Kadjita Asumbisa

Cognitive control based decision-making correlates with c-Fos activation in the anterior cingulate cortex

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Supervisor: Prof. Clifford Kentros

Co-supervisor: Nils Borgesius

Norwegian University of Science and Technology (NTNU)
Faculty of Medicine
Department of Neuroscience

NTNU – Trondheim
Norwegian University of
Science and Technology

ABSTRACT

Flexibility in decision-making is usually linked to cognitive control. This is believed to be an essential element required for economically adaptive behaviors in an ever-changing environment. With a flexible decision making system, organisms are able to rapidly adjust their actions for optimal outcomes in the face of competing alternatives. Studies show that this kind of flexibility in decision-making is usually impaired in persons with neuropsychiatric conditions such as schizophrenia, depression and substance abuse (Murphy et al., 2001; Rahman, B, R, Rogers, & Robbins, 2001). Cognitive rigidity has often been linked to damage, lesion or inactivation of the anterior cingulate cortex (ACC) (G. B. Bissonette, E. M. Powell, & M. R. Roesch, 2013). This medial frontal cortex structure is believed to hold representations of effort-reward outcomes, needed to drive optimal decision-making (Rushworth, Walton, Kennerley, & Bannerman, 2004). Accordingly, rat lesions or inactivation of this region have been shown to result in animals becoming less willing to invest high efforts for large rewards (Hosking, Cocker, & Winstanley, 2014; M. E. Walton, Bannerman, & Rushworth, 2002). However, little is known about ACC's activation associated with effort-reward based decision making when cognitive control is engaged in an intact brain. By using c-fos immediate early genes, we assessed the association between cognitive control based decision making on the effort T-maze, and its correlates with ACC activation. To test mice on the effort T-maze, this study first established an appropriate barrier/reward combination (LR arm=8cm[0.2ml] vs SR arm=5cm[0.05ml]) for mice on the effort T-maze; something that was lacking in the literature. In addition, our c-Fos result suggests that activation in ACC correlates with suppression of the less optimal response in the face of competing alternatives. The possible underlying mechanisms and future directions are further discussed.

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ABBREVIATIONS

AAV	Adeno-associated virus
ACC	Anterior cingulate cortex
AP-1	Activator protein site 1
BLA	Basolateral amygdala
CC1	Cognitive control group 1
CC2	Cognitive control group 2
ChR	Channel rhodopsin
CRE	cAMP response element
CREB	Calcium response-element binding protein
Ctrl	Control group
DAB	Diaminobenzidine
dACC	Dorsal anterior cingulate cortex
eNpHR	Halorhodopsin
fMRI	Functional magnetic resonance imaging
IEG	Immediate early gene
NAc	Nucleus accumbens
LE	Low effort
LR	Large reward
MAPK	Mitogen activated protein kinase
MRI	Magnetic resonance imaging
NGS	Normal goat serum
SR	Small reward
PBS	Phosphate buffered saline solution
TBS	Tris buffered saline solution
Tx	Triton X-100
vACC	Ventral anterior cingulate cortex

1 INTRODUCTION

1.1 Background

The ability to rapidly adapt one's decisions in an ever-changing environment is a hallmark of cognitive control. This is further characterized by being able to generate appropriate adjustments in response selection, biasing and maintenance of information relevant to the changing context. This kind of cognitive flexibility in decision-making is often lacking in persons suffering from neuropsychiatric disorders affecting the ACC (London, Ernst, Grant, Bonson, & Weinstein, 2000; Murphy et al., 2001). The stroop test and Wisconsin Card Sorting task present a classical approach for testing cognitive control in humans. Amongst rodents, a simple paradigm for testing effort-based decision making that engage cognitive control is the effort T-maze. The maze is designed in a shape of a "T" that offers animals a choice between a high reward obtainable at a high cost and a low reward obtainable at a low cost (Denk et al., 2005). Here, the ability to suppress the tendency to get small reward with little effort in favor of choosing the high effort arm for a higher reward represents some form of cognitive control. Several converging lines of evidence point to the ACC's involvement in cognitive control (Matsumoto & Tanaka, 2004; Ridderinkhof, Ullsperger, Crone, & Nieuwenhuiss, 2004; Ruff, Woodward, Laurens, & Liddle, 2001). However, most of what is known about the ACC's involvement in cognitive control based decision-making comes from functional imaging, lesion or inactivation studies (Hosking et al., 2014; Kennerley, Walton, Behrens, Buckley, & Rushworth, 2006; K Sasaki, Gemba, Nambu, & Matsuzaki, 1993; M. E. Walton et al., 2002). In as much as these approaches have provided some relevant insights, they come with several limitations. Whereas lesion and inactivation studies have the tendency to totally or partially disrupt the network activity of unintended circuits, fMRI provides low temporal resolution hence, making it difficult to match brain activity to behavior in time. Thus this current study employed c-fos staining to characterize the involvement of ACC in cognitive control based decision-making on the effort T-maze.

1.2 Anatomy and neuronal connections of the cingulate cortex

Paul Broca was amongst the earliest researchers to describe a broad band of tissue that enveloped the corpus callosum and parts of the ventral forebrain, and referred to it as *le grand lobe limbique* (Broca, 1878). This became known as the limbic lobe, deriving its name from the Latin word *limbus*, which means border (Lamendella, 1977). The dorsal portion of the limbic lobe is what has become known as the cingulate cortex (Allman, Hakeem, Erwin, Nimchinsky, & Hof, 2001). The cingulate cortex therefore comprised of the region that formed a *cingulum* or boundary around the corpus callosum.

In the 1940's MacLean's concept of the "triune brain" suggested that the brain of vertebrates evolved in a series of three concentric shells around a reptilian core (MacLean & Ashbrook, 1993; McLean, 1990). He referred to the innermost and outer shells as "paleo-mammalian" and "neo-mammalian" which comprised the cingulate cortex and the neocortex respectively. This became one of the earliest studies that distinguished the neocortex from the cingulate cortex. Following from that, Sanides (1970) developed a similar scheme for brain evolution that further pointed the cingulate cortex as having a more primitive laminar structure compared to the neocortex, implying it may have preceded it in evolution.

The cingulate cortex is mainly divided into anterior and posterior regions. It is instructive to know that these divisions are mainly used as a way of naming different regions of the cingulate cortex rather than based on strict cytoarchitectural differences. This notwithstanding, while layer IV is present in the posterior regions, it is lacking in the anterior region. Although ACC is known to lack a clearly defined layer IV, it has a well-developed layer V which projects strongly to many subcortical structures such as the thalamus (Allman et al., 2001).

The ACC forms a large region around the rostrum of the corpus callosum (Devinsky, Morrell, & Vogt, 1995), whose ventral and dorsal portions correspond to Broadman's area 24a and 24b respectively (Vogt & Peters, 1981). The dorsal part is mainly connected to the prefrontal and parietal cortex (M. Posner & DiGirolamo,

1998), allowing the ACC to have influence on motivational and spatial behaviors. By contrast, the ventral part is more strongly connected to the basolateral amygdala (BLA), nucleus accumbens (NAc), hypothalamus and the anterior insula (Devinsky et al., 1995; Rosene & Van Hoesen, 1977). Based on these connections of the ventral ACC, it is largely believed to be involved in reward related behaviors. Direct reciprocal projections from septum (Kemper, Wright, & Locke, 1972), subiculum (Meibach & Siegel, 1977) and parahippocampus (Petras, 1971) also arrive at the ACC. These connections with the hippocampal formation are also believed to be the channel for ACC's participation in memory related behaviors that facilitate cognitive control by using the history of behavioral outcomes to inform necessary adjustments (Petras, 1971). An intricately wired intrinsic network has also been shown to exist within the cingulate cortex (Jones, Groenewegen, & Witter, 2005), allowing for more integrated processing within the structure. In summary, the location of ACC, together with its widespread interconnectivity, makes it well positioned for the control of different aspects of behavior.

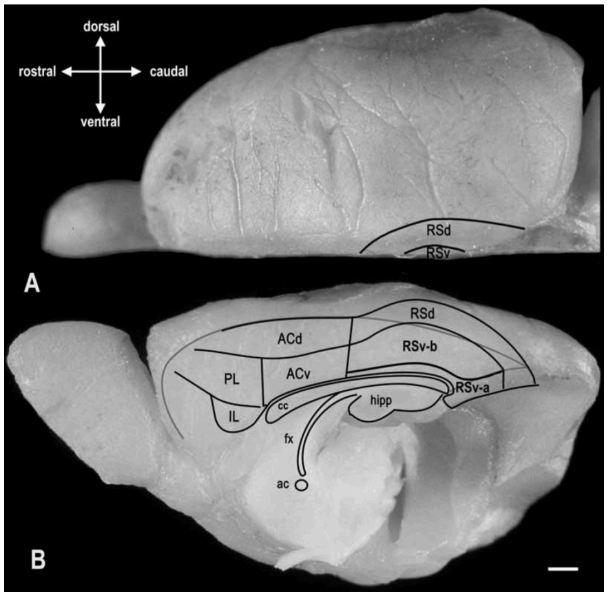

Figure 1.1 Anatomical location of ACC. The figure shows reconstruction of the cingulate cortex on photographs of the right hemisphere of the rat, represented in a dorsal (A) and a midsagittal view (B). The cerebellum and brain stem have been removed in order to show the ventro-caudal margins of the cingulate cortex. ACd= dorsal cingulate cortex, PL= pre-limbic cortex, IL=infralimbic cortex, RSd/v=dorsal and ventral retrosplenial cortex, cc-corpus callosum, hipp=hippocampus, fx=fornix (adapted from (Jones et al., 2005).

1.3 Functional properties of ACC

1.3.1 Decision making

Decision-making is a prerequisite for the continued survival of many organisms. Studies show that many people suffering from neurological diseases often have difficulties with decision making, especially in situations that require decisions based on evaluation of cost-benefit contingencies (Bechara, 2001; Rahman et al., 2001). For example, this difficulty is seen in neuropsychiatric patients suffering from drug addiction (London et al., 2000) and depression (Murphy et al., 2001). The concept of decision making has been defined by Rangel, Camerer, and Montague (2008) as a "cognitive process that involves the representation and assignment of values and probabilities to different options, the selection of an option based on this value assignment, the execution of specific behavior that is expected to lead to the desired outcome, the evaluation of the outcome and the learning and updating of the evaluation and action-selection."

Making decisions about impending actions may therefore require an understanding of the expected value of outcomes, relative costs incurred between choices, and the probabilities of achieving the possible outcomes. An essential part of decision-making is being able to use past experience to select the most optimal action from other competing ones (Kennerley et al., 2006). In most experimental set-ups, the decision making process is guided by cues or instructions (Ragozzino, Ragozzino, Mizumori, & Kesner, 2002; Yin & Knowlton, 2004). However, purely voluntary decisions are usually guided by the recent history associated with the available alternatives (Bayer & Glimcher, 2005; Sutton & Barto, 1998).

Studies examining the neural and neurochemical correlates involved in making decisions on how much effort to invest for rewards has implicated the anterior cingulate cortex (ACC), basolateral amygdala (BLA) and the dopaminergic projections arising from the nucleus accumbens (NAc) (Hauber & Sommer, 2009). M. E. Walton et al. (2002) showed that rats with lesions that affected the entire ACC were unwilling to invest more effort for a higher reward on the effort T-maze. This finding was further corroborated by Hosking et al. (2014) where infusion of

baclofen-muscimol into ACC decreased all animals preference for the high reward option on a rodent cognitive effort lever pressing task. Using the effort T-maze task with rats, Salamone, Cousins, and Bucher (1994) showed that rats with dopamine depletions in the NAc no longer choose the arm that required high effort for a higher reward. Deficits in effort based decision making has also been shown in animals with BLA and ACC lesions (Floresco & Ghods-Sharifi, 2007), suggesting that interaction between these structures is necessary for optimal decision making in cost-benefit situations.

Although these studies, together with other functional imaging studies point to the involvement of ACC in effort related decision making (Hampton & O'Doherty, 2007) the exact contribution of ACC to effort based decision making is still poorly understood (Hauber & Sommer, 2009). However, studies have shown that neuronal projections from BLA to ACC convey information about the reward magnitude associated with the alternative responses. Thus, ACC's involvement in effort based decision making may be related its role in representing cost-benefit contingencies that are constantly integrated to guide optimal decision-making (Kennerley et al., 2006; Rudebeck, Walton, Smyth, Bannerman, & Rushworth, 2006; Sanfey, Rilling, Aronson, Nystrom, & Cohen, 2003; M. E. Walton, Bannerman, Alterescu, & Rushworth, 2003).

1.3.2 Cognitive control

Decision-making can also engage elements of cognitive control; requiring the suppression of a standard stimulus driven response (Frith, 2001; M. I. Posner & Petersen, 1989) in favor of an alternative as seen in the Wisconsin Card Sorting task (Berg, 1948) and Stroop test (Treisman & Fearnley, 1969). According to Botvinick, Cohen, and Carter (2004) cognitive control refers to "a set of functions serving to reconfigure the cognitive system for the performance of a specific task, especially in challenging and non routine situations."

Conflict monitoring has been proposed as a possible function that must be recruited for successful execution of cognitive control. Studies suggest that the ACC encodes the occurrence of conflicts in information processing and thus, may initiate the

trigger responses needed for behavioral adjustments in cognitive control (Botvinick, Braver, Barch, Carter, & Cohen, 2001; Koch, Gade, Schuch, & Philipp, 2010). In most of the studies that have implicated the ACC in conflict monitoring and cognitive control, the association is often related to tasks that require the inhibition of a learned prepotent response. ACC's involvement in response inhibition has been observed in several cognitive tasks such as the stroop task (George et al., 1994), the go/no-go paradigm (Casey et al., 1997; Kazuo Sasaki, Gemba, & Tsujimoto, 1989) and in the Simon task (Hübner & Mishra, 2013), as well as in other tasks that place demands on response inhibition for optimal decision making (Bush et al., 1999; Taylor, Kornblum, Minoshima, Oliver, & Koeppe, 1994).

Pardo, Pardo, Janer, and Raichle (1990) were amongst the earliest to report the activation of ACC during incongruent trials on the Stroop task. In the Stroop test, participants may be presented with a mismatch between the color the word refers to and the color in which the word is displayed in (i.e. *red displayed in red* for congruent trial and *red displayed in green* for incongruent trial). By employing positron emission tomography (PET), Pardo et al. (1990) showed that activation in the ACC was significantly higher during performance on incongruent trials than congruent trials on the Stroop task. The finding of greater activation on incongruent trials has also been reported in other studies (Carter, Mintun, & Cohen, 1995; George et al., 1994).

Casey et al. (1997) have used fMRI to demonstrate ACC's involvement in response inhibition on the GO/NO-GO paradigm. In this study, subjects had to view a series of presented letters, and were required to press a bottom with each stimulus presentation except when the presented letter was X. The experimental set-up consisted of a GO condition, defined by 100% presentation of non-target stimulus (non X's) and a NO-GO condition- defined by the presentation of 50% non-target letters (i.e. X's). Upon several rounds of testing on the GO condition, the button pressing became a prepotent response to the stimulus presentation. However, when the participants were moved unto the NO-GO block, containing a mix of target and non-target letters, activation in ACC was seen to be strongest. The level of ACC

activation in the GO/NO-GO condition may therefore be associated with the participants need to suppress the button pressing prepotent response for successful performance on the no-go condition.

In the performance of these cognitive tasks, the strongest activation in the ACC often occurs on incongruent trials at the level of response selection (Pardo et al., 1990; Turken & Swick, 1999). However, ACC may not be directly involved in response selection but instead, sends signals to other frontal structures to exert the control needed for behavioral modification (M. E. Walton, Croxson, Behrens, Kennerley, & Rushworth, 2007). Hence, suggesting that ACC may be involved in suppressing or overriding representations of prepotent responses that is later relayed to other frontal structures for behavioral modification.

1.4 Functional organization and the c-fos proto-oncogene

Dating back to the early days of brain research, several strides were made in the pursuit of functionally mapping out the brain. For instance, in the latter part of the 19th century, research on the brain took a giant leap. With improved technologies came more pragmatic ways of examining brain tissue. Key amongst the developments was Golgi's silver stain (Golgi, 1873), allowing for a more systematic microscopic way of examining cells; both in the normal brain as well as in pathological brains. Santiago Ramón y Cajal pushed the frontiers of brain science further by using Golgi's staining technique to painstakingly draw the structure of the stained brain cells as seen through a microscope (Cajal, 1888).

This development pioneered an increasing interest in the functional organization of the brain, leading to the "neuron doctrine". Proponents of this theory proposed that the brain was made up a vast collection of cells that interacted at a very microscopic level to give rise to behavior. Studies on speech comprehension (Wernicke, 1974), speech production (Broca, 1865), visual recognition (Lissauer, 1890), and voluntary movement (Fitsch & Hitzig, 1870; Hughlings, 1958) all provided support for the localized functional organization of the brain. Localization of brain function therefore became a prominent theme in that era.

However, the concept of localization of function started becoming unpopular at the turn of the century. Most of what contributed to this functional localization notion was based on studies concentrated on primary and secondary sensorimotor areas (Brett, Johnsrude, & Owen, 2002), for which lesion or activation labeling is relatively straightforward. But, localization of function becomes less straightforward when considering brain activity in complex behaviors such as spatial navigation (Moser, Kropff, & Moser, 2008) working memory (D'Esposito et al., 1995), decision-making (Glimcher & Fehr, 2013) and cognitive control (van Veen & Carter, 2006). The reason being that, such complex behaviors involve the recruitment of a network of cells from many different brain regions.

As a result, the recent trend adopted for mapping out the functional organization of the brain has therefore become more focused on identifying the contribution of different cell population to the final behavior. Hence, in trying to understand the underlying neuronal mechanisms for complex cognitive behaviors such as decision-making and cognitive control, researchers tend to focus more on the contributing circuitries as well as the level of activation in the different areas involved.

By using immediate early gene (IEG) staining techniques several studies have successfully labeled functionally defined subsets of neurons involved in different behaviors. Popular amongst IEG neuronal markers are c-fos, Arc/Arg3.1 and zif268/egr-1. The aforementioned markers have been widely used to define subsets of functionally active cells. For instance, c-fos has been used to show the involvement of hippocampal CA1 and perirhinal cortex in recognition of place and objects, respectively (Mendez, Arias, Uceda, & Arias, 2015). Arc has also been used to investigate synaptic plasticity in several brain regions (Guzowski, McNaughton, Barnes, & Worley, 1999; Izumi, Ishimoto, Yamamoto, Nishijo, & Mori, 2011; Mikuni et al., 2013). Furthermore, Zif268 has been used to show neuronal apomorphine-evoked whisker behavior activation in the rat barrel cortex (Filipkowski, Rydz, & Kaczmarek, 2001) and fear associated memory retrieval in amygdala and hippocampus (Strekalova et al., 2003). All these neuronal markers continue to

contribute significantly to the quest to map out the functional organization of the brain.

However, due to the stereotypical induction of c-fos in cells that are activated by different stimuli, it has become the most widely used IEG for functional anatomical mapping (Barros et al., 2015; Bullitt, 1990; Mendez et al., 2015; Stanciu, Radulovic, & Spiess, 2001). The c-fos protein is a member of the Fos family. Three other major proteins of the Fos family have been identified, namely; FosB, Fra-1 and Fra-2. Nonetheless, c-fos transcription has been shown to have very low detectable mRNA and protein expression under basal conditions (Hughes, Lawlor, & Dragunow, 1992; Krukoff & Khalili, 1997) as compared to FosB (Herdegen et al., 1995) and the FRAs (Honkaniemi, Kononen, Kainu, Pyykönen, & Pelto-Huikko, 1994). Hence, making it the ideal tool for functional mapping of neurons that are activated by a given behavior.

The proto-oncogene c-fos forms a heterodimer with members of the Jun family of transcription factors which in turn binds DNA at AP-1 at the promoter and enhancer regions of target genes, converting extracellular activities into changes in the expression of genes (Chiu et al., 1988). Upon activation of genes that contain the AP-1 complex, there is expression of genes that encode neuronal activities such neurotransmitters, depolarization, increase in Ca^{2+} influx and elevated levels of intracellular Ca^{2+} (Fields, Eshete, Stevens, & Itoh, 1997; Gaiddon, Loeffler, & Larmet, 1996; Ghosh, Ginty, Bading, & Greenberg, 1994). The peak expression of c-fos has been shown to last between 1-3 hrs following stimulus presentation and gradually disappears by 4-6 hrs (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Ding, Carver, Terracio, & Buggy, 1994; Stanciu et al., 2001). The c-fos proto-oncogene is known to possess two distinct Ca^{2+} detectors: (1) Calcium influx through voltage dependent Ca^{2+} channels and (2) Calcium influx through ligand gated Ca^{2+} channels. Calcium influx through the former induces the calcium response-element binding protein (CREB) phosphorylation via the CAM kinase pathway, and induces c-fos via cAMP response element (CRE), whereas the latter detector leads to the activation of mitogen activated protein kinase (MAPK) transduction pathway, targeting serum

response element, SRE (West et al., 2001). These two distinct pathways for detecting calcium influx make c-fos induction a reliable predictor of neuronal activation.

1.5 Rationale and Aims

Several lesion and regional inactivation studies on the effort T-maze show that animals require an intact ACC to make decisions to expend effort to obtain a higher reward (Hosking et al., 2014; M. E. Walton et al., 2002). Thus the effort T-maze can be used to study the correlates of optimal decision-making in the ACC. Secondly, the effort T-maze paradigm can further be adapted to study cognitive control during decision-making. Since cognitive control usually requires the suppression of a prepotent response, animals can be trained to have a strong preference for a particular arm (prepotent response). With this training in place, different barrier combinations that may require suppression of the prepotent response can be introduced to assess cognitive control at different levels.

Although lesion and inactivation studies on the effort T-maze have provided us with some insights into ACC's involvement in effort based decision-making, the difficulty with making interpretations from such studies is that, they give information on broken, damaged brains. On the other hand, IEG staining gives information on unperturbed, fully functional brains. Thus to investigate how activity in the ACC correlates with effort based decision making and cognitive control, this study employed c-fos staining to:

1. Correlate the level of activity in the ACC to the number of LR (Large Reward) arm choices on the effort T-maze.
2. Assess the pattern of activation in the ACC when cognitive control based decision making on the effort T-maze is a function of: (a) reward, (b) effort, or (c) reward/effort.

2 METHODS

2.1 Animals

For this project, 12 male and 18 female C57BL/J mice aged 8-12 weeks, and weighing 28-32 grams were used. Mice were housed individually in transparent plexiglas cages in a room with 12hr light and dark cycles. Mice were handled 10mins each for 5 days to acclimatize them to human handling. During this period the mice had free access to food and water. However, to increase the motivation of the mice for the training and testing phase, mice were diet restricted to 90% of their ad libitum body weight. All through the training and testing phase, the animals were weighed daily to ensure that their body weight never came below 90% of their initial weight. All experimental procedures on the animals were carried out in compliance to the Norwegian Regulations on Animal Research (Forsøksdyrutvalget).

2.2 Apparatus

An effort T-Maze based on the design described by M. E. Walton et al. (2002) was used (Figure 2.1). It was made of a wire mesh, grey painted wood and dark plastic walls and 5cm wide corridors. The maze stood on stands of 30cm high. Each arm also had a length of 30cm. The barriers were constructed in a form of 3D right-angled triangles with wire mesh. The mice had to climb the vertical side of the barrier and descend at the end with the slope to obtain the reward. The barrier heights used in this experiment were 5cm, 7cm, 8cm and 10cm all with different angles of slope depending on the barrier height. Two barriers were constructed for all the four different heights, resulting in a total of eight constructed barriers.

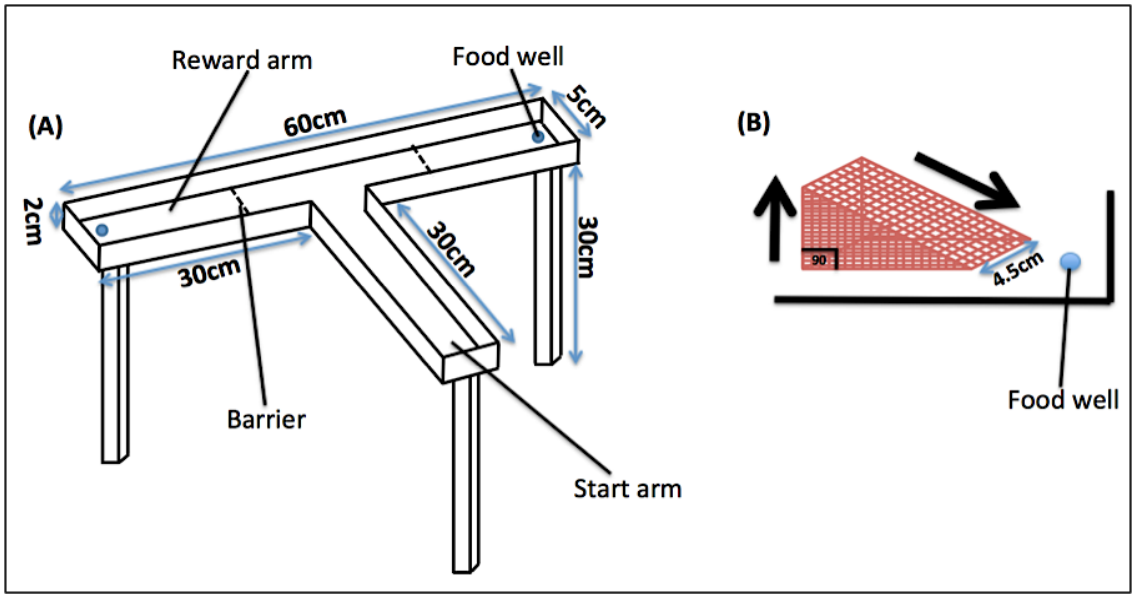

Figure 2.1 Effort T-maze. The figure shows the dimensions of the (A) Elevated effort T-maze constructed out of wood and (B)3D wire mesh barrier used.

2.3 Behavioral Training

The habituation and discrimination training protocol used in this study was similar to what Bardgett, Depenbrock, Downs, Points, and Green (2009) and M. E. Walton et al. (2002) described in their study based on the original from Salamone et al. (1994). For each cohort, three mice were trained with the left side as the large reward arm, and another three with the right side as the small reward arm. The large and small reward arm designation was maintained for each cohort throughout the remaining training and test trials.

2.3.1 Habituation

On the first day of the habituation training, each mouse was placed into the T-maze, allowed to explore the maze and consume the reward (0.2 ml chocolate milk) placed in both reward arms. The food wells were constantly refilled, and the mice removed after 10 minutes. On the next day, mice were placed into the maze with 0.2 ml chocolate milk in both arms. The trial ended when mice consumed the reward in both arms or after 150 seconds elapsed. The mice were given five trials for two more days. On Day 4 of the habituation training, all the mice were consuming the food reward on every trial.

2.3.2 Discrimination Training

Phase 1: Each mouse received five trials per day for two days. The pre-assigned large reward (LR) and small reward (SR) arms were filled with 0.2 and 0.05ml chocolate milk respectively. The inter-trial interval was approximately four minutes with two mice being run during the interval. The trials ended when the milk in both arms was consumed or after 150 seconds had elapsed.

Phase 2: In this phase, each mouse performed ten trials per day for two days. A wooden block was used to prevent access to the left or right arm prior to testing. Mice were pseudo-randomly forced into the LR and SR arm five times each. They were never forced into the same arm more than twice in a row. The trial ended after they had consumed the reward in the food cup or 90 seconds elapsed.

Phase 3: In the last phase, each mouse performed ten trials per day for two days. On trials five and ten, a wooden block was used to prevent access to the previously chosen arm to prevent the animals from adopting a side bias; if a mouse chose the left arm on trial 4, the left arm was blocked on trial 5 to force the mouse into the right arm and vice versa. The trial ended after each mouse had chosen an arm and consumed the reward from the cup or 90 seconds elapsed.

2.3.3 Barrier Training

Depending on the barrier combination to be tested, different barriers were placed in the middle of the goal arms prior to testing. The first day consisted of eight trials. For the first five trials, the trial ended after the animals had climbed the barriers to obtain the reward in both arms. However, the last three trials ended after the animals had chosen an arm, climbed the barrier, and consumed the reward. The subsequent days were similar to the last three trials of the first day, where trials ended after animals made a choice, and this also constituted the barrier testing.

2.4 Testing

Each test day started with two forced runs in opposite directions to serve as a reminder of the arm containing the LR and SR (M. E. Walton et al., 2002). After the forced trials, the main testing consisted of eight free choice runs and two forced

runs, making a sum of ten runs. On trials five and ten, animals were forced into the opposite arm visited in the previous trial, four and nine respectively. This also served as another measure to reduce their tendency to build a strong bias towards one goal arm. Baseline discrimination testing without barriers was performed on all cohorts during **Phase 3** of the discrimination training. Each cohort consisted of six mice. The testing for each cohort consisted of different experimental testing blocks.

2.4.1 Cohort 1

The first testing block for this cohort consisted of a 5cm barrier in the HR arm and no barrier in the SR arm. This was intended to assess decision-making based on initial effort-reward contingencies. After two days of testing, they were moved on to the second block that consisted of two 5cm barriers, one in each arm. The second testing block lasted for one day. Upon confirming that they could still discriminate between the LR and SR arms, barriers of 10cm and 5cm were placed in the HR and SR arms respectively. This constituted the last testing block for this cohort.

2.4.2 Cohort 2

To eliminate the likely influence of beginning the experiment with "effort required in the LR arm" and "no effort in the SR arm" (*see* Cohort 1- block A), the first testing block for Cohort 2 started with a 5 and 10cm barrier in the SR and LR arms respectively. Following from their performance, they moved unto testing block two with 10cm barriers in both the LR and SR arms to assess if their ability to discriminate between the LR and SR arms was still intact. Testing was done for two days in both experiments.

2.4.3 Cohort 3

To assess whether reducing the difference between the barrier heights for both arms will affect preference for the LR arm, the first testing block was made of a 5 and 7cm barrier in the SR and LR arms respectively. Following from two days of block B, testing block C sought to assess whether preference for the LR arm was based on the reduced barrier height difference (*relative effort*). To do this, the milk reward was equated in both arms, whilst the 5 and 7cm barriers still remained in their respective arms. Testing for the second block also lasted for two days.

2.4.4 Cohort 4

The experiment for this cohort sought to assess whether a wider difference between the two barriers in each arm will significantly affect preference for the LR arm, and at the same time correlate the number of LR arm choices with c-Fos expression in the ACC. Hence, the experiment consisted of a 5 and 8cm barrier. Testing was done for two days followed by perfusions.

2.4.5 Cohort 5

To assess the pattern of activation in the ACC when cognitive control based decision making on the effort T-maze is a function of reward, effort or a combination of the two, animals in this cohort were divided equally into three groups with slightly different training regimes (*see* Figure 3.6).

For all the groups in this cohort, the barriers were introduced a day after the completion of **Phase 3 Discrimination Training**, followed by perfusions. The groups were abbreviated as follows: ctrl (control), CC1 (cognitive control group 1) and CC2 (cognitive control group 2). CC1 was trained with 0.05ml chocolate milk in the SR arm and 0.2ml in the LR arm, then 5 and 8cm barriers were introduced into the SR and LR arms respectively. The ctrl group was also trained with 0.05 and 0.2ml chocolate milk in the SR and LR arms respectively, followed by introduction of 8cm barriers in both arms. The last group, CC2, was trained with 0.2ml chocolate milk in both arms, followed by the introduction of 5 and 8cm barriers in the SR and LR arms respectively.

2.5 Perfusion and Tissue Preparations

The expression of c-Fos has been shown to occur 1-3 hrs following stimulus presentation or treatment (Ding et al., 1994; Girard-Joyal et al., 2015; Stanciu et al., 2001). Based on that, perfusions were done within 60-90mins following the last runs of Cohort 4 and 5. After the behavioral testing, the mice were deeply anesthetized with isoflurane, and intraperitoneally injected with an overdose of pentobarbital (100mg/kg). Upon pinching the toes and tail to confirm the absence of consciousness, the mice were perfused. The rib cage was cut open and the sternum pulled away to open up the breast cavity. A needle connected to a tube running with

normal saline solution was inserted into the left ventricle of the heart, followed by an incision at the right atrium. After 3mins at 8-9ml/min, the solution passing through the tube into the animal's circulation was switched to freshly made 4% paraformaldehyde (PFA in PBS, pH 7.4) for another 3mins to fixate the brain. After adequate perfusion as evidenced by twitches in the tail and limbs, as well as whitening of the major organs, the animal was decapitated, and the brain extracted. The extracted brain was then post fixated in PFA for 24hrs at 4°C and stored in Dimethyl Sulfoxide (DMSO) for cryo-protection until sectioning. Forty-micrometer (40µm) coronal sections were later obtained with a microtome and placed into three series in an alternating order for each brain.

2.6 Immunohistochemistry

Six free-floating tissue sections from the first series of each mouse were rinsed in 0.1M of phosphate-buffered saline (PBS; pH 7.4). After several PBS rinses, the sections were incubated in a blocking solution of hydrogen peroxide (H_2O_2) and methanol for 20mins. Following additional rinses, first in PBS and then in Tris-buffered saline with 10% Triton-X (TBS-Tx, pH 8.0), the sections were further blocked with 20% normal goat serum (NGS) in TBS-Tx for 30minutes. The sections were then incubated in at 4°C for 48hrs in a solution containing rabbit anti c-Fos polyclonal antibody (Santa Cruz K-25, 1:1000), 5% NGS and TBS-Tx. After washing in a solution of TBS-Tx to remove any unbound antibody, sections were incubated for 90min in the secondary antibody (biotinylated goat Anti-rabbit lgG (1:2000) solution in TBS-Tx, followed by 90mins incubation in the Vectastain ABC system (Vector Vectastain ABC kit Elite Pk-6100 standard; Vector Laboratories, Burlingame, CA, USA). The sections were again washed with TBS-Tx to remove unbound antibodies. Then, the sections were incubated in freshly prepared diaminobenzidine (DAB) solution for 4mins followed by rinses in Tris HCL (pH 7.6) to halt the reaction. The tissue sections were then mounted on gelatinized microscope slides and cover slipped.

2.7 Image Processing and Cell Counting

The region of interest and the neuroanatomical coordinates used for counting cells in the ACC were based on the nomenclature by Paxinos and Franklin (2004) ranging between +0.38mm and +0.74 mm from bregma. The c-Fos positive nuclei were defined based on homogenous grey-black stained elements with a well-defined border. Scanned images of the sections were obtained using Axio Scan.Z1 v1.0. Processing and analyses of the images were performed through a series of automated steps using ImageJ32. First, images were converted to gray scale (8 bit) and binarized using maximum entropy thresholding method (Wong & Sahoo, 1989). Third, images were smoothed and holes in the cell bodies filled with the binary function; overlapping nuclei were also separated through watershed segmentation (Vincent & Soille, 1991). Fourth, the polygon selection tool was then used to mark out the delineated regions for automatic cell counting. Finally, automatic cell counts from two uniformly stained sections from each animal was obtained with the imageJ particle analyzer plugin and expressed as the mean count for each animal and subsequently, each group. Cell counting occurred without foreknowledge of group allocation although the tendency of this to bias counting was already eliminated with the automatic counting procedure adopted.

3 RESULTS

Several studies show that the ACC is involved in decision-making involving cognitive control (Botvinick et al., 2004; Ridderinkhof et al., 2004; Ruff et al., 2001). However, most of these studies used MRI and fMRI, which may not provide enough precision due to the low temporal and spatial resolution inherent in these approaches. Also, rat lesion studies using the effort T-maze have added to the building evidence that ACC is involved in cognitive control (M. E. Walton et al., 2002). To further explore the activation of ACC during effort-based decision making involving cognitive control, this study first set out to determine the correct barrier combinations to be employed on the effort T-maze for experimentation on mice.

We conducted several behavioral experiments to establish the barrier combinations that mice were willing to invest effort for the large reward. The barrier combination that provided the desired behavioral result was reached after testing Cohort 4. Upon establishing this, mice from Cohort 4 and 5 were tested using that barrier combination and later perfused for c-Fos staining. Hence, the results from Cohort 1-3 are purely behavioral results showing the choice pattern of mice on the effort T-maze with different barrier combinations.

3.1 Effect of Different Barrier Combinations on Decision Making

The first testing block for each cohort in the effort T-maze started with no barriers in the reward arms. This served as a baseline control for discrimination learning, followed by the introduction of different barrier combinations.

To analyze the behavior results, non-parametric statistical tests (Kruskal Wallis H and Mann-Whitney U) were used due to the non-normal distribution of the dependent variable (number of LR arm choices). Also, to asses if LR arm choices varied between day 1 and 2 for each testing block, a within block analysis for days was conducted. The results showed that there was no significant difference between day 1 and 2 within each testing block for all cohorts, $p > 0.05$.

3.1.1 Cohort 1: 5cm Barrier vs No Barrier Test

Analysis of the behavior results for Cohort 1 using the Kruskal Wallis H test showed that there was a statistically significant effect of barrier size on the LR choices, x^2 (3)= 19.56, p= 0.00. The results from further pairwise comparisons with Bonferoni correction are summarized in Table 3.1.

The first testing block with no barriers showed that mice had learned to discriminate between the LR and SR arms, and had a high preference for the LR arm (median LR choices=8 in 8 runs). The second testing block then sought to assess the effect of a barrier in the LR arm and no barrier in the SR arm. Hence, a 5cm barrier was introduced into the LR arm. It was hypothesized that this would not affect preference for the LR arm, due to the higher reward being in the LR arm. However, this led to a significant decline in the number of LR choices, p=0.00. Following this, a third testing block consisting of two 5cm barriers, one in each arm was introduced. This was aimed at assessing the stability of the earlier discrimination learning established from the first testing block. The significant increase in the number of LR arm choices here showed that mice still had a stable memory and preference for the LR arm, p=0.00. Upon confirming this, 10 and 5cm barriers were placed in the LR and SR arms respectively for the next testing block. This block was introduced to assess the number of LR arm choices when an effort is required in both arms. The results showed a non-significant gradual decline in the number of LR arm choices from the previous block. Taken together, these results suggest that placing one barrier in the LR arm and no barrier in the SR arm leads to mice choosing the SR arm.

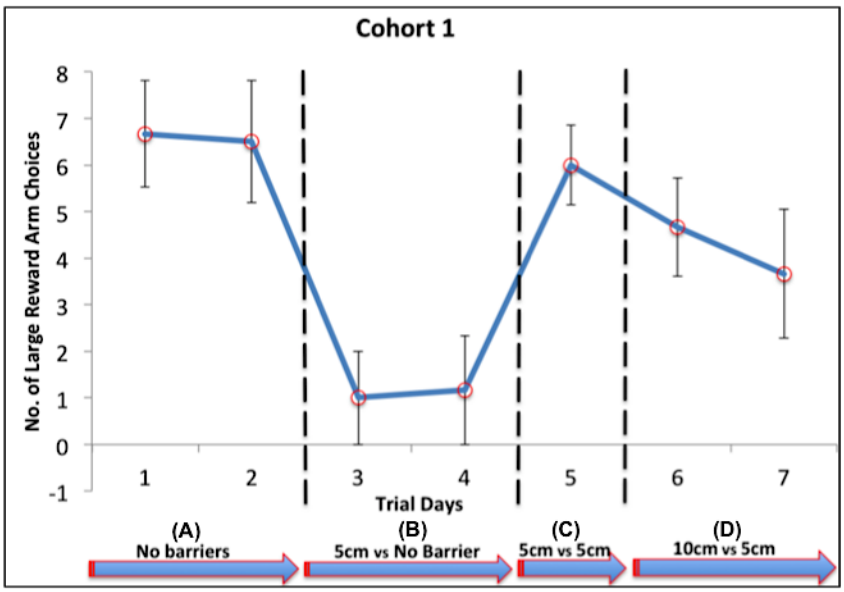

Figure 3.1 Cohort 1: Effect of Barrier Size on Large Reward (LR) arm Choices (Mean ± SEM). The Mean ± SEM represents the LR arm choices from 6 mice in 8 trials for each data point with different barrier combinations. The first testing block only tested the decision to choose between LR and SR arms with no effort required (No Barriers). The second block had a 5cm barrier in the LR arm and No barrier in the SR arm; implying that the SR arm choice will not involve any effort. Followed by trial blocks of 5cm vs 5cm and 10cm vs 5cm, both requiring some level of effort.

Table 3.1 Mann-Whitney U Summary Statistics for Cohort 1

Cohort 1: Testing blocks		**U**	**sig**	
(A) No barriers (median=8)	**(B) 5cm*no barrier (median=0)**	**10**	**.00**	**p < .0125#**
	(C) 5cm*5cm (median=6.5)	**20**	**.107**	**p > .0125**
	(D) 10cm*5cm (median=4)	**34.5**	**.024**	**p > .0125**
(B) 5cm*no barrier (median=0)	**(C) 5cm*5cm (median=6.5)**	**6**	**.002**	**p < .0125#**
	(D) 10cm*5cm (median=4)	**27**	**.005**	**p < .0125#**
(C) 5cm*5cm (median=6.5)	**(D) 10cm*5cm (median=4)**	**24**	**.257**	**p > .0125**

Bonferoni corrected alpha

From Table 3.2, the pairwise comparisons using Mann-Whitney U for testing blocks (A)*(B), (B)*(C) and (B)*(D) showed statistically significant difference. On the other hand, testing blocks (A)*(C), (A)*(D) and (C)*(D) were not significantly different.

3.1.2 Cohort 2: 10cm vs 5cm Barrier Test

Following from the results in Cohort 1, a second cohort was set-up to test the effect of having barriers in both arms. This was also intended to provide a more robust basis for testing effort-based decision-making since making a choice to either arm required some level of effort that could be measured. In Cohort 1, experience from blocks B and C may have affected D. Hence, in Cohort 2, the 10cm vs 5cm barrier combination was the first block to be introduced to prevent the influence of previous barrier experience.

Kruskal Wallis H test reported a significant effect between barrier size and large reward arm choices, $x2\ (2)= 21.19$, $p= 0.00$. Hence, further pairwise comparison was performed using the Mann-Whitney U. Table 3.2 shows the summary statistics and test of significance.

The first testing block for Cohort 2 also showed that mice had a strong preference for the LR arm (median LR choices=8 in 8 runs). The second testing block for this cohort was similar to testing block B for Cohort 1. However, for this cohort an effort was required for choosing either arm as opposed to choosing between a LR arm requiring an effort and a SR arm that required no effort. Hence, a 5 and 10cm barrier was placed in the SR and LR arms respectively. This resulted in a significant decrease in the number of LR arm choices, $p=0.00$. Following this testing block, 10cm barriers were introduced to both the LR and SR arm for the next testing block. This was done to assess: (1) the stability of the memory for the designated rewards in the LR and SR arms and (2) willingness to climb 10cm barrier for the reward. The results showed a slight but not significant increase in the number of LR arm choices from the previous testing block, suggesting they still had a stable memory and were willing to climb a 10cm barrier for the reward.

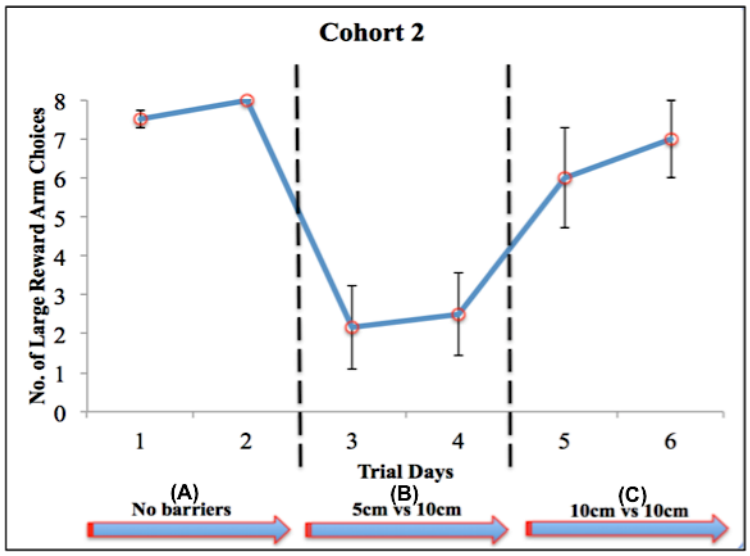

Figure 3.2 Cohort 2: Effect of Barrier Size on Large Reward (LR) arm Choices (Mean ± SEM). The Mean ± SEM represents the LR arm choices from 6 mice in 8 trials for each data point with different barrier combinations. The first testing block only tested decision to choose between LR and SR with no effort required (block A). The second block tested the effect of different levels of effort for the SR arm (5cm) and the LR arm (10cm). Followed by a final testing block consisting of a 10cm barrier in the LR and SR arm.

Table 3.2 Mann-Whitney U Summary Statistics for Cohort 2

Cohort 2: Testing blocks		**U**	**sig**	
(A) No barriers (median=8)	(B) 5cm*10cm(median=1)	1.5	.00	$p < .016^{\#}$
	(C) 10cm*10cm(median=5.5)	18	.001	$p < .016^{\#}$
(B) 5cm*10cm(median=1)	(C) 10cm*10cm(median=5.5)	36	.036	$p > .016$

Bonferoni corrected alpha

Testing blocks (A)*(B) and (A)*(C) showed statistically significant difference, whereas testing blocks (B)*(C) did not show a statistically significant difference. However, the median number of LR arm choices in testing block C (median=5.5) was higher than testing block B (median=1).

3.1.3 Cohort 3: 7cm vs 5cm Barrier Test

Here again, the baseline testing with no barriers showed a high number of LR arm choices. Testing from previous cohorts suggested that although animals are able to climb the 10cm barrier in the LR arm, willingness might have been reduced due to the associated reward ratios. Hence, a 7 and 5cm barrier combination was used for this cohort to assess the effect on LR arm choices. The result from testing block A showed no significant difference from testing block B, p=0.56. Thus, suggesting that

the 7cm barrier was considered worth the assigned high reward. To further confirm this, it was hypothesized that when the reward is equated in the arms, mice should begin to choose the LE (Low Effort) arm. This constituted the testing block shown in Figure 3.3(B). However, the results showed that preference for the LE arm was below 50%, suggesting that the 2cm difference between the barriers could be too small for differentiation.

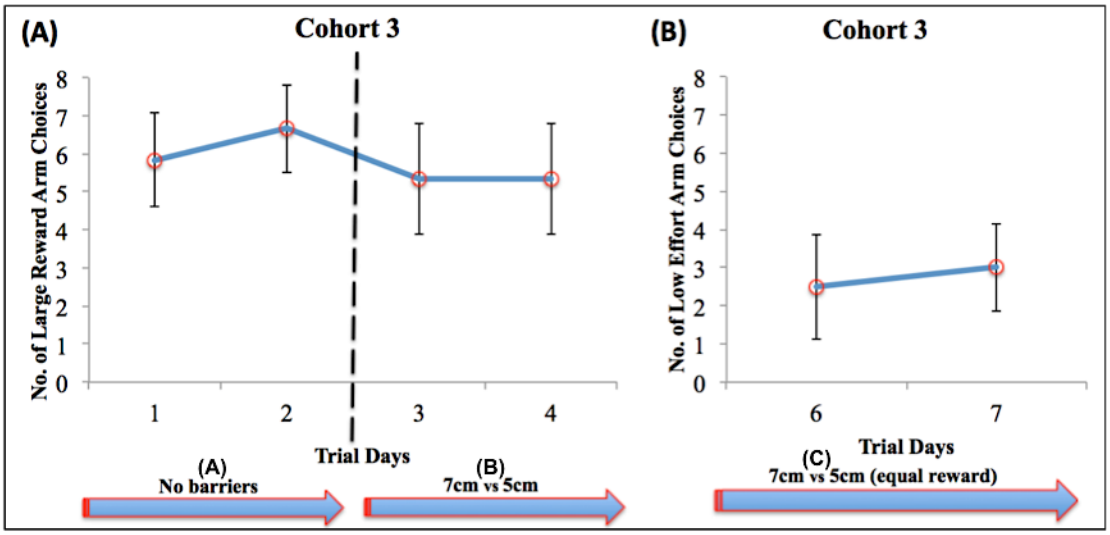

Figure 3.3 Cohort 3: Effect of Barrier Size and Reward on arm of Choice (Mean ± SEM) .The Mean ± SEM represents the LR arm choices from 6 mice in 8 trials for each data point with **(A)** no barriers, 7cm vs 5cm barriers for the LR and SR arms respectively and **(B)** 7cm vs 5cm barriers with equal rewards in both arms. Trial day 5 is missing from the figures because it was used to train mice to the change in reward scheme. All the trial blocks for figure **(A)** had 0.05 and 0.2ml chocolate milk reward in the SR and LR arms respectively whereas figure **(B)** contained 0.2ml chocolate milk in both arms.

Man-Whitney U showed that there was no significant difference between testing block A (median= 8) and B (median=8), U=64, p=0. 561.

3.1.4 Cohort 4: 8cm vs 5cm Barrier Test

Results from the last testing block in Cohort 3 suggested that 2cm difference between the LR and SR arm barriers was too small for differentiation. Hence, the barrier testing for Cohort 4 comprised of 8 and 5cm barriers in the LR and SR arms respectively. The results from day 1 of this testing suggested that mice were willing to choose the LR arm with 8cm barrier (Figure 3.4). Hence, mice were tested again on day 2 with the same barrier combinations and perfused for c-Fos staining.

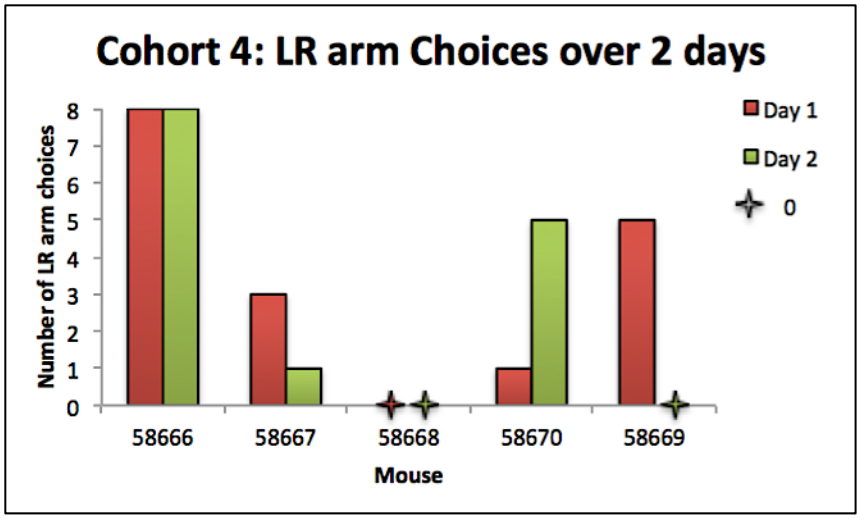

Figure 3.4 Cohort 4: Number of LR arm choices over 2 days with 8 and 5cm barriers. The figure shows the number of LR arm choices (out of 8 runs) for each mouse when 8 and 5cm barriers were placed in the LR and SR arm respectively.

3.2 c-Fos Results

A popular approach for labeling functionally defined neurons is the use of IEG's. Among the known IEG's, c-Fos has been shown to reliably label functionally defined neurons due to its stereotypic induction in response to different behaviors and stimuli (Barros et al., 2015). Hence, c-Fos staining was adopted to: (1) assess the correlates of LR arm choices with the number of activated cells in ACC and (2) assess c-fos activation at different levels of cognitive control. Functional imagining studies have linked activation in ACC to effortful decision making in instances were effort/reward contingencies must be integrated to inform decision (M. E. Walton, Devlin, & Rushworth, 2004). Based on this, we predict that animals that make LR arm choices will show more activation in the ACC.

The c-Fos staining from Cohort 4 was analyzed to assess the relationship between c-Fos positive cell counts and the number of LR arm choices. Since the two variables of interest for this cohort could be measured on interval scales, the Pearson Product-Moment Correlation was used for the analysis. For Cohort 5, the data was analyzed to ascertain if differences in the number of c-Fos positive cell counts differed between the three groups (ctrl, CC1 and CC2) in dACC and vACC. Multivariate variance analysis, MANOVA, was used for this analysis because the data consisted of two dependent variables measured on an interval scale and three categorical independent variables.

3.2.1 Cohort 4: c-Fos expression correlates with LR arm choices

Upon establishing the correct barrier combinations from Day 1 testing of this cohort, testing was continued for another day with the 8 and 5cm barriers. Perfusions and c-Fos staining followed this. The number of LR arm choices on Day 2 was then correlated with the number of c-Fos positive cells in the ACC as shown in Figure 3.5(B).

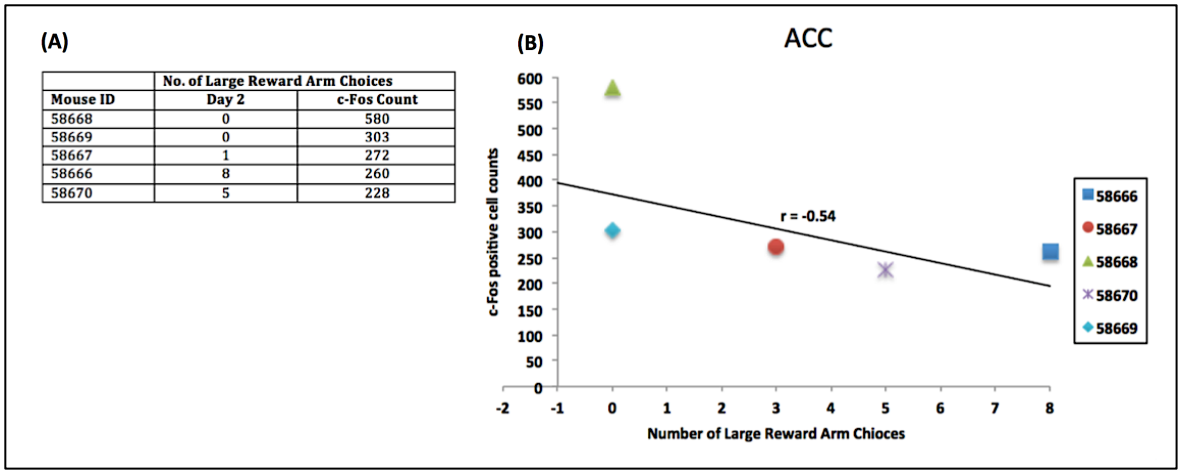

Figure 3.5 The figure shows (A) Summary of day 2 LR arm choices and c-Fos counts for individual animals (B) Pearson product moment correlation between the number of LR arm choices and level of c-Fos activation in ACC.

Although the results from the Pearson correlation was not statistically significant, it showed a strong negative relationship between large reward arm choices and the number of c-Fos positive cells in the ACC, r (3) = -0.54, p= 0.34. Hence, the more LR arm choices, the less activation of c-Fos and vice versa. Surprisingly, this is not what we hypothesized.

3.2.2 Cohort 5: c-Fos expression across different experimental groups

Upon establishing the 8 and 5cm barriers as being appropriate for the testing, different experimental groups were set up to assess neuronal activation when cognitive control is engaged at different levels. c-Fos expression for Cohort 5 was assessed 90min after the introduction of different barrier combinations to the three experimental groups (Figure 3.6). For ctrl, 8cm barriers were placed in both arms after being trained with 0.05ml and 0.2ml reward in the SR and LR arms respectively. For CC1, a 5cm barrier was placed in the SR arm and an 8cm barrier in the LR arm after being trained with 0.05ml and 0.2ml reward in the SR and LR arms respectively. Finally, for CC2, an 8cm barrier was placed in the "LR arm" and a 5cm

barrier in the "SR arm" after being trained with 0.2ml reward in both arms. The reward training for all the groups lasted for 6 days, followed by one day of barrier testing. In ctrl, cognitive control was a function of the reward, whereas in CC1 and CC2, cognitive control was a function of the reward/effort and only effort respectively.

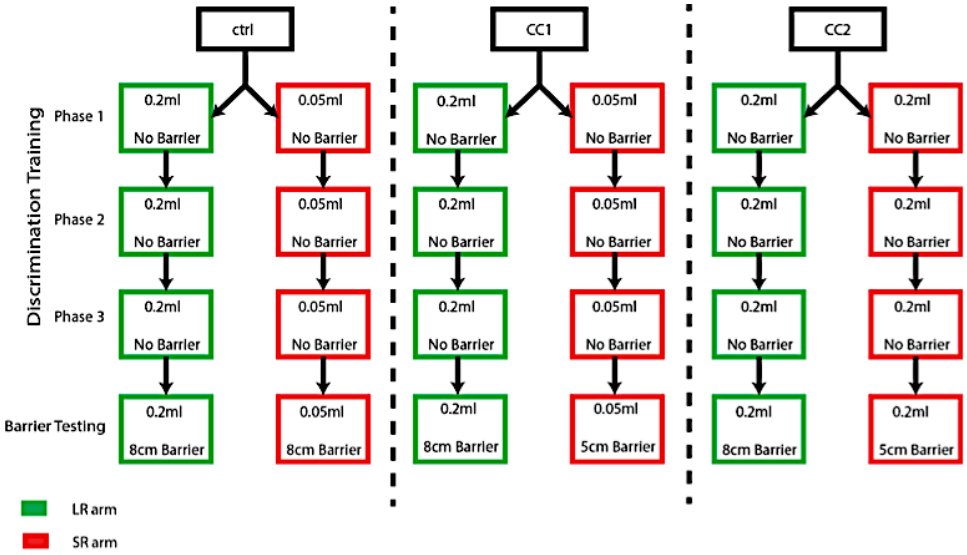

Figure 3.6 Schematic diagram of training and testing protocols for the different groups in Cohort 5. The green and red highlights in the figure represent the pre-designated LR and SR arms respectively. From the figure-**ctrl**: control group, **CC1**: Cognitive control group 1 and **CC2**: Cognitive control group 2.

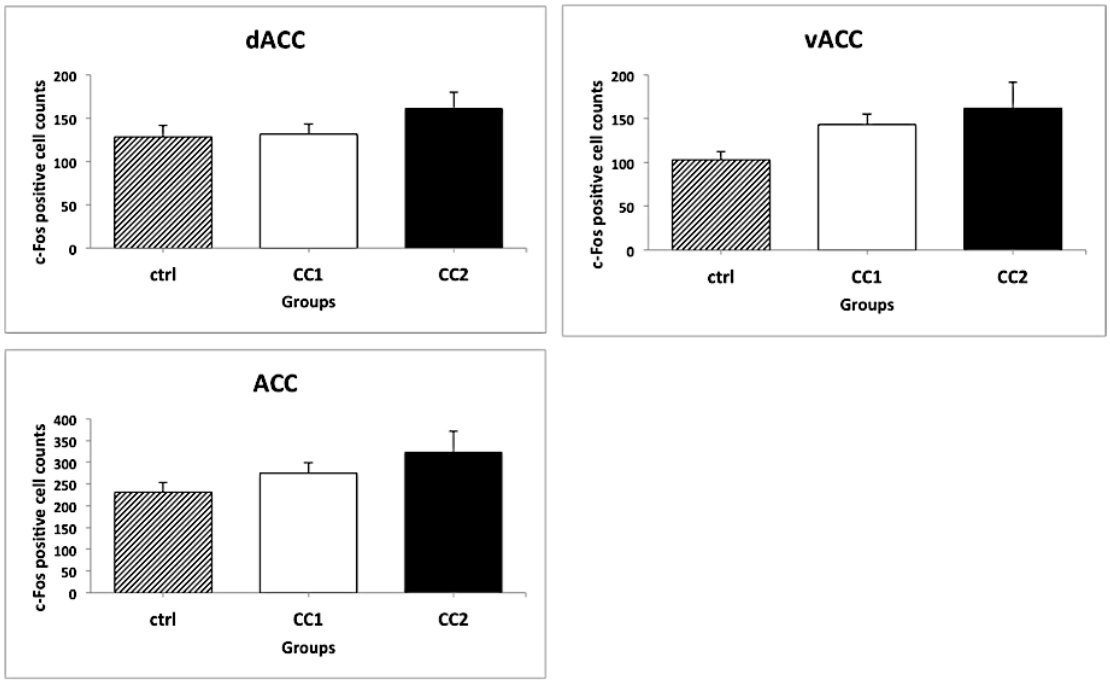

Figure 3.7 Effect of the different experimental conditions on the c-Fos-positive cell counts. The ER group showed the highest number of c-Fos positive cells in both dACC and vACC. The CC group also showed higher c-Fos positive cells compared to the EB group in both regions. The cell count difference between EB and the other groups (CC and ER) was more prominent in vACC. However, none of the observed differences reached statistical significance, $p>0.054$.

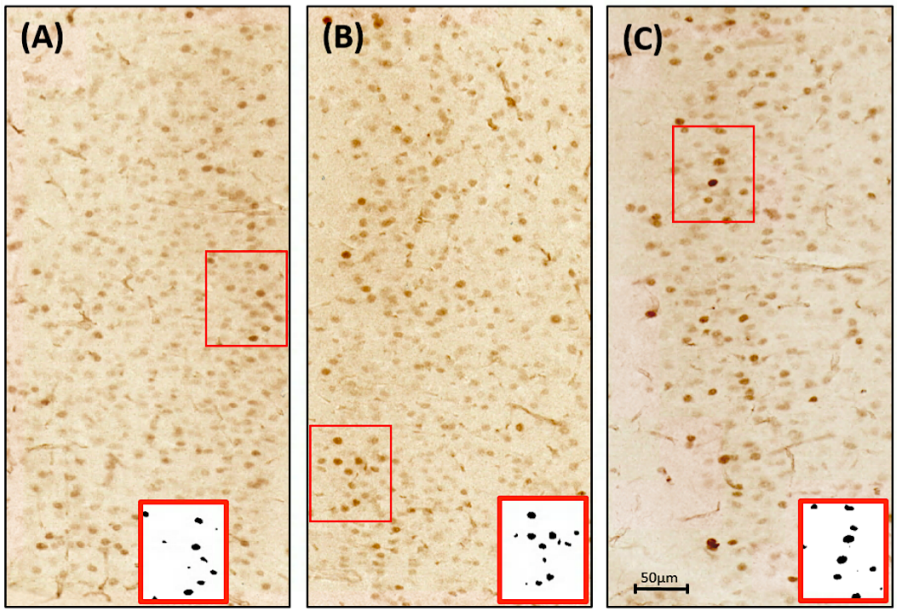

Figure 3.8 Representative scans of coronal sections showing the c-Fos-positive cells in the ACC from the groups in cohort 5; (A) ctrl (B) CC1 and (C) CC2. The quantification of c-Fos immuno-positive cells was performed after sections were processed into binary images as depicted in red squares at the corner of the figures shown.

Multivariate variance analysis, MANOVA, showed that there was an almost statistically significant difference in the number of c-Fos positive cells in ACC between the groups [$F(4, 14) = 3.02$, $p = 0.054$; Wilk's $\Lambda = 0.29$, partial $\eta 2 = 0.46$] Since the MANOVA showed no significant differences between the groups across all the dependent variables, further post hoc tests were not conducted. However, the results were examined for possible trends between the groups.

Within the dACC, the observed trend indicated that the CC2 group (M=161, SEM=14.8) showed the highest number of c-Fos positive cells followed by CC1 group (M=131.5, SEM=14.8) and ctrl group (M=128.3, SEM=17.1). This trend was also consistent for the groups within the vACC. However, the mean differences reported here were much larger, indicating a much stronger difference between the groups. The reported means and standard errors for the groups are as follows: CC2 (M=162.3, SEM=20.3), CC1 (M=143, SEM=20.3) and ctrl (M=103, SEM=23.4). Also, the partial eta-squared for the dACC and vACC were 0.26 and 0.32 respectively, indicating the vACC had a much stronger effect size. In addition, examination of the ACC as a whole, and comparing the number of c-Fos counts in the different groups showed that the trend remained preserved (Figure 3.7).

Putting it all together, the c-Fos results suggest that; (1) c-Fos activation in the ACC negatively correlates with LR arm choices and that (2) c-Fos activation in ACC is highest when cognitive control based decisions favor an equally rewarding choice that requires less effort.

4 DISCUSSION

4.1 Summary of main findings

Cognitive control is an element of decision-making that is largely affected in persons suffering from depression, substance abuse, anxiety disorder and schizophrenia (Bechara, 2001; London et al., 2000; Murphy et al., 2001; Rahman et al., 2001). Studies have shown that decision-making on the effort T- maze is ACC dependent and involves cognitive control in rats (Hosking et al., 2014; Rushworth et al., 2004; M. E. Walton et al., 2003). Here we show that, on the effort T- maze, mice are more willing to climb the high barrier to get the large reward with 8 vs 5cm barrier combination. Thereby establishing a protocol for a mouse version of the effort T-maze that was previously unavailable. Thus, making the study of effort based decision making now possible in all genetic mouse models. In addition, our c-Fos data suggests that levels of activation in the ACC correlate with suppression of the less optimal response in favor of the other competing choice response on the effort T-maze.

With the rewards fixed at 0.05ml chocolate milk for the SR arm and 0.2ml for the LR arm, barrier testing with Cohort 4 revealed that mice were more willing to climb the barrier in the LR arm when 8 and 5cm barriers were placed in the LR and SR arms respectively, compared to 10cm barrier in the LR arm and 5cm barrier in the SR arm. The c-Fos staining also revealed a strong negative correlation between c-Fos positive cells in ACC and LR arm choices. In addition, the observed c-Fos trend for Cohort 5 showed that, activation was highest in CC2, where cognitive control was a function of the barrier [LR=8cm(0.2ml) vs SR=5cm(0.2ml)], and least for ctrl, where cognitive control was a function of the reward [LR=8cm(0.2ml) vs SR=8cm(0.05ml)], with CC1 [LR=8cm(0.2ml) vs SR=5cm(0.05ml)] being marginally higher than the ctrl group. In CC1, cognitive control was a function of the reward and effort. Hence, CC2 depicted a classical cognitive control set-up, where we expected to see the highest c-Fos activation. However, that was not the case in this study. The possible reasons are discussed further.

4.2 Effect of barrier combination on arm choices

Prior to arriving at the barrier combination where mice were willing to climb the high barrier for the large reward, Cohort 1-4 was tested using various barrier combinations. The first testing block for all the cohorts was with no barriers, and mice always showed a strong preference for the LR arm. After testing block A, subsequent testing with different barrier combinations was introduced to assess effort-based decision making on the maze. When the difference between the efforts in terms of barrier size was 5cm (Cohort 1: 5cm vs no barrier, Cohort 2: 10cm vs 5cm), preference for the LR arm was reversed. This finding was consistent with M. Walton, Croxson, Rushworth, and Bannerman (2005) where they report a reversal in LR arm choices in rats on the effort T-maze following an increase in the energetic demands associated with the LR arm. In that study, the ratio of food reward was 1: 2 for the SR and LR arms respectively. With this fixed reward ratio, initial testing showed that rats tended to prefer to put in more effort to obtain the LR when a 30cm barrier was placed in the LR arm with no barrier placed in the SR arm. However, in the current study, the food reward ratio was set at 1:4 (SR: LR), with no barrier in the SR arm and a barrier of 5cm in the LR arm. Surprisingly, animals from Cohort 1 did not show any preference for the LR arm with the increased reward ratio and the relatively smaller barrier as compared to M. Walton et al. (2005) which may be accounted for by the level of motivation. Alternatively, this may be as result of the differences in how the two species (rats and mice) perceive and respond to what constitutes a reward worth investing higher effort.

Motivation and homeostatic states are known to widely influence decisions in cost/benefit situations (Bautista, Tinbergen, & Kacelnik, 2001; Paulus, 2007). In the study by M. Walton et al. (2005), rats were maintained at 85% of their free-feeding weight, whereas in this current study mice were maintained at 90% of their free feeding weight. Hence, motivational levels of the mice in this study could have contributed to their unwillingness to climb the 5cm barrier for the LR. Follow up testing blocks with equal barriers in both arms showed that when the effort required to obtain the reward was equated between the arms, mice returned to choosing the LR arm, ruling out insensitivity to effort and reward. Hence, their

motivational levels may have influenced the unwillingness to climb the 5cm barrier for the large reward.

To assess the choice pattern when the efforts are reduced, Cohort 3 was tested with 5 and 7cm barriers in the SR and LR arms respectively. This resulted in a strong preference for the LR arm. Surprisingly, when the rewards were equated in the SR and LR arms for the next testing block, mice continued to show a strong preference for the "LR" arm. This continued preference for the "LR'" arm despite the fact that it was no longer the most economically optimal choice may be explained by the tendency of animals to attribute a higher value to one of two stimuli associated with similar rewards if they previously had to work hard to obtain the reward associated with that stimulus, as confirmed in previous studies (Clement, Feltus, Kaiser, & Zentall, 2000; Denk et al., 2005; Kacelnik & Marsh, 2002). Alternatively, this may suggest that the mice did not perceive the difference in effort needed to climb a 5 or 7cm barrier. Upon increasing the LR arm barrier to 8cm with the SR arm barrier still fixed at 5cm, preference for the LR arm across the six mice in Cohort 4 was normally distributed and did not suggest any general trend. Hence, making this barrier combination the ideal one for testing how activation of cells in ACC correlates with different LR arms choice patterns.

4.3 Competition by mutual inhibition

The anterior cingulate cortex is widely known to hold representations of different courses of action during the performance of effort-reward tasks (Emeric et al., 2008; Ito, Stuphorn, Brown, & Schall, 2003). Flexibility in decision-making is believed to depend on the ACC's ability to update these representations to reflect changes in the effort-reward contingencies (Kennerley et al., 2006; Rushworth, Hadland, Gaffan, & Passingham, 2003). It is therefore not strange to assume the presence of different subpopulation of ACC cells representing LR and SR arm choices on the effort T-maze. At the network level, c-Fos activation associated with the SR arm choices in Cohort 4 may be accounted for by the concept of competition by mutual inhibition.

According to this network phenomenon, representations of each available alternative inhibit each other until activity remains in one, for a course of action to

be executed (Hunt et al., 2012). Based on this, different subpopulations representing the LR and SR arm choices may interact to inhibit each other until activity remains in one of the subpopulations. Additionally, the difficulty in decision-making associated with choice of arm may also influence how activity will emerge from the interactions of the sub-populations. For instance, if the choice between the arms is difficult, the two sub-populations may be co-active for a much longer period before one wins, resulting in more activated cells. However, the level of activation in this situation may be more related to task difficulty than choice patterns. Another caveat in using this phenomenon to account for the c-Fos expression pattern in Cohort 4 is that, we do not expect to see any significant difference in c-Fos expression between animals that make SR and LR arms choices. This being that, if the sub-populations representing the LR arm choices successfully inhibits the sub-population representing the SR arm choices or vice versa, c-Fos activation levels in response to arm choices should be anti-correlated, assuming the sub-populations are of similar sizes. Based on this limitation, the mechanism of competition by mutual inhibition may not adequately account for the strong negative correlation between LR arm choices and c-Fos expression, as well as the c-Fos expression pattern in Cohort 5.

4.4 Response inhibition

A more plausible phenomenon worth considering as accounting for the results from Cohort 4 and 5 is the concept of response inhibition. Response inhibition simply refers to processes involved in inhibiting or suppressing actions that are no longer required (Verbruggen & Logan, 2008). As intimated earlier, ACC is believed to hold representations of different courses of action and their corresponding outcomes, which are constantly updated to enhance flexibility during the performance of cost-benefit tasks. In the study by M. E. Walton et al. (2002), lesion to ACC did not totally impair LR arm choices because when reward ratio between the LR and SR arms was changed from 4:2 to 5:1, lesioned animals return to choosing the LR arm. Hence, the c-Fos expression patterns may represent a subpopulation of cells that are activated after the animal has suppressed a previously learned behavior for an alternative that appears more optimal. This suggestion is consistent with ACC's implicated role in performance monitoring (Emeric et al., 2008; Ito et al., 2003). In the current study, mice from Cohort 4 showed a strong preference for the LR arm during initial

trials with no barriers. However, upon introduction of the barriers, mice that perceived the effort not worth the reward had to strongly suppress or inhibit their initial LR arm preference for the SR arm and vice versa. This is consistent with studies that suggest that ACC is involved in the selection of competing alternatives (Kennerley et al., 2006; Pardo et al., 1990) and inhibition of learned responses that have become automatic based on a pre-existing plan (Gregory B Bissonette, Elizabeth M Powell, & Matthew R Roesch, 2013; Swick & Jovanovic, 2002). Representations of response inhibition in the ACC may therefore account for the strong negative correlation between the c-Fos expression and the number of LR arm choices, such that, the more a learned response is suppressed, the stronger the activation within that subpopulation.

Similarly, with respect to Cohort 5, the high c-Fos expression of CC1 and CC2 groups compared to the control group could be related to the representations of the different levels of response inhibition that were engaged. Prior to the introduction of the barriers, animals in CC2 made random choices to the reward arms because both arms were equally rewarding and did not require any effort. However, the introduction of two different barriers (LR=8cm vs SR=5cm) required that they deliberately suppress choice runs to the "LR" arm so that they can go to the equally rewarding "SR" arm that required less effort. This may therefore explain why this group showed the highest c-Fos expression. For the CC1 group, since animals were trained with different rewards (LR arm=0.2ml vs SR arm=0.05) during the no barrier-testing block, animals developed a strong preference for the LR arm. Upon introduction of different barriers to the arms (LR arm= 8cm vs SR arm=5cm) one mouse reverted to the SR while the other continued to choose the LR arm. The change in choice pattern suggested some element of cognitive control; requiring the suppression of LR arm choices in favor of the SR arm. Although the mouse that reverted to the SR arm had more c-Fos positive cells in the ACC (278.5 vs 271), the observed difference was too small to be fully accounted for by the theory of response inhibition. In the control group, animals did not have to suppress any behavior because the introduction of equal barriers after days of no barrier testing with different rewards (LR arm=0.2ml vs SR arm=0.05ml) implied that choice

behavior was still a function of the reward, and not the effort. So mice in this group did not have to suppress any learned response because all mice continued to visit the LR arm. This may suggest that ACC activation is purely a function of effort and not reward. However, it would be too hasty to draw such a conclusion since the rewards were held constant throughout the entire training phase, allowing animals to form stable memories of the reward locations. Hence, making it difficult to make definite attributions of ACC activation solely to effort. To fully test this using c-Fos staining, a reversal of this protocol where mice are simply trained to discriminate between a high barrier and low barrier with the introduction of rewards for one day, followed by perfusion can be implemented.

Although these findings agree with the literature on the role of ACC in response inhibition, they are at variant with some fMRI studies that show that activation in the ACC is associated with effortful decisions when there are competing alternatives with varying reward outcomes (Mulert et al., 2008; M. E. Walton et al., 2004). However, it is worth noting that, the low temporal resolution of fMRI studies can make it difficult to time stamp activation to behavior with precision. Moreover, response inhibition during cognitive control forms a part of other processes going on during cognitive control, which may be difficult for fMRI to tease out. Thus, based on our study, we propose that activation in ACC is more associated with "optimal" decision-making in the face of competing alternatives, and not just with effortful decisions.

Cognitive control requires the successful suppression of learned responses that are no longer optimal based on the changes in context. Hence, the pattern of c-Fos expression could therefore represent a subpopulation of cells in the ACC that are activated during the suppression of an already learned reward guided response for optimal decision making. This is further corroborated by a large number of studies that also show selective activation of ACC in fMRI during incongruent trials where a prepotent response must be inhibited for optimal decision-making (Carter et al., 1995; Pardo et al., 1990; K Sasaki et al., 1993; Taylor et al., 1994).

5 CONCLUSION

In conclusion, the results suggest that c-Fos activation in the ACC correlates with suppression of one response in favor of the other during cognitive control based decision-making. Difficulties in exercising cognitive control in decision making has often been associated with damage to the ACC (Fellows & Farah, 2005; Swick & Jovanovic, 2002). Hence, an addition to our understanding of its contribution to the decision making process is critical to developing effective interventions. Multiple lines of evidence have robustly suggested that ACC holds representations of effort-reward contingencies that guide optimal decision-making (Kennerley et al., 2006). However, not much is known about the unique contribution of this region to the overall decision-making output. Thus, by employing new technologies such as optogenetics, and the use of mouse models of psychiatric diseases, future studies should be able to further elucidate the functional role of this structure in the decision-making circuitry, as well as set the stage for the development of effective interventions for affected neuropsychiatric patients.

5.1 Confounding factors

There are of course possible confounding factors that need to be acknowledged before interpreting the results and drawing conclusion. For example, due to the high sensitivity of the ACC to pain (Bullitt, 1990; Johansen, Fields, & Manning, 2001; Navratilova et al., 2015; Rainville, Duncan, Price, Carrier, & Bushnell, 1997; Yan et al., 2012), imperfect anesthesia before and during perfusion could contribute to the c-Fos activation in the ACC. This was unlikely to have happened during perfusion as great care was taken to prevent this. However, prior to perfusion, toe pinching was applied to check the depth of the anesthesia. This might have influenced the result. It is also important to take into account that the results from this study are correlational and do not imply a causal relationship. Moreover, expression of c-Fos has been found to be activated by both GABA-ergic and glutamatergic neurons (D'Alessandro & Harrison, 2014; Staiger et al., 2002). Hence, making it difficult to determine the activated cell types in this study as well.

5.2 Future directions

Most of what is known about the ACC's involvement in cognitive control is based on fMRI, inactivation and lesion studies. As intimated earlier, these approaches come with a myriad of caveats. Hence, for future studies, it will be interesting to employ optogenetics, which may allow one to overcome some of the aforementioned limitations. This technique allows for a more specific manipulation and identification of neuronal populations without directly interfering with untargeted regions. By way of adeno-associated viruses (AAVs) viral vectors, microbial opsins such as halorhodopsin (eNpHR) and channelrhodopsins (ChR) can be expressed in cells in the ACC for selective inhibition and excitation respectively. Thereby making it possible to assess the effect of selective neuronal manipulation at different time points while animals make choice runs on the effort T-maze. Now that we have established an effort T-maze protocol for mice, one can also use Cre-expressing mouse lines. This makes it possible in combination with Cre-dependent AAV viruses to limit the expression of the opsins to specific neuronal subtypes and thus investigate the causal relationship at an even finer scale. Furthermore, the rigidity in decision-making found in persons suffering from many psychiatric diseases can be addressed using mouse models of these diseases on the effort T-maze.

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Appendix A

Experiment on the involvement of ACC in error coding

Background

In connection with ACC's implicated role in conflict monitoring and cognitive control, multiple imaging studies show that it also plays an important role in error detection (Botvinick et al., 2001; Coles, Scheffers, & Holroyd, 2001; Scheffers, Coles, Bernstein, Gehring, & Donchin, 1996). This suggestion has primarily been based on observations of the error related negativity (ERN) potential in the ACC following commission of errors (Henderson et al., 2006). In addition, tetrode recordings in mice also suggest that ACC contains cells that respond to errors. The evidence comes from a study by Weible, Rowland, Monaghan, Wolfgang, and Kentros (2012) where they discovered that following familiarization of mice to two objects, the removal of one object caused some cells to begin firing at the absent object's location (Fig. S 1). These cells may be involved in signaling errors, a discrepancy between expectation and outcome, a claim that is supported by previous studies using electroencephalography (EEG) in humans. Thus to identify the true identity of these cells, this study set out to identify "error" cells in the ACC and to record from them in two different error related contexts using the T-maze and object removal task.

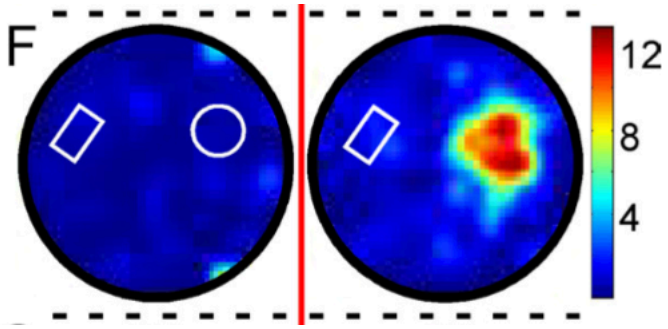


Figure S 1 Responses of ACC neurons to object removal. Rate maps of ACC neurons before (first column) and after (second column) removal of one object (adapted from Weible et al. (2012).

Hypothesis

True error cells, signaling discrepancies between expectations and outcomes will have similar firing properties across different error related contexts.

Methods

To achieve this, ACC implanted mice were trained to perform a simple rewarded alternation in a T-maze based on the protocol by Deacon and Rawlins (2006). In this task, each trial consists of a sample and choice run (Figure S2). Animals were required to make choice runs to the arm opposite the sample arm for each trial to obtain the reward.

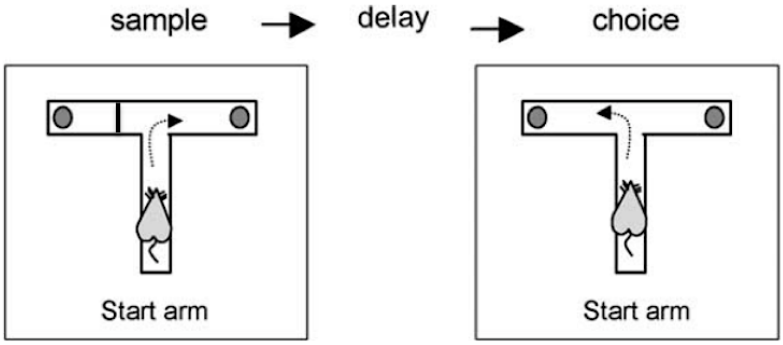


Figure S 2 Rewarded T-maze alternation. On the first or sample run, the mouse is placed on the stem of the T-maze and forced to sample a pseudoorandomly pre-determined arm for the reward. The mouse may then be removed from the maze for a delay period. After the delay, the mouse is returned to the stem of the maze, and, will be rewarded again upon choosing the alternate arm of the T-maze (adapted from Dudchenko (2004).

The maze was set up to establish a strong reward expectation on a particular arm, then switching or omitting the reward on random trials. In addition to the T-maze task, mice were trained on an object removal task based on the protocol used in Weible et al. (2012). Mice were then recorded from on the object removal task, following removal of one object. Then subsequently, on the T-maze with expected rewards randomly switched or omitted to generate errors.

However, prior to implanting the mice for recording, testing was done on the T-maze to show that mice can perform the rewarded alternation task (Figure S3). Upon establishing this, we tested the effect of time delays on alternations to assess the span of their working memory on the task (Figure S4.).

Results from tetrode recordings

Due to time constraints, we were unable to analyze the data from the recordings.

Behavior results: T-maze

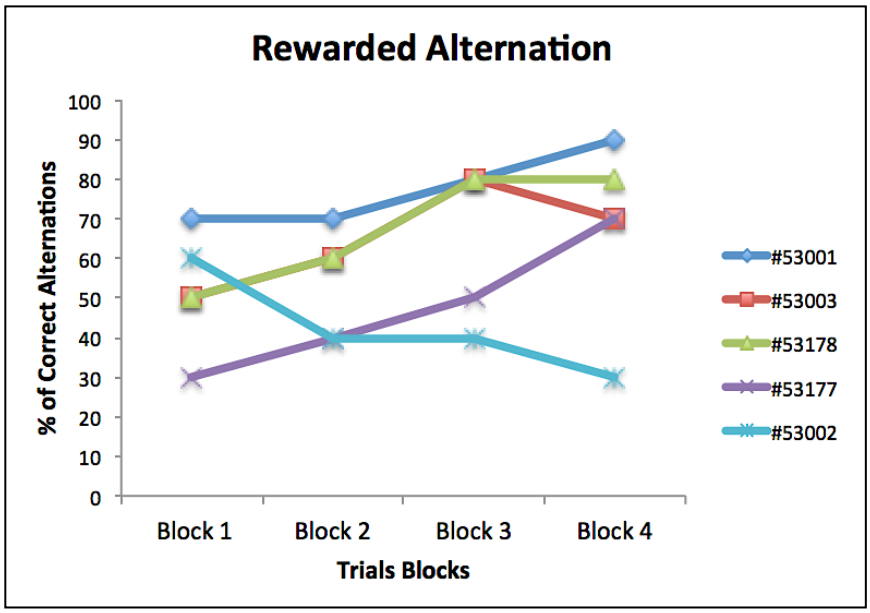


Figure S 3 The number of correct alternations for 5 animals over 4 days of testing. Each data point represents the number of correct alternations in 10 trials. Testing blocks 1, 2, 3 and 4 correspond to day 1,2,3 and 4 respectively. The chance level was set at 50% correct alternations. Hence #53002 was considered to be performing at chance level, and as a result was excluded from the delayed alternation test.

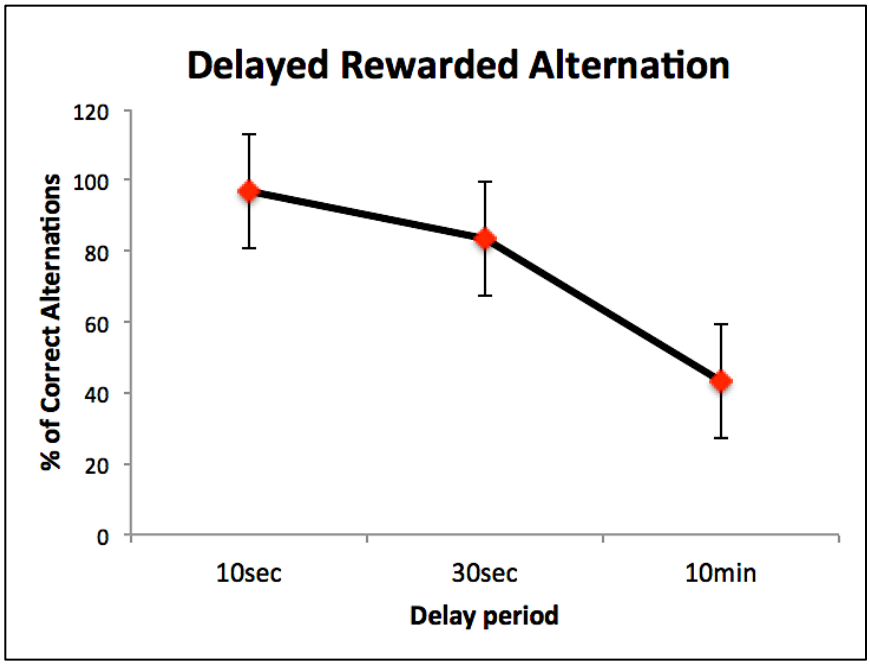


Figure S 4 The effect of delay on correct alternations on the T-maze. The figure shows the average performance of 4 mice on the alternation task with different delay intervals. From the figure, we see that the percentage of correct alternations continuously falls with increased delay periods.

Appendix B

Protocols

Behavioral training and testing protocols

Effort T-maze protocol (adopted from Bardgett et al. (2009) and M. E. Walton et al. (2002)

Day	Procedure
1-4	Habituation
5-6	Discrimination Training: Phase 1
7-8	Discrimination Training: Phase 2
9-10	Discrimination Training: Phase 3
11	Barrier training
12-	Barrier testing

Rewarded T-maze alternation protocol (adopted from Deacon and Rawlins (2006)

1. Habituation: This is done by filling food wells in the T-maze with chocolate milk, and putting animals in for about 3mins. Replenish the reward if necessary. This is done four times with gaps between exposures of at least 10 min.
2. Allow individual animals to run from the start arm with one goal arm blocked by a wooden block. Equal numbers of left and right runs are given. Ten trials in all (5 to the left and 5 to the right). This is repeated for two days
3. Set up for a trial run by baiting the sample and choice arms with reward, with access to the correct choice arm denied by a block.
4. Place the animal in the start area. Allow the animal to run to the sample arm and consume all of the reward.
5. When the animal has consumed all the reward, return it to the start arm and remove the block/raise the door to allow it to choose one. Allow time to consume the reward if correct. If it chooses incorrectly, remove it after a 150sec; ensure that it has definitely discovered that the sample well is empty.

Immunohistochemistry for c-Fos (DAB protocol)

1 Rinse sections 3 X 10mins in 0.125M phosphate-buffered saline (PBS).
2 Block in 2 X 10min in hydrogen peroxide (H_2O_2) in methanol.
3 Rinse 3 X 10min in PBS.
4 Rinse 3 X 10min in Tris- buffered saline with 10% Triton-X (TBS-Tx).
5 Block with 20% Normal Goat Serum (NGS).
6 Incubate in primary antibody, rabbit anti c-Fos polyclonal (1:1000) in 5% NGS and TBS-Tx for 48hrs at 4°C.
7 Rinse 3 X 10mins in TBS-Tx
8 Incubate in secondary antibody, biotinylated goat anti-rabbit IgB (1:2000) in TBS-Tx
9 Prepare ABC solution and leave on bench.
10 Prepare diaminobenzidine (DAB) solution.
11 Rinse 3 X 10mins in TBS-Tx
12 Incubate in ABC solution for 90mins
13 Rinse 3 X 10mins in TBS-Tx
14 Rinse 2 X 4min in Tris HCL
15 Incubate in freshly prepared DAB solution for 5mins
16 Rinse in Tris HCL to halt the DAB reaction.
17 Mount sections on glass slides and allow to dry
18 Coverslip mounted sections with entellan and xylene

Appendix C

Optimization of c-Fos antibody

To assess the validity and reliability of the antibody in detecting neuronal activation, a positive control experiment was set-up. In this experiment, 3 out of 6 animals from a home cage were introduced into a different cage with new items. Sixty to ninety minutes following this, animals from both groups were perfused and stained for c-Fos positive cells in the hippocampus. The induction of c-Fos in the hippocampus following introduction of animals to novel environments is a well-documented observation (Kempermann, Kuhn, & Gage, 1997). Based on this, it was hypothesized that the highest c-Fos expression will be seen in the brains of the new cage animals. Two different batches of the c-Fos antibody from Santa Cruz were tested on the brains from this experiment to determine the best batch for the ACC experiment.

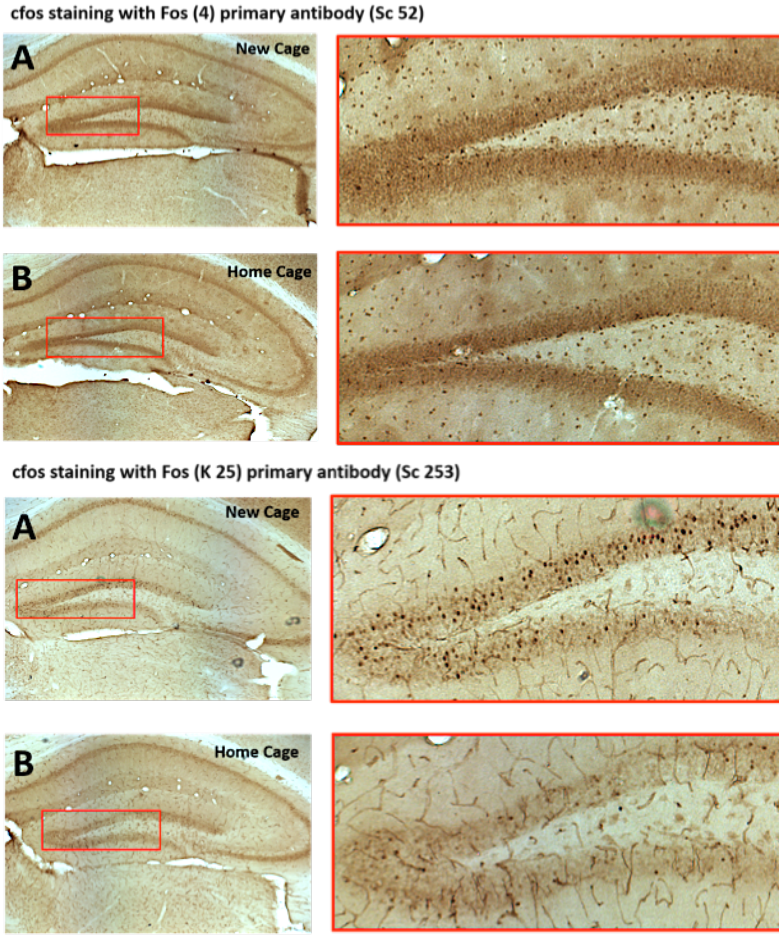


Figure S 5 Example of stained images with two different c-Fos antibodies. The figure shows staining on the hippocampi brains (dentate gyrus) from the aforementioned experiment; (A) New cage and (B) Home cage controls. Staining with Fos (K-25) antibody shows higher c-Fos positive cells in (B) compared to (A), as hypothesized. However, staining with Fos (4) did not show any difference between (A) and (B).

Appendix D

Image processing and automatic cell counting with Image J

Automatic cell counting was adopted in this study primarily to avoid the inconsistencies associated with the manual and semi-automatic counting approaches. Although advanced stereological counting approaches are available for more accurate counting, they usually come in handy in studies where the absolute counts are of primary importance. However, in this study, the focus was on the relative counts between the experimental groups. Thus, necessitating the use of the automatic cell counting approach.

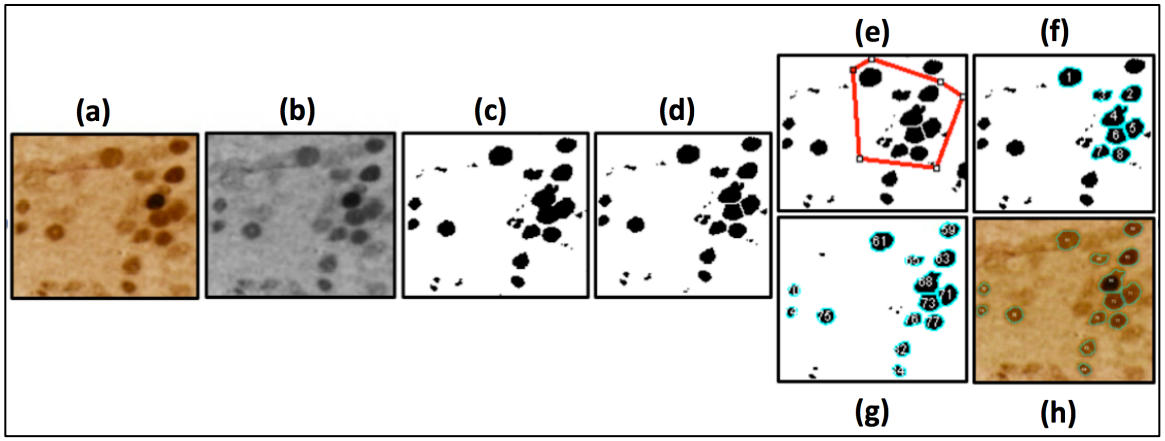


Figure S 6 Sequence of processing for automatic cell counting in Image J32.

(a) Raw scanned image

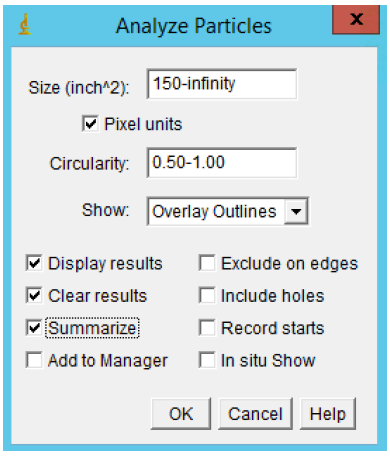
(b) Raw image binarized (converted to 8 bit grayscale)

(c) Maximum entropy threshold applied

(d) Watershed function applied to separate overlapping cells

(e) Using the polygon tool to select the region of interest by tracing pre-delineated regions performed in Illustrator

(f) Overlay outlines of cells automatically counted within the selected region based on set parameters in the particle analyzer window.



(g) Overlay outlines of cells automatically counted from the entire view based on set parameters in the particle analyzer window.

(h) Reverted image showing the overlay count outlines on the original sample image for verification of what the particle analyzer counted based on the set parameters.

Appendix E

Examples of stained images from Cohort 4

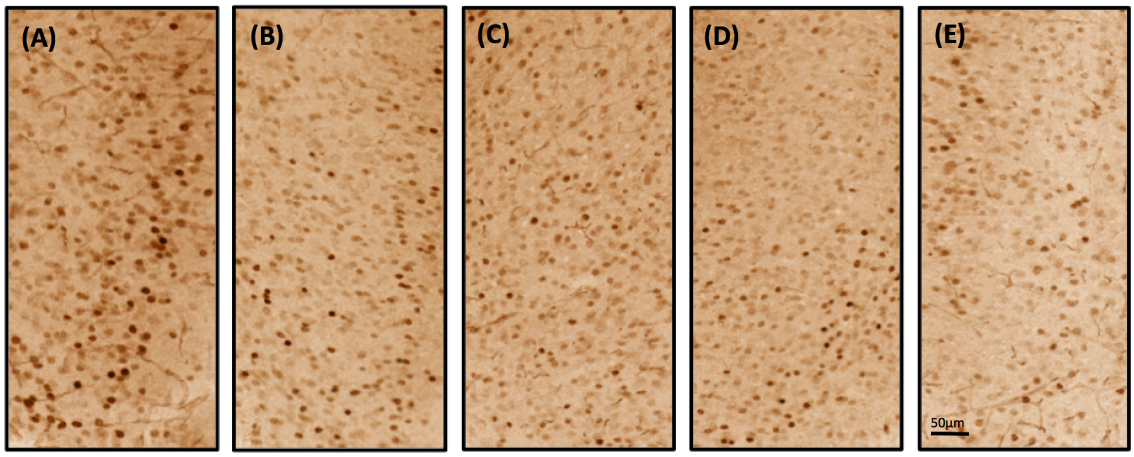


Figure S 7 Representative scans of coronal sections showing the c-Fos-positive cells in the ACC from the animals in cohort 4; (A) #58668 (B) #58669 (C) #58667 (D) #58666 (E) #58670.

Appendix F

Summary c-Fos counts for individual mice and arm choices after introduction of barriers

Mouse ID	Group	c-Fos counts	LR arm	SR arm
#53573	ctrl	223	100%	-
#58657	ctrl	235.5	100%	-
#58656	CC1	271	100%	-
#58659	CC1	278.5		70%**
#58672	CC2	333	-	100%**
#58658	CC2	313.5	-	100%**

**switch from prepotent arm choices