difference in time spent and entries made in setup 3 (time $p = 0.02$; entries $p = 0.02$). The results indicated that the mice had a preference for the closed arms only in setup 3, which meant that only setup 3 was working and that this setup had to be used in all further EPM experiments.

3.1.2 Electrophysiological results from the elevated plus maze and open field

Based on the hypothesis that bull's-eye cells coded for dangerous places and annulus cells coded for safe places, these cells should fire on the open and closed arms of the EPM, respectively. Therefore, to identify these cells and their firing pattern on the EPM, the mice (n = 8 males, n = 1 female) were recorded in an OF before being recorded on the EPM (for reference see paragraph 2.3.1 *Behavioral and electrophysiological procedure*). The recordings yielded 42 units that passed the cluster selection criteria and could be used in further analysis. Unpaired t-tests on unsmoothed rate maps, investigating whether the difference in firing rate between the center and perimeter of the OF were statistically significant ($p < 0.05$), were used to categorize the neurons (Weible et al., 2012). The cells categorized as bull's-eye cells (n = 4) had a significantly higher firing rate in the center of the OF compared to the perimeter; annulus cells (n = 22) had a significantly lower firing rate in the center compared to the perimeter; unspecific cells (n = 9) did not have a statistically significant ($p > 0.05$) difference in firing rate between the center and perimeter (Fig. 3.2). The cells were categorized into non-specific (NS) bull's-eye (n = 4) or annulus (n = 3) cells if they had the same characteristics as the bull's-eye or annulus cells in one OF session, but had the characteristics of an unspecific cell in another OF (Fig. 3.3).

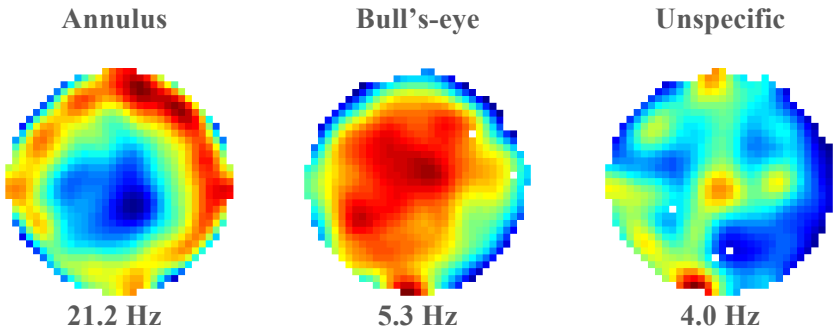

Figure 3.2 Examples of the annulus, bull's-eye and unspecific cells recorded in the open field. All cells that had a significantly ($p < 0.05$) higher firing rate around the perimeter compared to the center were counted as annulus cells, while all cells that had a significantly higher firing rate in the center compared to the perimeter were counted as bull's-eye cells. Unspecific cells did not have a significant difference ($p > 0.05$) in firing between the center and perimeter. If two OF sessions had been recorded, the firing patterns had to be significant in both sessions in order for a cell to be counted as a bull's-eye or annulus cell. That is, if the difference in firing between the center and perimeter was significant in only one of the OF sessions the neurons were categorized as non-specific annulus or bull's-eye cells. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

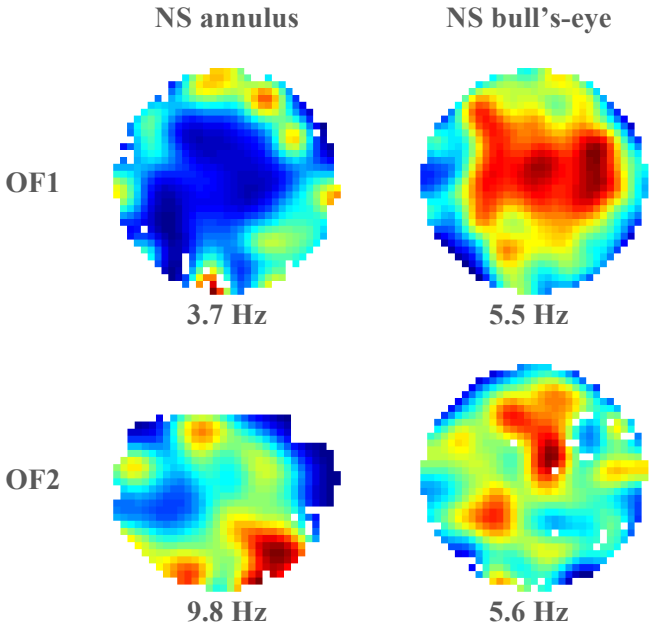

Figure 3.3 Examples of the non-specific annulus and bull's-eye cells recorded in the open field. The NS annulus cells had a statistically significant ($p < 0.05$) higher firing rate around the perimeter compared to the center in one of two OF sessions, while NS bull's-eye cells had a significantly higher firing rate in the center compared to the perimeter in one of two OF sessions. In this case: NS annulus OF1 $p < 0.01$, OF2 $p = 0.32$; NS bull's-eye OF1 $p < 0.01$, OF2 $p = 0.10$. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

By visual inspection of the EPM rate maps the neurons were categorized according to their firing pattern on the EPM. Figure 3.4 gives an overview of the different types of neurons recorded in the OF sessions including where these neurons had their peak firing located on the EPM.

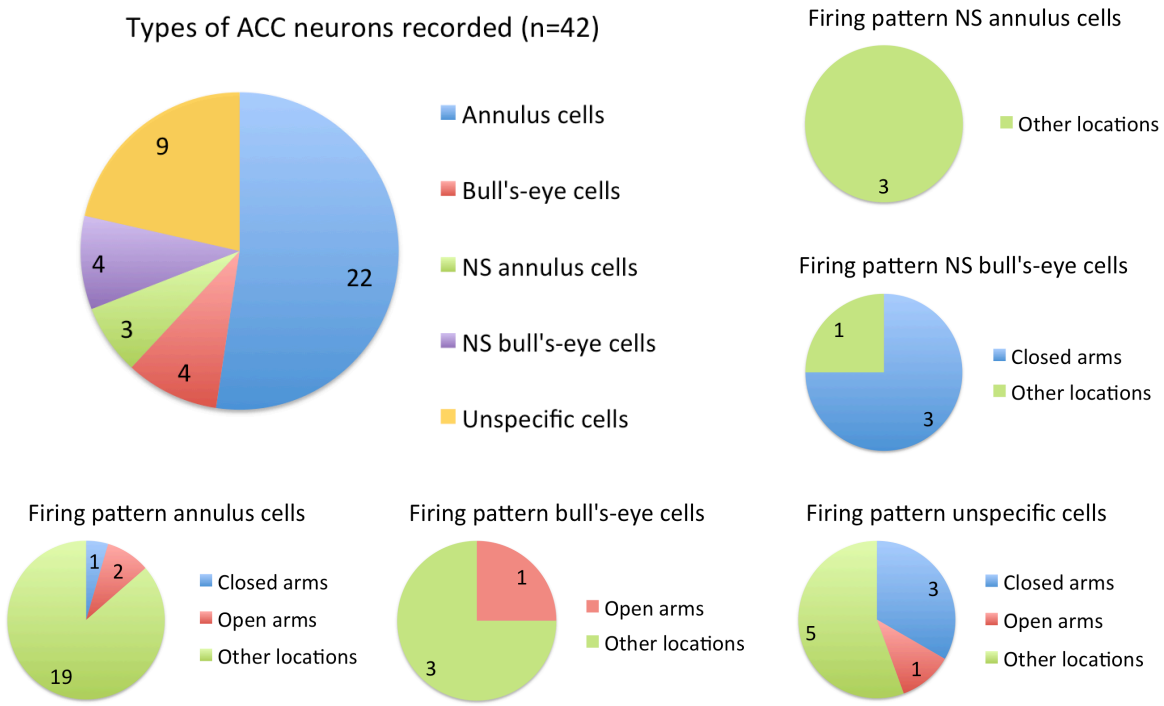

Figure 3.4 Overview of the ACC neurons recorded in the open field including which firing pattern these neurons had on the elevated plus maze. The numbers indicate the number of neurons present in each category. All cells categorized as annulus (n = 22) or bull's-eye (n = 4) cells had a statistically significant ($p < 0.05$) higher firing rate around the perimeter than in the center or in the center than around the perimeter, respectively. The unspecific cells (n = 9) never had significantly different ($p > 0.05$) firing rates between the center and perimeter. The NS annulus (n = 3) and bull's-eye (n = 4) cells had the same characteristics as the annulus and bull's-eye cells in one OF session, and as unspecific cells in another OF session. Of all neurons, 21 (12 annulus; 3 bull's-eye; 3 NS annulus; 1 NS bull's-eye; 2 unspecific) had non-interpretable EPM rate maps because the mice never entered the open arms. These cells were therefore categorized in "other locations" on the EPM.

In this study one annulus cell located its peak firing on the closed arms and could therefore be coding for safety (Fig. 3.5). In addition, one bull's-eye cell located its peak firing on the open arms and could therefore be coding for danger (Fig. 3.6). This strengthened the hypothesis that some annulus cells code for safety and some bull's-eye cells code for danger.

However, of the remaining annulus cells two fired on the open arms, while 19 fired on unspecific places of the EPM. The three remaining bull's-eye cells all fired on unspecific locations. Three NS bull's-eye cells fired in the closed arms of the EPM, while the remaining four NS cells fired in other locations. Of the unspecific cells three fired in the closed arms, one fired in the open arms and five on other locations of the EPM. But, twelve annulus, three bull's-eye, three NS annulus, one NS bull's-eye and two unspecific cells were categorized as firing on other locations on the EPM because these cells came from mice that did not enter the open arms. Hence, these EPM rate maps were un-interpretable and it remains unclear what

these cells could have been coding for. Taken together, the results indicate that although some of the bull's-eye and annulus cells qualified as coding for danger and safety not all did. The danger and safety cells were actually a minority of the total bull's-eye and annulus cells. More specifically, of the annulus cells only 5 % (1/22) qualified as coding for safety while 95 % (21/22) did not, and of the bull's-eye cells 25 % (1/4) qualified as coding for danger while 75 % (3/4) did not.

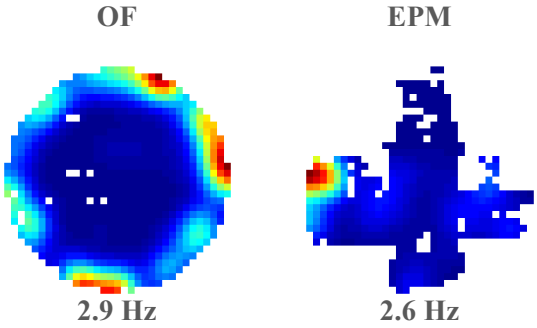

Figure 3.5 Firing patterns of an ACC annulus cell that could code for safety. The neuron fired significantly more around the perimeter than the center in both OF sessions ($p < 0.01$), which is consistent with the firing properties of annulus cells. Because the neuron also located its peak firing in one of the closed (horizontal) arms of the EPM it could code for safety. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

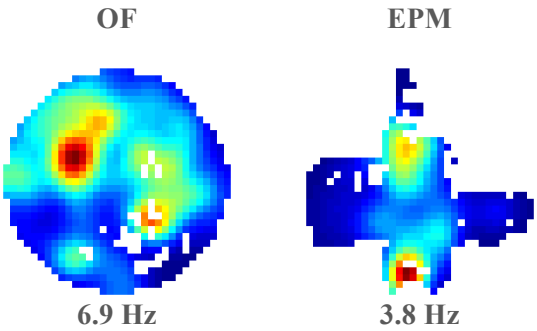

Figure 3.6 Firing patterns of an ACC bull's-eye cell that could code for danger. The neuron fired significantly more in the center than the perimeter in both OF sessions ($p < 0.01$), which is a property that is consistent with bull's-eye cells. The same neuron also had its peak firing located on the open (vertical) arms of the EPM and could therefore be coding for danger. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

3.1.3 Electrophysiological results from the open field and elevated field

The downside with using the EPM to investigate whether annulus cells code for safety is that some annulus cells might code for the wall they are firing around. Therefore, when an annulus cell fires in the closed arms of the EPM we do not know whether the cell is coding for safety or a wall. Similarly, when a bull's-eye cell fire on the open arms we do not know if it is coding for danger or an open space. Therefore some mice were recorded on an elevated field without walls after the open field. If the annulus cells coded for safe locations they should

relocate their peak firing from the perimeter of the OF to the center of the EF. Likewise, if the bull's-eye cells coded for dangerous locations they should relocate their peak firing from the center of the OF to the perimeter of the EF. From three male mice 46 units passed the cluster selection criteria and were used to generate rate maps. Unpaired t-tests of the firing frequencies on unsmoothed rate maps were then used to categorize the neurons on both the OF and EF. The neurons were categorized as "annulus" in either fields if they had a statistically significant ($p < 0.05$) higher firing rate around the perimeter compared to the center, and as "bull's-eye" if they had a significant higher firing rate in the center compared to the perimeter. Neurons categorized as "unspecific", did not have significantly different ($p > 0.05$) firing rates between the center and perimeter. Figure 3.7 gives an overview of the firing properties of the neurons recorded over the two tasks.

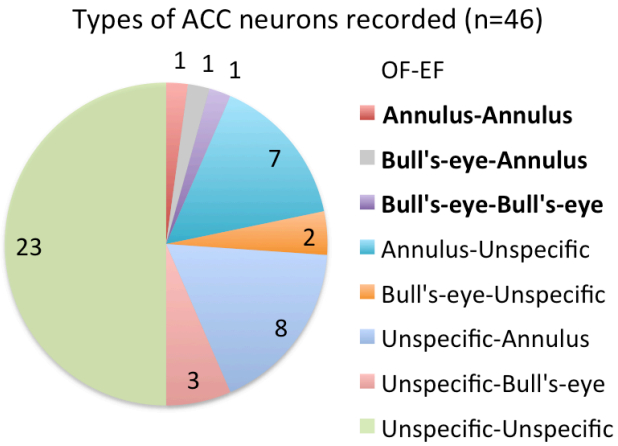

Figure 3.7 Firing properties of ACC neurons recorded on the open- and elevated field. The neurons were categorized according to their firing pattern on both the OF and EF. The numbers indicate the number of neurons present in each category. "Annulus": the neuron had statistically significant ($p < 0.05$) higher firing around the perimeter compared to the center. "Bull's-eye": the neuron had statistically significant higher firing in the center compared to the perimeter. "Unspecific": the neuron had no significant ($p > 0.05$) difference in firing between the center and perimeter. E.g. "bull's-eye-annulus": bull's-eye firing pattern in the OF - annulus firing pattern on the EF. No neurons had a firing pattern that corresponded to "annulus-bull's-eye".

This categorization of the neurons showed that one cell could be coding for danger. This "bull's-eye-annulus" cell fired as a bull's-eye cell on the OF, but relocated its peak firing to the perimeter on the EF (Fig. 3.8). Thus, based on rodent's aversion of open and elevated fields (Montgomery, 1955), it fired in locations considered to be dangerous in both tasks. Because no neurons acted like "annulus-bull's-eye" cells, who relocated their peak firing from the perimeter on the OF to the center of the EF, no neurons were found to be coding for safety in these recordings.

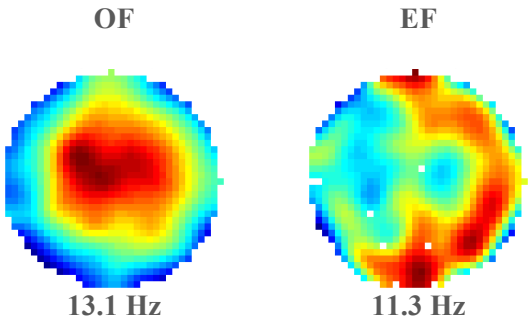

Figure 3.8 Firing patterns of the ACC cell that could code for danger. The neuron had a firing pattern that corresponded to a bull's-eye cell in the OF ($p < 0.01$) and an annulus cell on the EF ($p < 0.01$). More specifically the neuron shifted its peak firing from the center of the OF to the perimeter of the EF, which are both considered to be dangerous locations of the fields. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

Of the remaining 45 neurons one was an "annulus-annulus" cell (Fig. 3.9), which always located its peak firing around the perimeter. This neuron could therefore be a border cell similar to those of the entorhinal cortex as described by Solstad et al. (2008). In this case the border cell coded for the OF wall and EF edge. There was also found one "bull's-eye-bull's-eye" cell, which fired in the center of both fields (Fig. 3.10).

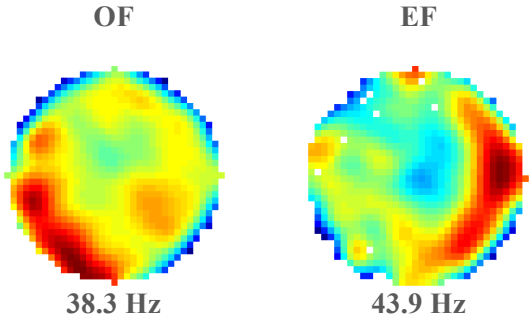

Figure 3.9 The ACC annulus-annulus/border cell. The neuron had a significant higher firing rate around the perimeter compared to the center in both tasks (OF $p < 0.01$, EF $p = 0.01$). This meant that the neuron could be a cell that coded for borders, which in this case were the wall of the OF and edge of the EF. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

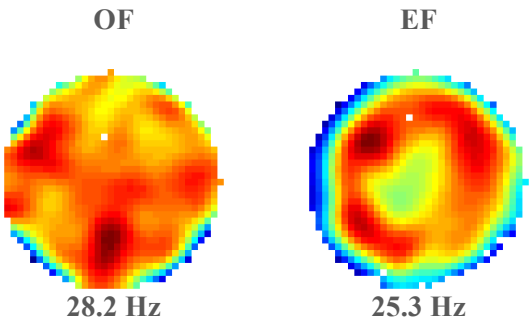

Figure 3.10 The ACC bull's-eye-bull's-eye/center cell. This neuron had a significant higher firing rate in the center compared to the perimeter of both fields (OF $p = 0.01$, EF $p < 0.01$), which meant that the cell could be a center-coding cell. Peak firing frequency indicated in hertz (Hz) and in warmer colors.

The last 43 neurons had insignificant differences in firing between the center and perimeter in either one or both tasks and were therefore categorized as unspecific in one or both tasks. Figure 3.11-3.13 shows examples of what was considered as unspecific cells.

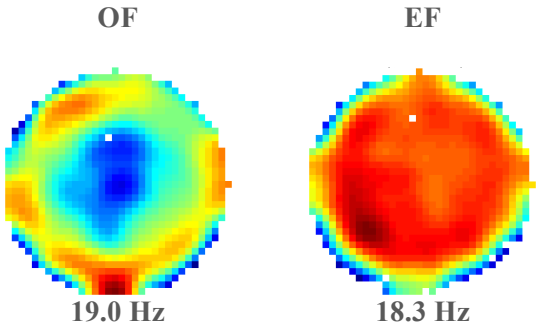

Figure 3.11 ACC annulus-unspecific cell. This neuron appeared to be a cell coding for safety because it shifted its peak firing from the perimeter of the OF to the center of the EF, but the difference in center-perimeter peak firing was only significant in the OF-session (OF $p = 0.01$, EF = 0.54). Peak firing frequency indicated in hertz (Hz) and in warmer colors.

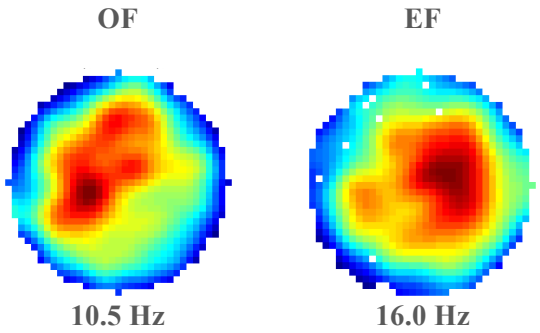

Figure 3.12 ACC bull's-eye-unspecific cell. This neuron appeared to be a bull's-eye-bull's-eye cell because it had its peak activity located in the center of both fields. However, the difference in center-perimeter peak firing was only significant in the OF-session (OF $p < 0.01$, EF = 0.07). Peak firing frequency indicated in hertz (Hz) and in warmer colors.

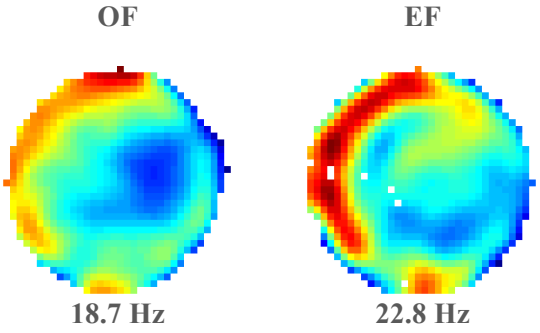

Figure 3.13 Unspecific-unspecific cell. The neuron had a firing pattern similar to an annulus-annulus cell, but the difference in firing between the center and perimeter was not statistically significant in either tasks (OF $p = 0.21$, EF $p = 0.13$). Peak firing frequency indicated in hertz (Hz) and in warmer colors.

Taken together, the OF-EF results showed that only 7 % (3/46) of the all the recorded cells were coding for something specific; danger, borders or center. That is, 93 % did not have a consistent firing pattern over more than one session. However, 50 % (23/46) of the cells never had a specific firing pattern. Thus, from the cells that had a specific firing pattern in at least one of the sessions 13 % (3/23) coded for danger, borders or centers. After analyzing the OF sessions from the OF-EPM recordings it was also shown that the cells could change their firing pattern within the same type of session, e.g. from OF1 to OF2. Therefore we recorded two sessions of both the OF and EF (OF-EF-EF-OF) for two animals to test the stability of the firing patterns. From these sessions 13 neurons were found, but none of them had a significant firing pattern over all four sessions (OF1/OF2-EF1/EF2): ten were OFunspecific-EFunspecific, one OFannulus-EFunspecific, one OFunspecific-EFannulus, one OFunspecific-EFbull's-eye (also included in total count fig. 3.7). Furthermore, 50 % (5/10) of the unspecific-unspecific neurons acted like an annulus or bull's-eye cell in one of the OF sessions or one of the EF sessions (while being unspecific in the remaining three sessions). Therefore, these two-session recordings indicated that one should be cautious about interpreting the results from the single OF and EF, given the 50 % false positive rate.

3.1.4 Cell types recorded on the elevated plus maze, open and elevated field

Table 3.1 and 3.2 summarizes the main findings from the EPM, OF and EF recordings.

Table 3.1 Percentages of functional cell types recorded in the open field and on the elevated plus maze. OF: percentage of total number of cells recorded. Unspecific cells include NS annulus, NS bull's-eye and unspecific cells. EPM: percentages of annulus and bull's-eye cells that qualified as coding for safety and danger, respectively.

	Annulus cells	**Bull's-eye cells**	**Unspecific cells**
OF	52 % (22/42)	10 % (4/42)	38 % (16/42)
EPM	Safety: 5 % (1/22)	Danger: 25 % (1/4)	-

Table 3.2 Percentages of cells that had a specific firing pattern in both the open and elevated field. Of the neurons that had a specific firing pattern in at least one of the sessions (OF or EF) 13 % (3/23) coded for danger, border or center. No neurons were found to be coding for safety.

	Danger cell	**Center cell**	**Border cell**
OF/EF	4 % (1/23)	4 % (1/23)	4 % (1/23)

3.2 The anterior cingulate cortex and cognitive control

3.2.1 Behavioral results from the olfactory- and visual discrimination learning

Previous studies have shown that the ACC is involved in cognitive control. However, this hypothesis is mainly based on lesion studies (e.g. Walton et al., 2009, Rudebeck et al., 2006) and imaging studies (e.g. Milham and Banich, 2005). Therefore, we wanted to record from mice during a cognitive control task to identify electrophysiological correlates of cognitive control in the ACC. We trained the mice to discriminate between three olfactory and three visual cues, where one of each type predicted a food reward in distinct environments. To test cognitive control, both visual and odor cues were presented but only one type could be used to find the reward depending on the environment. The mice therefore had to suppress their response to one type of sensory cue. For summary of the procedures see figure 2.3. This paragraph presents the results from the discrimination learning, while the results from the cognitive control task is presented in paragraph 3.2.2.

A Shapiro-Wilks test ($p > 0.05$) and visual inspection of the normality plots showed that the mean performances were approximately normally distributed, while the chance level and variables within each mean were not normally distributed (Shapiro-Wilks $p < 0.05$). Therefore a SLR analysis, WSR test and MWU test were used to investigate whether the mice had learned to use the cues to guide them to the reward.

In the ODL1 (Fig. 3.14 A; n = 10 males; cues vanilla [= reward], raspberry and lemon) the SLR showed that there was no statistically significant linear relationship ($p > 0.05$) between number of days and number of errors, or between number of days and latency to find the reward. The slopes were estimated to be -0.08 ($p = 0.55$) and -80.50 ($p = 0.27$) respectively, but these were not significant. The WSR test showed no significant difference in number of errors made on day 3 of training compared to the control/day 4, where the target was switched to raspberry (errors 1.2 ± 0.1 and 1.4 ± 0.1, mean ± SEM for day 3 and control respectively, $p = 0.53$), or in latency to find the reward on day 3 compared to the control (time 48 ± 30 s and 49 ± 30 s, mean ± SEM for day 3 and control respectively, $p = 0.94$). In addition, a MWU test showed that the mice made significantly more errors than the chance level (chance level = 1.0, calculations in appendix C) on both day 3 ($p = 0.04$) and on the control ($p < 0.01$). Taken together, this indicates that the mice did not learn to use the olfactory cues to find the reward.

In the VDL1 (Fig. 3.14 B; n = 4 males; cues X [= reward], Δ and □) the SLR also showed that there was no statistically significant linear relationship ($p > 0.05$) between number of days and number of errors, or between number of days and latency to find the reward. The slopes were estimated to be −0.01 ($p = 0.94$) and −14.30 ($p = 0.20$) respectively, but were not significant. The MWU test showed that there was no significant difference between number of errors made on day 4 and the chance level (errors 0.9 ± 0.2 [mean ± SEM] and 1.0 for day 4 and chance level respectively, $p = 0.56$). Hence, the mice did not learn to use the visual cues to find the reward.

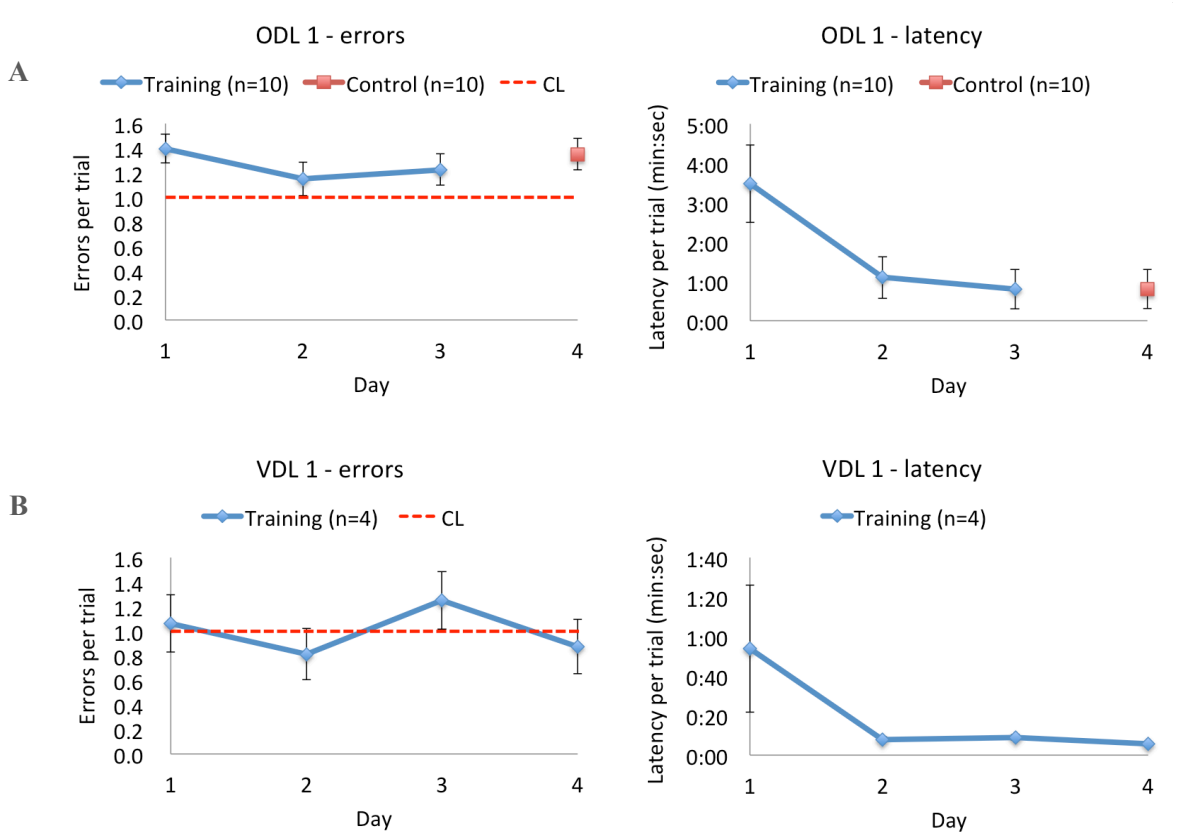

Figure 3.14 Behavioral results (group mean ± SEM) from the olfactory- and visual discrimination learning 1. There was no statistically significant linear relationship ($p > 0.05$) between number of days and errors, or between number of days and latency in either the ODL1 (**A**) or the VDL1 (**B**). There was also no significant difference in latency and errors on day 3 ODL1 (time 48 ± 30 s; errors 1.2 ± 0.1) compared to the ODL1 control (time 49 ± 30 s; errors 1.4 ± 0.1). However, both the ODL1 day 3 errors and ODL1 control errors were significantly larger ($p < 0.05$) than the chance level (1.0, CL). In addition, there was no significant difference in errors made on day 4 of the VDL1 (0.9 ± 0.2) compared to the chance level. All these factors indicated that the mice did not learn to use the cues in the ODL1 or VDL1.

Based on the outcome of the ODL1 and VDL1 we realized that the learning protocols had to be changed. Therefore we prolonged the learning period and covered the rewards with wood

chips. Digging in wood chips meant that the mice had to make a higher effort to find the reward, which could contribute to focusing more and eventually learning the difference between the cues. A pilot study investigating if covering the reward with wood chips would make the mice use the cues was performed. The results (Fig. D.1, appendix D) indicated that use of wood chips, in fact, made the mice focus more on the cues.

The SLR showed that there was a statistically significant negative linear relationship between number of days and errors per trial, and between number of days and latency to find the reward in the ODL2 (Fig. 3.15 A; n = 4 males, n = 8 females). The slopes were estimated to −0.07 ($p < 0.01$) and −11.04 ($p < 0.01$) respectively, and were found to be significant. This showed that the mice improved their performance, but it did not test if they actually used the cues. Therefore, they were presented with a control experiment ("ODL2 control") where the target scent was switched (from vanilla to raspberry). The WSR test showed that the number of errors and latency on ODL2 day 10 was significantly different from the errors and latency on the ODL2 control (errors 0.3 ± 0.1 and 1.0 ± 0.1 [$p < 0.01$]; time 11 ± 1 s and 35 ± 11 s [$p = 0.04$], mean ± SEM for day 10 and control, respectively). In addition, the MWU test showed that there were made significantly fewer errors on day 10 ODL2 compared to the chance level (chance level = 1.0, calculations in appendix C, $p < 0.01$), while errors made on the control were not significantly different from chance level ($p = 1.00$). Taken together, these results indicate that the mice did learn to use the olfactory cues to guide them to the reward.

In the VDL2 (Fig. 3.15 B; n = 2 males, n = 2 females) the SLR showed that there was no statistically significant linear relationship between number of days and errors to find the reward, or between number of days and latency. The slopes were estimated to −0.04 ($p = 0.11$) and −0.81 ($p = 0.09$) respectively, but were not significant. However, there was a trend towards better performance. The WSR test showed that day 10 was not statistically significant different from the control (target switched from X to Δ), either with regards to number of errors or latency (errors 0.1 ± 0.1 and 0.4 ± 0.1 [$p = 0.16$]; time 8 ± 1 s and 12 ± 4 s [$p = 0.19$], mean ± SEM for day 10 and control, respectively). But, the MWU test showed that the number of errors made on day 10 was significantly smaller than chance level ($p < 0.01$), which suggested that the mice did not pick cups randomly. However, the control was also significantly smaller than the chance level ($p < 0.01$), which indicated that they did not use the visual cues to find the reward.

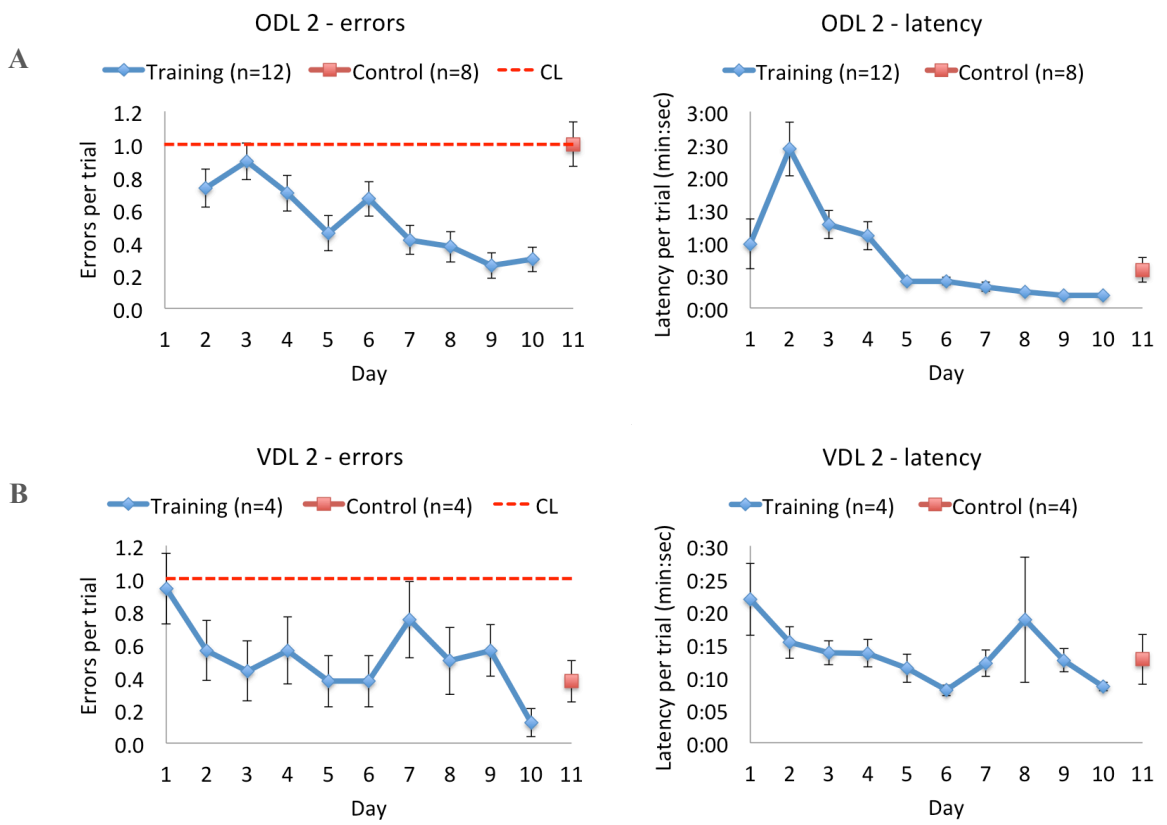

Figure 3.15 Behavioral results (group mean ± SEM) from the olfactory- and visual discrimination learning 2. A) ODL2: There was a statistically significant ($p < 0.05$) negative linear relationship between number of days and errors (slope: −0.072), and between number of days and latency (slope: −11.042). Day 10 was also significantly different from the control (day 11) both with regards to errors (day 10: 0.3 ± 0.1, control: 1.0 ± 0.1) and latency (day 10: 11 ± 01 s, control: 35 ± 11 s). In addition, the errors made on day 10 were significantly different from the chance level (1.0, CL) while the errors made on the control were not. **B)** VDL2: there was no statistically significant linear relationship ($p > 0.05$) between number of days and errors to find the reward, or between number of days and latency. Day 10 was not significantly different from the control, either with regards to number of errors (day 10: 0.1 ± 0.1, control: 0.4 ± 0.1) or latency (day 10: 8 ± 1 s, control: 12 ± 4 s). In addition, both the number of errors made on day 10 and errors made on the control was significantly smaller ($p < 0.01$) than the chance level. Hence, the results indicated that the mice used the cues in the ODL2 but not in the VDL2.

Based on these results a group of mice proceeded to the CCT2. For optimal performance on this test the mice should have learned to use the visual cues. However, one part of the test could still work because they learned the olfactory cues, which acted as distractors in the visual chamber. Before moving on to the CCT the mice's performance in the olfactory task was investigated to ensure that they still remembered to use vanilla as the target cue, after the ODL2 control and the 11 days of VDL2. These results (Fig. D.2, appendix D) indicated that they still used vanilla as the target. Despite poor performance on both the ODL1 and VDL1 one group also proceeded to the CCT1.

3.2.2 Behavioral results from the cognitive control test

Some of the mice that had completed both the ODL and VDL proceeded to the CCT. Depending on if the mice had been trained in the ODL/VDL 1 or 2 they were tested in the CCT 1 or 2 respectively, for detailed procedures see figure 2.3. In short ODL/VDL 1 had three/four days of training with no addition of wood chips in the cups, while ODL/VDL 2 had ten days of training with addition of wood chips in the cups. The CCT results were divided in mean performance in olfactory chamber (visual distractors), visual chamber (olfactory distractors) and complete test (mean performance in both chambers). This was done to be able to compare the performance in both CCT chambers with the last day of training in the discrimination learning (e.g. CCT1 olfactory chamber with day 3 ODL1), and to compare the complete test with the chance level. The CCT was considered to work when the mice made significantly more errors in the CCT olfactory/visual chamber than the last day of training but at the same time made significantly fewer errors than chance level on the complete test.

A Shapiro-Wilks test ($p < 0.05$) and visual inspection of the normality plots showed that the variables within each mean and the chance level were not normally distributed. Therefore, a WSR test was used to compare the performance in the CCT with the performance on the last day of training in the discrimination learning, while a MWU test was used to compare the CCT performance with the chance level.

In CCT1 (Fig. 3.16; n = 4 males) there was no significant difference between the errors made in the olfactory chamber compared to errors on day 3 ODL1 (errors 1.1 ± 0.2 and 1.2 ± 0.1, mean ± SEM for olfactory chamber and day 3 respectively, $p = 0.47$), or olfactory chamber compared to the chance level (1.0, $p = 0.76$); between the errors made in the visual chamber compared to errors on day 4 VDL1 (errors 0.7 ± 0.2 and 0.9 ± 0.2, mean ± SEM for visual chamber and day 4 respectively, $p = 0.35$), or visual chamber compared to the chance level ($p = 0.18$); or between the chance level and complete test errors (0.9 ± 0.2, $p = 0.36$). However, the mice spent significantly less time in the CCT olfactory chamber compared to day 3 ODL1 (time 6 ± 1 s and 48 ± 30 s, mean ± SEM for olfactory chamber and day 3 respectively, $p = 0.03$), while there was no significant difference in time spent in the visual chamber compared to day 4 VDL1 (time 4 ± 1 s and 6 ± 1 s, mean ± SEM for visual chamber and day 4 respectively, $p = 0.23$). The results indicate that the mice did not use the cues to find the reward, and that the CCT1 was not working. This was not unexpected considering the

results from the ODL1 and VDL1 already suggested that the mice did not use the cues to find the reward. Therefore, no recordings were conducted in the CCT1.

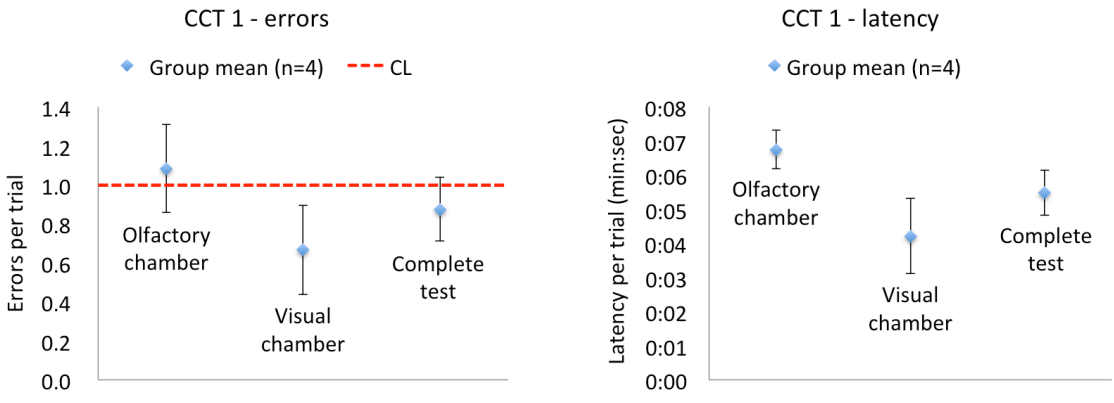

Figure 3.16 Performance (group mean ± SEM) of the mice in the cognitive control test 1. There was no significant difference between the chance level (1.0, CL) and the mean errors in the olfactory chamber (1.1 ± 0.2), visual chamber (0.7 ± 0.2) or complete test (0.9 ± 0.2, mean performance in both chambers). There was also no significant difference between olfactory and visual chamber errors and the errors made on the last day of training in the ODL1 (1.2 ± 0.1) and VDL1 (0.9 ± 0.2). The mice spent significantly less time in the CCT olfactory chamber (6 ± 1 s) than on day 3 ODL1 (48 ± 30 s), while they did not have a significant difference in time in the visual chamber (4 ± 1 s) and day 4 VDL1 (6 ± 1 s). These results indicated that the CCT1 did not work.

In the CCT2 (Fig. 3.17; n = 2 males, n = 2 females) the number of errors made in the olfactory chamber was not significantly different from the errors made on day 10 ODL2 (errors 0.4 ± 0.2 and 0.3 ± 0.1, mean ± SEM for olfactory chamber and day 10 respectively, $p = 0.74$), but was significantly lower than the chance level (1.0, $p = 0.01$). However, significantly more errors were made in the visual chamber than on day 10 VDL2 (errors 0.7 ± 0.2 and 0.1 ± 0.1, mean ± SEM for visual chamber and day 10 respectively, $p = 0.04$). In addition, the visual chamber errors were not significantly different from the chance level ($p = 0.18$). The complete test errors (0.5 ± 0.1) were significantly lower than the chance level ($p < 0.01$). The WSR tests also showed that there was no significant difference between the latency in the olfactory or visual chamber and day 10 ODL2 or VDL2, respectively (time 16 ± 7 s and 11 ± 1 s [$p = 0.33$]; 13 ± 2 s and 8 ± 1 s [$p = 0.12$], mean ± SEM for olfactory chamber and day 10 ODL2; visual chamber and day 10 VDL2, respectively). The fact that the mice did not learn to use the cues in the VDL2 had impact on the CCT2 olfactory chamber, because they did not get confused when the visual cues acted as distractors (no significant change in CCT2 olfactory chamber and day 10 ODL2). However, the mice got confused when the olfactory cues acted as distractors in the CCT2 visual chamber. This was most likely a result of the reward being paired with a new scent (vanilla never paired with target visual cue

X in the CCT: paragraph 2.3.2 *Cognitive control test*), because the mice did not learn to use the visual cues in the VDL2. The complete test errors were significantly below the chance level, but this was a result of the mice not getting distracted in the olfactory chamber. If the test had worked properly there should have been a significant change between the last day of training and the CCT olfactory/visual chamber, but at the same time the complete test errors should have been below the chance level. Because the test did not work properly no recordings were made in the CCT2.

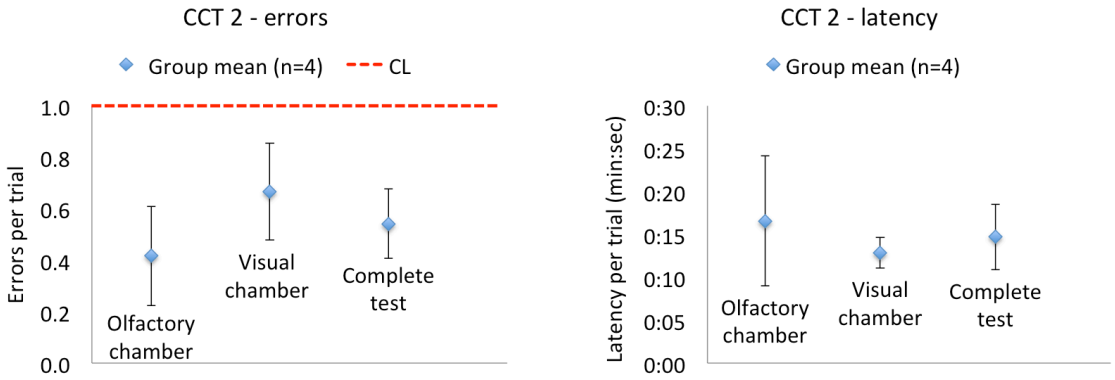

Figure 3.17 Performance (group mean ± SEM) of the mice in the cognitive control test 2. Only the number of errors made in the olfactory chamber (0.4 ± 0.2) and the number of errors made on the complete test (0.5 ± 0.1, mean performance in both chambers) was significantly lower ($p < 0.05$) than the chance level (1.0, CL). The errors made in the olfactory chamber were not significantly different from day 10 ODL2 (0.3 ± 0.1), which is consistent with being significantly lower than chance. The errors made in the visual chamber (0.7 ± 0.2) were significantly higher than the errors made on day 10 VDL2 (0.1 ± 0.1) and not significantly different from the chance level. On this test the mice did not spend significantly different amounts of time in the olfactory chamber (16 ± 7 s) and day 10 ODL2 (11 ± 1 s), or in the visual chamber (13 ± 2 s) and day 10 VDL2 (8 ± 1 s). Altogether, this indicated that the CCT2 did not work properly.

3.2.3 Electrophysiological results from olfactory discrimination

Studies have shown that cells in the ACC code for physical objects (Weible et al., 2012, Weible et al., 2009). In addition, it has been shown that certain parts of the brain, as the hippocampus, contain cells that code for physical qualities as scents (Muzzio et al., 2009). Because the hippocampus projects to the ACC (Hoover and Vertes, 2007), we wanted to investigate if ACC neurons also could code for these physical qualities. Two male mice were recorded from in this task. Here they performed four 10-minute trials of the olfactory discrimination task with wood chips added in the cups (for reference see paragraph 2.3.4). Six units passed the selection criteria, but none of these had a firing pattern that could resemble coding for a specific scent.

Chapter 4

Discussion

4.1 Summary of main findings

4.1.1 The elevated plus maze, open field and elevated field

The EPM setup 3 with black walls and bright lights was found to be functional and was therefore used in all EPM recordings. The majority of cells recorded in all experiments had unspecific firing patterns, which suggests that the ACC is an integrating structure that fires in response to input from other brain areas. However, a minority of the cell count also had specific firing patterns of which some qualified as coding for safety and danger. This suggests that the ACC also has specific functions. The open field recordings from the OF-EPM experiment yielded 42 units of which 52 % (22/42) were categorized as annulus cells, 10 % (4/42) as bull's-eye cells and 38 % (16/42) as unspecific cells. Furthermore, the firing pattern of these neurons on the EPM showed that 5 % (1/22) of the annulus cells qualified as coding for safety, while 25 % (1/4) of the bull's-eye cells qualified as coding for danger. In the OF-EF recordings 50 % (23/46) of the cells always had an unspecific firing pattern. Therefore, of the cells that had a specific firing pattern in at least one session 13 % (3/23) coded for something specific: 4 % (1/23) qualified as coding for danger, 4 % (1/23) for borders and 4 % (1/23) for centers. No neurons were found to be coding for safety in the OF-EF recordings. See also table 3.1 and 3.2 in paragraph 3.1.4.

4.1.2 Discrimination learning and cognitive control task

The mice did not learn to use the olfactory cues in the ODL1 or the visual cues in the VDL1. They did learn to use the olfactory cues in the ODL2, but not the visual cues in VDL2. Because they did not learn to use all cues the CCT1 and 2 became non-functional and therefore no recordings were conducted in the CCT. Two animals were recorded in the ODL2 but no scent coding neurons were found.

4.2 Methodological considerations

Because of the small sample sizes in the described experiments the results become inconclusive. Nevertheless, the results may show a trend of the population's response but more data is required to confirm this. The use of both female and male mice in the experiments may also give inconclusive data because male and female mice respond

differently to different tasks (Walf and Frye, 2007, Zhu et al., 2006, Bothe et al., 2005). This means that they might respond differently to being placed on the OF, EF and EPM or learning the olfactory and visual tasks. For example, female mice have shown more exploratory behavior in the OF compared to male mice (Bothe et al., 2005). In addition, female mice with low estradiol and progestin levels spend less time on the open arms of the EPM compared to males. But female mice with high estradiol and progestin levels spend more time on the open arms of the EPM compared to both males and females with low levels of estradiol and progestin (Walf and Frye, 2007). Therefore, the use of females may not tell us anything about the male part of the population and vice versa.

Otherwise, data from behavioral experiments are often hard to replicate and compare across laboratories because small differences in protocols may have large effects on the animal's behavior. A large variety of factors affect the behavior of the animals, from housing conditions and handling from laboratory technicians to experimental settings and conditions of the experimental room e.g. noise, humidity, smells (Deacon, 2006, Zhu et al., 2006). Measures were taken to handle all individuals as similar as possible, but experience showed that the mice had different characteristics despite being genetically identical. Therefore, some mice needed more handling prior to the experiments in order to be calm in the presence of the experimenter. However, during experimental sessions all animals were handled in the same way.

In these tasks, all 15 male and 10 female mice were single housed. Single housing is considered a stressor that might cause anxiety and depression in mice, which could further affect their behavior in the current study (Walf and Frye, 2007). However, because the mice were food restricted during the ODL, VDL and CCT they had to be single housed. This was to avoid food competing behavior that could result in less dominant mice getting less food than the minimum allowance set by the Norwegian Food Safety Authority. Studies show that male mice are less affected than female mice in being single housed (Hunt and Hambly, 2006). The first week after separating the female mice they were therefore group housed for an hour a day in an attempt to gradually habituate them to isolation. The male mice were not regrouped the first week after separation as this could cause territorial fighting (Deacon, 2006).

Other important considerations when conducting different behavioral experiments is the order of testing as different tasks may affect each other, e.g. use of fear conditioning may affect exploration in other tasks (Bothe et al., 2005). At the same time one might consider doing experiments that test natural anxiety at an early stage when the mice are most anxious, and test cognitive abilities at a later stage when the mice have become more habituated (Deacon, 2006). In the behavioral experiments the order of testing was: EPM → ODL → VDL → CCT, while in the recording experiments the order was OF → EPM or EF (→ ODL2). However, all animals were handled extensively prior to the start of the experiments in order to diminish anxious behavior towards the experimenter. Hence, the measured anxiety depended mainly on the setup of the apparatus. A study by Lapin (1995) have also showed that handling of mice prior to EPM exposure do not affect behavior on the maze, but the author also argues that different types of handling might result in sensitization or habituation of anxious responses. Further on, higher habituation rates have been positively correlated with learning abilities of mice (Light et al., 2011).

4.3 Safety and danger coding neurons in the anterior cingulate cortex

4.3.1 Behavioral results

The data from these experiments were hand scored, meaning that the data is not as precisely scored as in experiments where automated data programs scores the behavior. However, in this experiment we were only looking for the functional setup where mice had significantly more time and entries on the closed than open arms, and not the effect of some given anxiogenic or anxiolytic agent where the error margins are smaller. This was also the reason why other anxiety signs (e.g. rears, head dips, defecating, stretch-attend posture; Walf and Frye, 2007) were not counted. The data showed that the mice did not have a preference for the closed arms in setup 1 where both the open and closed arms were dimly lit, or in setup 2 where the open arms were dimly lit and the closed arms were dark. In setup 3 where the open arms were brightly lit and the closed arms were dark they had a preference for the closed arms. Hence, there was a bigger safe-danger difference between the open and closed arms in setup 3 than in setup 1 and 2. This is consistent with literature showing that mice prefer darker to brighter locations (Deacon, 2006, Wax and Goodrick, 1975). Nevertheless, the results are both in agreement and disagreement with previous EPM studies. Certain studies show that anxiety effects on the EPM, i.e. open arm aversion and closed arm preference, is dependent on levels of illumination (e.g. Lee and Rodgers, 1990), while others show that the anxiety effects are independent of levels of illumination (e.g. Jones and King, 2001, Pellow et

al., 1985). But as mentioned in paragraph 4.2, behavioral data is hard to compare across laboratories because other factors may also contribute in anxiety effects on the EPM.

In the non-functional setups 1 and 2 only male mice were used, while in the functional setup 3 one male and six females were used. As mentioned in paragraph 4.2, the females may have affected the EPM results. However, the male mouse in setup 3 showed the same preference for the closed arms as the female group (male time 195 s and 40 s, entries 8 and 5 for closed and open respectively; females mean time 177 s and 42 s, entries 17 and 7 for closed and open respectively). Observations of male mice exploring setup 3 during the electrophysiological recordings also showed that these mice preferred the closed arms. The behavioral results from the electrophysiological recordings are not included in the functional setup results due to different handling procedure prior to the experiment and the mice being connected to recording equipment during exploration of the maze.

4.3.2 Recordings on the open field and elevated plus maze

The idea behind these recordings was if bull's-eye cells, which fire in the center of the OF, also fired on the open arms of the EPM they could code for dangerous places. Conversely, if annulus cells, which fire around the perimeter of the OF, also fired in the closed arms of the EPM they could code for safe places. The open field recordings yielded 42 units of which 52 % qualified as annulus cells, 10 % qualified as bull's-eye cells and 38 % qualified as unspecific cells. The percentage of bull's-eye cells is in agreement with previous studies showing small populations of bull's-eye cells in the ACC. After recording two OF sessions, Weible et al. (2009) categorized 13 % (12/95) of the recorded cells as bull's-eye cells, while Weible et al. (2012) categorized 8 % (22/281) of the recorded cells as bull's-eye cells. However, the percentages of annulus and unspecific cells are not in agreement with Weible et al. (2012) where only 4 % (11/281) of the cells were annulus cells and the remaining 88 % (248/281) were unspecific with regards to the OF. One can imagine that these numbers will even out when recording more cells, as this study recorded only a small proportion of ACC neurons compared to Weible et al. (2012). More specifically, this study recorded approximately 15 % (42/281) of the number of cells recorded by Weible et al. (2012).

Of the annulus cells recorded in the current study 5 % qualified as coding for safety, while 25 % of the bull's-eye cells qualified as coding for danger. Hence, this study shows that the danger and safety coding cells are a minority of the total bull's-eye and annulus cell count.

Admittedly, no statistics were performed on the EPM rate maps due to limited time to make a custom MATLAB code that were able to make and perform statistics on a plus shaped matrix. Nevertheless, the results show a trend towards danger and safety coding bull's-eye and annulus cells. There is also reason to believe that certain neurons in the ACC code for danger and safety because of a number of studies showing that the ACC is involved in affective coding (for reviews see Etkin et al., 2011, Shackman et al., 2011, Bush et al., 2000), this is further discussed in paragraph 4.3.4.

The EPM and OF tasks can also be connected to a cost-benefit paradigm in addition to safety and danger. That is, the EPM and OF have safe and dangerous zones and because mice have a natural desire to explore novel environments (Lister, 1987) these tasks could elicit cost-benefit paradigms. Because the ACC has been shown to be involved in cost-benefit decision-making (Walton et al., 2009, Kennerley et al., 2006, Rudebeck et al., 2006), the firing correlates of bull's-eye and annulus cells seen on the EPM and OF could also correlate to cost-benefit decision-making in addition to safety and danger.

4.3.3 Recordings on the open field and elevated field

Because the "safety annulus" cells fired both around the perimeter/wall of the OF and in the closed EPM arms we do not know if this cell codes for safety or walls/physical boundaries. Similarly, we do not know if the "danger bull's-eye" cells fired in the center and on the open arms because it was coding for danger or an open space. Therefore the firing properties of the annulus and bull's-eye cells were investigated on an open field without walls. Again, based on the mice's natural aversion for open and elevated spaces (Montgomery, 1955) the safety coding annulus cells should relocate their peak firing from the perimeter of the OF to the center of the EF, while the danger coding bull's-eye cells should relocate their peak firing from the center of the OF to the perimeter of the EF.

In this study 93 % (43/46) of the recorded cells did not have a consistent firing pattern over more than one session, which is consistent with previous studies showing that 88 % of the ACC neurons recorded in the OF have unspecific firing patterns (Weible et al., 2012). 50 % (23/46) of the cells recorded in this study had a specific firing pattern in at least one session, and of these 13 % (3/23) had specific firing patterns in both the OF and EF of which approximately 4 % (1/23) had the OF-EF firing pattern that could correspond to coding for danger. Therefore, of the four OF bull's-eye cells one could code for danger. Meaning that

25 % (1/4) of the OF bull's-eye cells recorded could be classified as "danger bull's-eye cells". This is consistent with the OF-EPM results also showing that 25 % (1/4) of the bull's-eye cells coded for danger.

The remaining 9 % of the cells with specific firing patterns in both fields had firing patterns that resembled coding for borders (≈ 4 %, 1/23) and centers (≈ 4 %, 1/23). Border cells have previously been shown in the medial entorhinal cortex, parasubiculum (Solstad et al., 2008) and claustrum (Jankowski and O'Mara, 2015), where they fired in response to an environmental border (wall or edge) independent of shape and size. Further on, a study by Sugar and Witter (2016) have suggested that the RS is important for the development of border cells in the parahippocampal region. ACC is reciprocally connected to the RS (Jones et al., 2005), entorhinal cortex (Jones and Witter, 2007) and claustrum (Zingg et al., 2014), which can suggest a role of the ACC in coding for borders. Another fact that supports the hypothesis about the ACC in border coding is that the (safety) annulus cells reported in the OF-EPM results all fired in close proximity to environmental borders. In addition, the annulus cells reported by Weible et al. (2012) increased their firing around objects placed in an OF. This is a property similar to border cells of the medial entorhinal cortex and parasubiculum that increase their firing relative to newly placed borders in an OF (Solstad et al., 2008). Interestingly, Jankowski and O'Mara (2015) reported both border, object and place cells in the claustrum. The border and object cells have also been described in the ACC, i.e. annulus and object cells (Weible et al., 2012), while place cells have been reported in the hippocampus (Moser et al., 2008). Because these structures are connected to each other (Zingg et al., 2014, Jones and Witter, 2007), the fact that the mentioned properties appear in several of these structures may suggest that the ACC, claustrum and hippocampus are cooperating in decoding this spatial information.

Taken together, the OF-EF results showed that only 7 % (3/46) of the all the recorded cells were coding for something specific; danger, borders or center. However, when two animals were recorded in two sessions of both the OF and EF the results indicated a 50 % false positive rate of the firing patterns of the ACC neurons. Therefore one should be careful about interpreting the single OF-EF sessions.

Is the center of the elevated field safe?

No neurons were found to be coding for safety in the OF-EF recordings. A reason might be that the EF only has a small or non-existing safe zone because mice fear elevated and open spaces (Montgomery, 1955). If both the center and perimeter of the EF could be considered dangerous, the recorded bull's-eye-bull's-eye/center cell would also be a danger-coding cell, which would further strengthen the hypothesis that the bull's-eye cells could code for dangerous locations. Also, if both the center and perimeter were dangerous locations on the EF, one could hypothesize that the border between these areas (the intermediate zone) would be the safest. In this respect, it is interesting that the EF firing pattern of the OF-EF center cell (fig. 3.10) resembles an intermediate zone cell, although this was not statistically significant. However, it might have been significant if the EF had a larger diameter. Therefore, future studies investigating safety-coding cells on the EF are encouraged to use a larger EF and include the intermediate zone in their data analysis.

4.3.4 The anterior cingulate and safety and danger coding summarized

The ACC is involved in affective coding (Bush et al., 2000) and because safety and danger are affective aspects of an environment there is also reason to believe that the ACC is involved in safety and danger coding. This coding might arise from the connectivity between the ACC and several structures that are involved in processing different aspects of affective stimuli. Examples of these structures are the amygdala, nucleus accumbens, insula and hypothalamus (Rolls, 2015, Bissiere et al., 2008). The results discussed in the previous paragraphs also support the hypothesis that the ACC is involved in safety and danger coding.

The majority of cells recorded in both the OF-EPM and OF-EF experiments had an unspecific firing pattern in one or more sessions, in addition to the OF-EF recordings showing a 50 % false positive rate. Furthermore, of the total neurons recorded 31 % (27/88) had an unspecific firing pattern in one session and a specific firing pattern in another session. This could mean that there is a possibility that some ACC neurons are "soft-wired" pluripotent cells that show different firing patterns depending on each environment (similar to the place cells of the hippocampus; Moser et al., 2008). In addition, 33 % (29/88) had specific firing patterns in both sessions. Therefore there is also a possibility that other ACC neurons, e.g. the bull's-eye and annulus cells, are more "hard-wired" showing the same firing pattern over several distinct environment (similar to the grid cells of the entorhinal cortex; Moser et al., 2008). This could mean that the ACC both integrates information coming from other cortical structures and

therefore codes information in different cells each time, and have some specific functions where information is coded in the same cells every time.

4.4 The involvement of the anterior cingulate cortex in cognitive control

4.4.1 Behavioral results from olfactory- and visual discrimination learning

Learning procedure 1

The mice did not learn to use the olfactory and visual cues in the ODL1 and VDL1. This is not consistent with previous studies showing that mice learn to associate olfactory cues with a reward within 12 trials and visual cues with a reward within 16 trials (Matzel et al., 2011, Kolata et al., 2007, Matzel et al., 2003). The reason that those mice learned to use the two types of cues, while the mice in the current study did not may depend on a variety of factors. As described in paragraph 4.2 many factors, from handling to experimental settings, can affect the behavior and therefore learning abilities of mice (Deacon, 2006). Moreover, the former studies used CD-1 mice, while the current study used C57BL/6 mice. These mice have genetically different background that may affect their innate behavior. For example, C57BL/6 mice are more susceptible to cage induced stereotypical behavior (e.g. bar-mouthing and circling the cage) than CD-1 mice. Further on, this stereotypical behavior may influence their behavior in different tasks, including exploratory behavior, novelty seeking behavior and choice based behavior (Novak et al., 2016). Another reason might be that the low number of cups without any barrier hiding the reward makes guessing too efficient compared to learning the olfactory and visual cues. Hence, it would be easier for the mice to guess where the reward was located, rather than making an effort to learn the cues.

Learning procedure 2

In the ODL2 the mice learned the olfactory cues over ten days when wood chips was added in the food cups. This is consistent with the study by Muzzio et al. (2009), which the current study's procedure was based on. This means that the addition of wood chips in the food cups and prolongation of the learning period reinforced the learning of the mice. The pilot study investigating whether wood chips would help the mice learn the cues (Appendix D: supporting behavioral results for the ODL) also showed a trend towards learning the cues over three days (12 trials) with addition of wood chips in the cups. By digging in wood chips the mice had to invest greater effort to find the reward compared to when the reward was exposed in the cups. Animals rarely want to invest greater effort to retrieve the same reward as they get when investing low effort (Brosnan and de Waal, 2014, Walton et al., 2003).

Therefore one can hypothesize that the mice learned the cues because they did not want to invest a greater effort than necessary (by digging in the non-target cups) to retrieve the reward. With regards to gender of the mice, Muzzio et al. (2009) used only male C57BL/6 mice. Here, a combination of male (n = 4) and female (n = 8) C57BL/6 mice were used. Because the mice in both studies learned the cues, the gender of the mice does not seem to have impacted these learning tasks. This is not in agreement with Walf and Frye (2007), Zhu et al. (2006) and Bothe et al. (2005) that have found large effects of gender on behavioral studies.

In the VDL2 the mice did not learn to use the visual cues, as there was no significant linear relationship between number of days and number of errors. The small sample size may have affected this because days 7-9 are outliers with regards to the trend towards learning. Thus, training an additional group of mice may have resulted in a significant negative relationship. Conversely, both the performance on the last day of training and the control was significantly below chance level. The fact that the last day of training was significantly below chance level suggests that the mice actually learned the cues. But the control also being less than the chance level indicated that the mice used some other cue(s) than the visual cues to find the reward. Because the ODL and VDL setups were exactly the same except for the chamber color and the cues, and because the mice learned the olfactory cues in the ODL2, these other cues would have to be easier to learn than the visual cues but harder than the olfactory cues. There were some slight differences between the target and non-target cups that may have been used as cues: 1) the non-target cups, but not the target cup, had wire mesh under the wood chips. Therefore, it can be hypothesized that the mice could smell or feel the wire mesh in the two non-target cups. When the mice investigated the cups before they started digging they sometimes placed their paws on the edge of the cup. If they did this on the non-target cups the wood chips would have been stable as a result of being placed on top of the wire mesh, while on the target cup the wood chips would start to slide downwards under their paws; 2) sticky tack was used to fasten the cues on or above all cups but in the non-target cups extra sticky tack was used to fasten the wire mesh. The sticky tack used to fasten the wire mesh was changed every day, while the one used to fasten the visual cues was not. Therefore another cue that the mice could have used to locate the reward is the smell of the sticky tack in the non-target cups. Especially smells are likely to form cues as mice to a large degree are driven by their sense of smell (Kolata et al., 2007).

Why do the mice never learn the visual cues?

The learning of the visual cues is dependent on the mice's visual competence. The results discussed in the previous paragraphs showed that the mice never learned to use the visual cues to guide them to the reward. For the group of mice performing learning procedure 2 this means that the mice did not learn to use the visual cues despite already having learned the olfactory cues in a very similar task. However, this could be expected as mice are more driven by their sense of smell than their vision (Kolata et al., 2007). The C57BL/6 mouse has also been shown to be susceptible to eye abnormalities and poor vision that may arise as early as birth (Jackson-Laboratory, 2016). This could have led to the mice not being able to distinguish between the gray shapes of the visual cues and the white walls of the visual chamber. Still, mice have previously been shown to learn visual cues. In successful visual cue trainings Kolata et al. (2007) used complex black and white visual cues, while Matzel and Kolata (2010) used different colored led lights to compose the visual cues. These cues could therefore have been more prominent than the current study's cues, which may have enhanced the discrimination learning. The mentioned studies also used CD-1 mice. Based on the efficacy of this mouse in learning both the olfactory and visual cues in the previous studies (without wood chips added in the cups), the CD-1 mouse appears to be a better model than the C57BL/6 mouse for learning discrimination tasks.

4.4.2 Behavioral results from cognitive control task

Neither the CCT1 nor CCT2 worked. The tasks are considered to work if the mice make significantly more errors in the chambers compared to the last day of training, but still perform significantly below the chance level. Performing on the chance level implied that the mice guessed where the reward was placed.

It was not unexpected that the CCT1 did not work, as the mice did not learn to use the olfactory or visual cues. Nonetheless, the mice did spend significantly less time before finding the reward in the CCT1 olfactory chamber compared to the last day of training in the ODL1. This indicates that the mice have become more efficient and learned the task, i.e. learned that there is an accessible treat in one of the cups, without necessarily having learned the cues. Hence, in this case this finding means that they had become more efficient in guessing where the reward was placed.

Because the mice learned the ODL2 cues and associated some cue(s) with the reward in the VDL2 the CCT2 could still work as mixing up the cues could still confuse them. One could argue that the mice could have forgotten to use vanilla as the target scent after the ODL2 control and the 11 days of VDL2. Therefore, the performance of the mice was tested before moving on to the CCT. These results showed that the mice in fact still used vanilla as the target scent (see appendix D: supporting behavioral results for the ODL). The results from the CCT2 showed that adding the visual cues in the olfactory chamber did not impact the performance of the mice, further indicating that the mice did not learn the visual cues. Adding olfactory cues in the visual chamber did, however, confuse the mice. But as the number of errors was not significantly different from the chance level this implied that the mice only guessed where the reward was placed (similar to the ODL control as vanilla was paired with the non-target visual cues). Hence, there was no guarantee that selective attention guided the mice to the reward. Because the CCT was not working, no recordings were conducted in this task.

4.4.3 Recordings from olfactory discrimination

As previously mentioned, neural correlates of physical objects have been shown in the ACC (Weible et al., 2012, Weible et al., 2009), while neural correlates of physical qualities as scents have been shown in the hippocampus (Muzzio et al., 2009). Because the hippocampus project to the ACC (Hoover and Vertes, 2007) we wanted to investigate if ACC neurons also could code for these physical qualities. Two mice were recorded in the ODL2 in search for scent/target coding ACC cells. If the neurons had been coding for a specific scent its peak firing on the rate maps should have relocated in accordance with the relocation of the scent in the chamber. No neurons were found to be coding for scents. However, the presence of neurons with this firing pattern would have raised more questions about these neurons firing properties. For example, one could not be sure if the cells coded for a specific scent, target, digging or eating the reward as these properties can be connected to cognitive and affective behaviors that are processed in the ACC (Bush et al., 2000).

There are some differences in which part of the ACC one should record from in search for neural correlates of selective attention and scent/target coding. Selective attention is a type of cognitive control, while scent/target coding can be connected to goal-directed behavior, which is another type of cognitive control (Amemori et al., 2015). Therefore, because the ACd is most related to cognition (Bush et al., 2000) one might consider targeting this region for

recording these tasks. Pathways by which the ACd could contribute in cognitive control are via projections to the prefrontal and parietal cortices and subcortical projections to structures involved in arousal as the amygdala, hypothalamus and locus coeruleus (Ebitz and Platt, 2015, Hoover and Vertes, 2007). Because the ACd also is interconnected with ACv (Jones et al., 2005) one might find neural correlates of cognitive control in this part as well. In addition, the neural correlates of cognitive control and scent/target coding could correspond to eating or finding the target (which are affective aspects of the target). Therefore one could also find such neuronal correlates in the affective ACv (Bush et al., 2000).

4.4.4 The anterior cingulate and cognitive control summarized

The ACC has been shown to be involved in selective attention tasks requiring cognitive control (Milham and Banich, 2005, Casey et al., 2000, Botvinick et al., 1999). To investigate how electrophysiological correlates of selective attention unfolds in the ACC mice were trained to distinguish between cues in two distinct environments. The learning of these cues was of great importance for the mice to be able to perform the selective attention task. However, not all cues were learned and this lead to a non-functional CCT. Therefore no recordings were conducted in the CCT. Hence, more research is required to make a functional VDL and CCT setup before recordings can be conducted from the ACC.

4.5 Future directions

Our findings raise several questions for future studies. Although the results and previous literature indicate that the ACC is involved in affective coding (Etkin et al., 2011, Shackman et al., 2011, Bush et al., 2000) such as safety and danger coding, it is still unclear whether the (safety) annulus cells code for safety or borders. If the safety cells had been found on the EF this would have strengthened the hypothesis that the OF annulus cells code for safety and not borders. However, no safety cells were found on the EF, which might be a result of both the EF center and edge being dangerous places. Therefore, future studies are encouraged to include the intermediate zone of the EF as a safe location and to increase the size of each zone (center, intermediate and surround) by making an OF and EF with larger diameter. If the annulus cells increase their firing frequency in the intermediate zone this would also strengthen the hypothesis that the OF annulus cells code for safety and not borders. Danger coding bull's-eye cells in both the OF-EPM and OF-EF experiments were found, but both the bull's-eye count and danger bull's-eye count were a minority of the total cell count. Thus, more experiments are needed to confirm that these cells truly code for danger. For example,

bull's-eye cells can be recorded in other experimental setups investigating anxiety e.g. the holeboard or light-dark box (for review see Kalueff et al., 2007).

With regards to future experiments using visual tasks for mice, experimenters are encouraged to use higher contrasts between the test chamber walls and the cues because mice are less driven by their vision than other senses (Kolata et al., 2007), e.g. use of led lights as described by Matzel and Kolata (2010). Because the C57BL/6 mouse also is susceptible to eye abnormalities (Jackson-Laboratory, 2016) one might consider using a strain that have been shown to learn to discriminate between visual cues as the CD-1 mouse (Matzel et al., 2011).

Future behavioral experimenters are also encouraged to use only male or female mice. The use of both genders in the current study does not seem to have affected the results to a large degree, but because of the many differences between male and female mice, the use of one gender or separating the genders in two test groups may give more coherent results (Bothe et al., 2005). Factors one might consider when deciding on gender could be that male mice are less affected by hormones but show more territorial fighting, while female mice are more affected by hormones but show less territorial fighting (Deacon, 2006). With regards to this and that male mice are less affected than female mice in being single housed (Hunt and Hambly, 2006), one might consider using male mice in behavioral experiments requiring single housing and female mice when group housing is needed. One should also consider whether other male and female characteristics could affect the study, e.g. hormonal cycle of the female mouse on the EPM as described in paragraph 4.2 (Walf and Frye, 2007).

A number of studies have examined electrophysiological correlates of ACC neurons and behavior. However, comparison of the data is complicated because in many studies the recordings were made in extensively trained animals, therefore making it difficult to differentiate between learned and innate neuronal responses (Weible et al., 2009). Future studies can assess this by looking at the exploratory state of the animal, because mice with previous knowledge of a experimental setup will not explore the setup in the same way as a novel setup (Bothe et al., 2005). Mice explore objects and environments by use of their whiskers. Therefore, by recording the neuronal activity in the whisker pad during exploration of an environment, one can investigate the animal's exploratory state. Exploratory whisking (when whiskers are swept over objects) is recognized by neuronal firing frequency of 5-15

Hz, while foveal whisking (when whiskers are thrust forward and palpate objects) is recognized by neuronal firing frequency of 15-25 Hz (Berg and Kleinfeld, 2003).

4.6 Conclusions

The ACC is best described as functionally diverse with evidence of a large variety of different coding, from cognitive to emotional. With regards to how neuronal correlates of selective attention unfold in the ACC, more experiments are needed to investigate this. These experiments should focus on making a functional cognitive control task; only when the task is functional, neural correlates of selective attention or other desired aspect of cognitive control can be recorded as mice are performing the task. The electrophysiological results from the safety and danger experiments suggest that the ACC both integrates information coming from other brain areas and have some specific functions. Many of the recorded cells had a shifting firing pattern over several recording sessions, which indicate that the same type of information can be coded in different cells each time it enters the ACC. Thus, the ACC can be viewed as an integrating structure that fires in response to input from other areas of the brain. Additionally, some ACC neurons as the bull's-eye and annulus cells had stable firing patterns over several sessions and this indicate that the ACC also may have specific functions. Some of these stable bull's-eye and annulus cells also qualified as coding for safety and danger. Therefore, this study strengthens the hypothesis that the ACC may be involved in safety and danger coding, and possibly also border coding.

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Appendix A: SOP food restriction at the KI/CNC

Prepared by SE/AÅ Approved by	Standard Operating Procedure **Animals on Food Restriction**	Version: 5 Date of preparation: 10.04.2012 Revisions: 05.02.2013 24.04.2015 by SE Next revision: Spring 2016

Background

Behavioral research often requires that an animal performs a task for which it receives a reward. The ultimate goal, however, is that the animals will get comfortable in the recording environment and willing to perform the task and accepting the reward without putting them on restricted feeding. Successful recording and healthy animals requires that the animal is familiar with the environment where the behavioral task/recording is to be done, familiar with the reward that will be offered and with being handled in a competent and confident way.

Implementation

Food restriction must be included in the application on animal experiments. The need for food restriction must be justified in the protocol.

All animals on food restriction must have a Food Restriction Sheet attached to their cage for as long as the restricted feeding takes place. The researcher is in charge of giving the animals the daily amount of food, checking and logging the animal's body weight and filling in necessary information on the sheet. The body weight must be checked and logged every day. In addition there is a separate column for the staff to make notes about the animals' body weight or other information.

Prior to surgery, all rats should be made familiar with humans, by repeated handling in a gentle and positive way. This should be performed by the researcher. In addition, both mice and rats should be introduced to the environment where the recording/behavioral task is going to take place. The purpose of this is to socialize the animal and to prevent it from being afraid of the recording environment.

Food restriction is not allowed the first 4-5 days post-surgery. Animals need extra nutrients to recover from surgery and we need to eliminate sources of stress during these critical days.

Sudden transition to small amounts of food is not allowed. A gradual adaptation to the period of less food available is better tolerated by the animal causing a lower stress response than a sudden restriction. **Adaptation to less food should happen over at least three days.** During this period, the animals should also be introduced to the food that will be offered as a reward.

Food restriction, rats (minimum requirements):

- Adaptation:
  - Day 1: give 12 pellets
  - Day 2 →: Reduce daily food by one pellet per day
- Minimum number of pellets per day to rats: **8**

Food restriction, mice (minimum requirements):

- Adaptation:
  - Day 1: Give 3 pellets

    - Day 2 →: Reduce daily food by half a pellet per day
- Minimum number of pellets per day to mice: **1**

Any need to give less food than mentioned above needs to be clarified with Siv Eggen.

The researcher must ensure that the sum of nutrients given as a reward and the amount of regular food given each day is sufficient to keep the animal in a healthy state. When possible, the rewards given should be sufficiently positive to the animal to keep it motivated to do the behavioural task with a minimum or no food restriction. The researcher should make an effort to find the least degree of restriction necessary, and make the animal familiar with the recording environment, so that the animal will be motivated to do the tasks.

Excess restriction will deprive the animal of the motivation as well as the nutrients, causing the animal to be unable and unwilling to perform the task and the researcher unable to obtain results.

Animals on food restriction should not be housed in pairs or larger groups, since this will most likely cause the dominant individual to eat more than its share, while the subdominant one(s) receive less than intended.

It is not allowed to have days with no food given to the animals. If the researcher is absent for some days, for instance on week-ends, instructions could be given to somebody else about how to feed the animals. Otherwise, the animals will receive *ad libitum* food during these days.

In cases where *ad libitum* feeding is intended, the researcher is responsible for feeding the animal before leaving the facility on the first day of the period of free access to food. During the period of *ad libitum* food, the food-restriction-sheet must be either folded together, or marked with “Ad lib” or “Free food” or similar, on the relevant days.

Animals on food restriction have to be weighed daily, ideally before experimental sessions. The body weight is to be logged on the food-restriction sheet. If the loss of body weight is more than 10 % of the body weight before the restriction started, the amount of food given must increase until the body weight has normalized. Loss above 15 % is not allowed, and requires that the animal is removed from the study or terminated.

> *Exception 1:* Calorie restriction in obese animals. The loss of body weight can exceed 15 % of the original body weight, but this has to be done in collaboration with the facility veterinarian to make sure the animal’s health is not at risk.
>
> *Exception 2:* Food-restriction in young animals (below 6 weeks of age). Young animals on food restriction must be monitored closely, and the period on food-restriction should be limited to a few days only. Weight loss must not be tolerated; t hey should rather increase in body weight showing a parallel curve to their non-deprived siblings.

For experiments where long-term food restriction (more than 4 weeks) is found to be necessary, an agreement should be made between the researcher and the local veterinarian about the degree of restriction and potential consequences for the animal, as well as discussing whether there could be alternatives to the long-term food restriction.

Appendix B: SOP surgical procedure at the KI/CNC

Prepared by MWF, CB and SE Approved by	Standard Operating Procedure **Surgical procedures at the Kavli Institute for Systems Neuroscience**	Date of preparation: 2010 Revisions: • 2012 by AÅ and SE • 07.05.2014 by AÅ and BBL • 02.12.2015 by SE Next revision: Spring 2016

General information

This SOP contains detailed information about performing surgery on rodents at the Kavli Institute for Systems Neuroscience and Centre for Neural Computation. The SOP is primarily designed for survival surgery, but most sections will also be relevant for acute procedures. Updated versions of this SOP and other SOPs are available on G:\guides\Standard operating procedures.

PRE-SURGERY

Pre-training

Before surgery, it is good practice to habituate the animals to the housing conditions and the food they will be given post-surgery.

The performance of a rat may be very much improved if they are pre-trained before the surgery (e.g. open field or more specific tasks).

Handling of rats must be done prior to surgery.

Food and water

Food-restriction is not necessary before surgeries. Rodents can't vomit, and hypoglycaemia is a major complication to surgery on rodents. Food-restriction will deplete the liver's glycogen reserves.

Surgery preparation (day before)

- **Check the health status of your animal.** Only healthy animals can undergo surgery: vivid eyes, normal posture, curious and exploring behavior.
- **Check that everything you need* is available** and prepared**
- **Clean and sterilize your instruments.** Heat sterilization: 180°C, minimum 2 hours. Equipment that cannot be heat sterilized should be UV-irradiated or disinfected with 3 % hydrogen peroxide (H_2O_2) and 70 % ethanol.

  * Cf instruments list
  ** If we are running empty of something, or instruments have been misplaced or are malfunctioning, please contact the facility staff. You should not borrow or take it

from other rooms or setups.

SURGERY

Starting procedures

- **Disinfect the surgery table and the equipment close to it** with 70 % ethanol or disinfectant wipes. Also, prepare one "brain cup" with 70 % ethanol, one with 3 % hydrogen peroxide (H_2O_2) and one with sterile saline. Prepare one syringe (10 ml for adult rats, 5 ml for mice and young rats) with sterile saline to be used during the surgery.

- For survival surgery: **Prepare a medication form for every surgery** and complete this form as much as possible before the surgery and during the following days.

- **Prepare a surgery protocol** and complete this during the surgery with information about the medications given and the level of gas anesthesia

- **Update mLIMS.** Make sure that the animal is reserved in your name, write the date of the surgery and the FOTS number (number system in the application form FOTS. Ask your supervisor if you are uncertain about this)

- **Check the body weight of your animal** and fill it in on the medication form (this is very important for calculating amounts of drugs to be given during the surgery and for the recovery period)

- **Prepare your instruments**

- **Turn on the heat pad** and check that it is working

- Check the level of isoflurane in the vaporizer. Refill if necessary. Check that all tubes in the anesthesia setup are connected properly and that there is no leakage or compression of the tubes. Check the pressure of the medical air.

- **Prepare the injectable drugs** that are to be administered before the procedure starts. See sections "Pain medication" and "Fluid therapy". For dosages, see the lists in the surgery rooms.

- **Anesthetize the animal**

  It is good practice to keep your animal in a quiet, not too bright environment before inducing the anesthesia to reduce the stress. Stressed animals will experience a rush of adrenaline, which will interact with the anesthetic agent. Put some blue paper in the induction box before placing the animal there.

  Isoflurane anesthesia: Use 5 % isoflurane to the induction chamber, and observe the animal carefully. It should be taken out from the chamber when the anesthesia has been induced. Move the animal to the tube mask, place it on a towel and reduce the

isoflurane level to around 3 %.

If you are using injectable anesthetics: The anesthesia may be induced with isoflurane in the induction chamber connected to a vaporizer. When the animal is anesthetized, it can be taken out from the induction chamber and the injectable anesthetics can be administered. You may inject the anesthetics while the animal is awake, if you have a good technique and are comfortable giving injections. Avoid stressing the animal while doing this. It takes 5 to 15 minutes for the anesthetics to be effective, even more if the animal is heavy. Slow, regular breathing and no responses to stimuli indicates a deep anesthesia.

- Shave the surgical field and brush the fur away.

- **Put on eye ointment** to protect the cornea

- **Give analgesics.** Give subcutaneous injections of analgesics as soon as the anesthesia has been induced. Give both Temgesic and Metacam/Rimadyl. For dosage, see the lists in the surgery rooms. For rat pups, you may avoid Temgesic as this might make the mother reject the pups after surgery. Still, give Metacam/Rimadyl to the animals.

- **Give local anesthesia** by sc infiltration of Marcain in the area where the incision is to be made. For dosage, see the list in the surgery room.

- Reduce the level of isoflurane to 2-2.5 %. Individual adjustments are needed, dependent on the depth of anesthesia.

- **Check the animal's reflexes** before fixating the head and starting the surgery. The animal should not respond to painful stimulus like pinching the skin between the toes. Repeat the toe pinch from time to time throughout the procedure.

- **Fix the head** in the stereotaxic frame. Make sure you have received sufficient training in this as incorrect fixation may result in brain damage or damage to the eyes. If you are uncertain, ask for help. Pay attention to the ears and the hazard of destroying the tympanic membrane. Do not start the surgery if the ear bars are placed so that the eyes of the animal start to pop out. This is a sign of incorrect placement of the ear bars, resulting in increased pressure in the head of the animal. The recovery will be slow, and you might get unintended eye or brain damage. (A trick to be sure that the ear bars are well placed is to see if the ears are draped over the ear bars.) Make sure that the scalp is flat and that the head can rotate in the AP plane but does not move in the ML or DV directions.

- After the animal is fixed in the frame, delineate a "close to sterile" area near the frame with a surgical drape. Here you can keep your instruments and equipment. Instruments with blood should first be dipped in hydrogen peroxide (to get rid of blood), then in ethanol and then in saline. Make sure that dirty hands, clothes, coughing etc. do not contaminate your instruments.

- **Clean the scalp with sterile NaCl and cotton, and then disinfect the skin with iodide.**

- During the surgery, only the tip of instruments should touch the exposed tissue. Make sure to keep the instrument tips sterile throughout the procedure. It is important to rinse the instruments with sterile NaCl to get rid of hydrogen peroxide and ethanol, before the instrument tip comes into contact with the tissue. You don't want to apply ethanol or H_2O_2 into the tissue.

Pain medication

The table below lists the kinds of medications that are to be given to the animals at different surgeries. For dosages, see lists in the surgery rooms/nursing rooms.
NSAID = Non-Steroid Anti-Inflammatory Drug – this is Metacam or Rimadyl

		Temgesic	NSAID	Marcain
Pre-surgery, adult mouse/rat		X	X	X
Pre-surgery, infant mouse/rat			X	X
8-12 hours post-surgery	Implantation	X		
	Injection	X		
24 hours post-surgery	Implantation	If needed*	X	
	Injection	If needed*	X	
48 hours post-surgery	Implantation	If needed*	If needed*	
	Injection	If needed*	If needed*	

* The "pain scoring sheet" may be used to determine if analgesics are needed. In case of doubt, assume that pain medications are needed and give analgesics to the animal.

Fluid therapy

For surgeries lasting more than 3 hours, the animal must receive supportive therapy with fluids. This is to compensate for the lack of water intake during the surgery and the hours of recovery. For shorter surgeries, fluid therapy should start if the animal does not regain intake of food/water within 4 hours after the surgery finished.

The fluids given should correspond to one day's water intake. The total amount can be calculated using this formula:

$$\frac{60\ ml}{1000\ g} = \frac{x\ ml}{BW\ (g)}$$

$$x\ ml = \frac{60\ ml\ *\ BW\ (g)}{1000\ g}$$

BW = Body weight. Max amount is 25 ml, even if the animal is heavy. The total amount should be divided into two administrations, where half the amount can be given at the beginning of the surgery and the other half towards the end. Use sterile NaCl and give this sc

in the area between the shoulder blades or along the back of the animal, max 5 ml per injection.

A list of amounts of fluids to animals with different body weight is included in the dosage list in the surgery rooms.

For fluid therapy of dehydrated animals, see SOP Fluid therapy.

During the surgery

- **Check reflexes** from time to time to assess the depth of anesthesia. Pinch the skin between the toes and look for a response. A response to painful stimuli should be absent. If the animal responds, adjust the isoflurane vaporizer until you have reached surgical level of anesthesia.
- Check the depth and regularity of the breathing. Slow, regular breathing with movement of the thorax and the abdominal muscles indicates a deep anesthesia. If the breathing gets too slow or irregular, indicating too deep anesthesia, reduce the isoflurane.
    - Normal undisturbed respiration: 90-180/min for mice, 70-115/min for rats
    - The frequency will drop during anesthesia, but should not drop more than 50 %.
    - Mucus membranes should be pink, not violet, blue or grey

- **Check the body temperature.** Touch hairless areas such as the toes, tail or ears. Put a towel or similar over the body of the animal if it appears to be cold. Hypothermia is a major cause of surgical mortality. However, remember that the use of heat pads or other heat sources may cause hyperthermia and burn wounds.
- As long as you have an open wound, remember to **apply sterile NaCl frequently to the tissue** to prevent the tissue from getting dry. Keep a large syringe filled with NaCl for this purpose.
- It is important to **clean and remove all bone debris from the skull surface and the wound** before starting the implantation of the drive and cementing with dental acryl. Rinse several times with NaCl
- Make sure to **mix the dental acryl well** before applying it. Before ending the surgery, be sure that the dental acryl is smooth and does not have sharp edges. A clean Q-tip can be used to “feel” the edges of the acryl. If you see or sense any sharp edges, **they must be removed using the drill** and the small drill head. Sharp edges may cut the skin around the implant. This will be uncomfortable for the animal and result in stress, wounds, possible infections and problems moving around during behavioral tasks.
- If you are suturing a wound, make sure the wound is closed without pulling the skin too tight. Tight stitches are painful and increase the risk of the animal trying to remove the stitches prematurely. Also, the knots must be secure and the technique aseptic. If you need instructions in wound closure, please contact the veterinarian.
- Check the anesthesia vaporizer, tubes and medical air system periodically in order to ensure a proper and steady level of anesthesia throughout the surgery.
- Do not leave an anaesthetized animal unattended. If you need to leave, make an appointment with someone who can look after the animal while you are away.

Sterility

Keep in mind during the whole surgery that the surgical site, the instruments and your hands need to stay as clean as possible during the procedure.

Remember that each time you touch the animal's fur or unclean surfaces outside the sterile area, you have contaminated your hands. Disinfect your gloves/hands to reduce the risk of contaminating the wound. Even with disinfected hands, you should not touch the wound or the tip of the instruments that come into contact with the tissue.

Clothes

Compulsory during surgical interventions

- One-piece suit or scrub suits
- Lab shoes
- Hairnet
- Mask

- Gloves are recommended

Optional

- Goggles
- Extra lab coat

Instruments

The delineated "close to sterile" area near the frame must be kept disinfected and close to sterile throughout the surgical intervention. Keep your instruments in a sterilized cup with 70 % ethanol or on the operation drape. Keep the sterile items under control.

After the instruments have contacted the wound, use a small cup with 3 % hydrogen peroxide to clean away the blood, then disinfect the instruments with ethanol, then rinse them in sterile saline, before using them again. If the surgical instruments need to be re-sterilized, wash them well before using the glass-bead sterilizer.

If you are implanting a drive, the drive should be placed in a holder during the surgery. The tetrodes/bundles should be lowered into a cup of ethanol for 10 minutes. The ethanol should reach above the lower edge of the outer cannula on Microdrives and above the lower half of the bundle on Hyperdrives/Versadrives/Tordrives. After the 10 minutes have passed, the tetrodes/bundles should be lowered in sterile water and kept there until you are ready for the implantation.

POST-SURGERY

Add some antiseptic around the implant to prevent foreign elements to enter the tissue. Injection with Temgesic 8-12 hours after the first injection.

Put the animal in a heating chamber for the immediate recovery phase. Do not leave the animal alone until reflexes and respiration rate (> 60/min) are reestabilished. The animal is usually very thirsty after surgery. It might be good to provide them pellets soaked in water and porridge when they wake up and are transferred to their home cage.

Never provide animals with water in a bowl before they are awake and are able to swallow by themselves. Do not place the animal in a cage with its cage mates before it is completely awake. There is a chance that sedated animals may suffocate if a group sleeps in a cluster. Exception: surgery on pups. Pups need maternal care.

24 hours post-surgery: Give another dose of NSAID. Give Temgesic too, if needed.

48 hours post-surgery: Implanted animals should get another dose of NSAID if needed.

The pain medication should continue for as long as necessary.

The animal must have free access to food and water the first 4-5 days post-surgery because of extra nutritional needs during the recovery, and to eliminate sources of stress during these days. If food restriction is necessary, it should not start until these first days have passed. See SOP Animals on Food Restriction for details. Do not deprive animals of drinking water without a special agreement with the veterinarian. Check the body weight of your animal every day postoperatively until you see that the animal is no longer losing weight – but begins to gain weight. Remember to complete the medication form with all the details of your surgery and leave it on the cage.

Never leave the city/region until 2 days post-surgery. Please enter the Institute at least once daily to check the status of the animal and give necessary medications for at least the first 2 days after surgery.

You are responsible for the animals that have been assigned to you. If for some reason you need to leave the city/region, please make an appointment with someone who can function as your substitute and respond if action is needed towards your animals. If this is the case, please leave a note on the cage or send an email to vetatech with information about your substitute, so that the staff knows who to contact.

Appendix C: Calculation of the chance level for the discrimination learning

Random choices of cups would result in the following probabilities within a single trial after the start point walls were removed:

$$P(pick\ target) = \frac{1}{3}, P(pick\ non\text{-}target\ 1) = \frac{1}{3}, P(pick\ non\text{-}target\ 2) = \frac{1}{3}$$

If the mouse where to pick a non-target cup on the first attempt to find the reward, the probabilities for the second attempt would be:

$$P(pick\ target) = \frac{1}{2}, P(pick\ non\text{-}target) = \frac{1}{2}$$

If the mouse picked a non-target cup on the second attempt as well, the probabilities for the third attempt would be:

$$P(pick\ target) = 1$$

However, these probabilities were only valid if the mouse never returned to a cup it had visited before on the same trial. Hence, to be able to compare the chance level with the errors made by the experimental group, returning to an already visited non-target cup was not counted as an error.

By using the above probabilities the expected number of attempts per trial (X) and the expected number of errors per trial ($X - 1$) was calculated in the following way:

$$P(X = 1) = P(correct\ 1st\ choice) = \frac{1}{3}$$

$$P(X = 2) = P(wrong\ 1st\ choice\ AND\ correct\ 2nd\ choice)$$

$$= P(correct\ 2nd \mid wrong\ 1st) \times P(wrong\ 1st) = \frac{1}{2} \times \frac{2}{3} = \frac{2}{6} = \frac{1}{3}$$

$$P(X = 3) = P(wrong\ 1st\ AND\ wrong\ 2nd\ AND\ correct\ 3rd)$$
$$= P(correct\ 3rd \mid wrong\ 1st\ AND\ wrong\ 2nd)$$
$$\times P(wrong\ 1st\ AND\ wrong\ 2nd)$$
$$= (correct\ 3rd \mid wrong\ 1st\ AND\ wrong\ 2nd) \times P(wrong\ 2nd \mid wrong\ 1st)$$
$$\times P(wrong\ 1st) = 1 \times \frac{1}{2} \times \frac{2}{3} = \frac{2}{6} = \frac{1}{3}$$

Leading to expected number of attempts:

$$X = 1 \times P(X = 1) + 2 \times P(X = 2) + 3 \times P(X = 3) = 1 \times \frac{1}{3} + 2 \times \frac{1}{3} + 3 \times \frac{1}{3} = 6 \times \frac{1}{3} = 2$$

Leading to expected number of mistakes:

$$X - 1 = 2 - 1 = 1$$

This means that if the group never learned to use the cues to guide them to the reward they would on average make 1 error per trial.

Appendix D: Supporting results for the olfactory discrimination

A pilot study was performed to investigate if covering the reward with wood chips would make the mice use the cues (Fig. D.1). Six mice trained in the ODL1 were used in this study where the reward was covered with wood chips on all four days (four trials/day, day 1: reward directly under surface of wood chips; day 2: reward in the middle of wood chips; day 3: reward on the bottom of the cup; day 4: control, target scent switched from vanilla to raspberry).

A Shapiro-Wilks test ($p < 0.05$) and visual inspection of the normality plots showed that the variables within each mean were not normally distributed. Therefore WSR and MWU tests were used to investigate whether there was a significant difference between the performance on day 3 and the control, and between day 3 and the chance level. The WSR test showed that there was no statistically significant difference between errors made on day 3 and the control, or between latency on day 3 and the control (errors 0.6 ± 0.1 and 1.0 ± 0.2 [$p = 0.06$]; time 19 ± 3 s and 20 ± 3 s [$p = 0.70$] mean ± SEM for day 3 and control respectively). However, the MWU test showed that there was made significantly less errors on day 3 compared to the chance level (1.0, calculations in appendix C, $p < 0.01$), while the errors made on the control was not significantly different from the chance level ($p = 0.76$). Therefore, there was a trend towards better performance in this pilot study than in the ODL1. In addition to adding wood chips in the cups in the ODL2, we also increased the number of days in the learning period to give the mice more time to learn the cues.

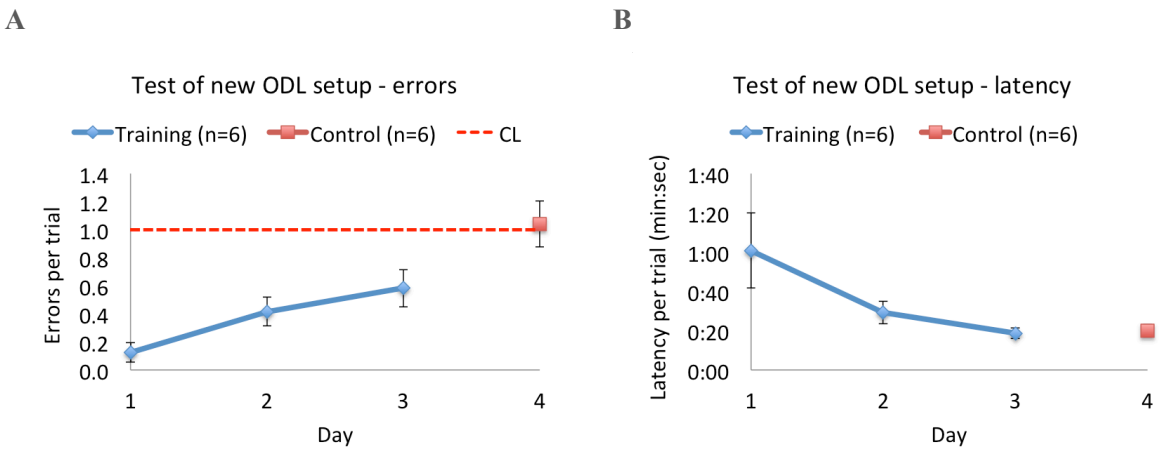

Figure D.1 Test of the new ODL setup with woodchips. There was no significant difference in performance between day 3 and the control, either with regards to **A)** number of errors (day 3: 0.6 ± 0.1, control: 1.0 ± 0.2) or **B)** latency (day 3: 19 ± 3 s, control: 20 ± 3 s). *Continued on page 86.*

Figure D.1 continued. However, the number of errors made on day 3 was significantly different than the chance level (1.0, CL), while the number of errors made on the control was not. Altogether, this indicated that there was a trend towards focusing more on the cues when wood chips were added in the cups.

Before moving on to the cognitive control test the mice's performance on the olfactory task was investigated. This was done to ensure that they still remembered to use vanilla as the target cue after the ODL2 control (switching target from vanilla to raspberry) and the 11 days of VDL2. Figure D.2 show the results from the entire ODL2 including this new control, named "control 2", for the four mice that proceeded to the CCT.

A Shapiro-Wilks test ($p < 0.05$) and a visual inspection of the normality plots showed that the variables within each mean were not normally distributed. Therefore the non-parametric tests were used to compare the performance on control 2 (day 12) with the chance level and the control (day 11). The WSR test showed that there was a significant difference in both errors and latency between day 12 and 11 (errors 0.1 ± 0.01 and 1.0 ± 0.2 [$p < 0.01$]; latency 10 ± 2 s and 33 ± 9 s [$p < 0.01$] mean ± SEM for day 12 and 11 respectively). Also, the MWU showed that there was a significant difference in errors made on day 12 and the chance level (1.0, calculations shown in appendix C, $p < 0.01$). The results indicated that the mice still used the correct cue and therefore they could move on to the CCT2.

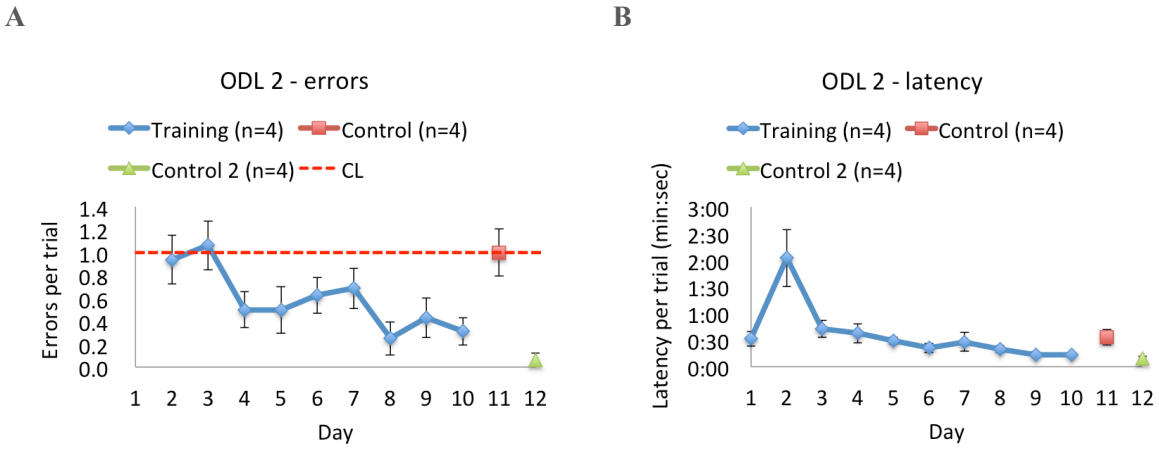

Figure D.2 Results from the entire ODL2 including control 2. "Control" (day 11) was the performance of the mice when the target scent was changed from vanilla to raspberry, while "control 2" (day 12) was the performance of the mice after ODL2 control and 11 days of VDL2. There was a statistically significant difference ($p < 0.05$) between day 12 and day 11, both with regards to **(A)** errors (day 12: 0.1 ± 0.01, day 11: 1.0 ± 0.2) and **(B)** latency (day 12: 10 ± 2 s, day 11: 33 ± 9 s). In addition, there was a significant difference between the errors made on day 12 and the chance level (1.0, CL). These results showed that the mice still used vanilla as the target cue after the ODL2 control and the 11 days of VDL2.