

Endocannabinoid Modulation of Post-Ischemia Depression

Marika Bonneville

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University of Ottawa's Institute of Mental Health Research at the Royal
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Faculty of Medicine
University of Ottawa

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Abstract

Post-ischemia depression (PID) is a condition that affects approximately 30% of survivors from stroke or cardiac arrest and has an important impact on patients' quality of life. Previous studies support important roles of the endocannabinoid (eCB) system in depression and brain ischemia. This study attempts to link all three variables together by investigating the role and mechanism of eCB signaling in the development of PID.

A global ischemia + hypotension model was used to induce a PID phenotype in CD1 mice. Three ischemic time frames were tested, and even though all three could induce significant cell death in the CA1 region of the hippocampus, only the 15-minute time point led to an increased immobility time on the forced swimming test (FST).

The main goal of this study was to investigate the effect of a cannabinoid type-1 receptor (CB1R) antagonist/inverse agonist, AM281, on the development of two depressive symptoms: anhedonia, measured with the sucrose preference test (SPT), and behavioral despair, measured with the FST. AM281 administration was able to significantly reduce the symptoms of anhedonia and behavioural despair.

Subsequently, the mechanism behind this antidepressant-like effect was investigated. Administration of bicuculline with AM281 did not significantly affect the antidepressant effect on the FST, therefore suggesting that AM281 does not act on GABAergic synapses. A similar protocol was adopted with NVP-AM077, where its administration combined with AM281 was able to block the effect of AM281, thus confirming the importance of glutamatergic synapses for the antidepressant effect of AM281. Furthermore, the administration of a TAT-GLUR2 peptide did not significantly affect the effect of AM281, implying that the astroglial cell-mediated LTD (long-term depression) at glutamatergic synapses is not involved in the antidepressant effects of AM281.

Finally, a bilateral intra-BLA (basolateral nucleus of the amygdala) administration of AM281 was able to reduce the immobility time on the FST.

In conclusion, these results highlight the important contribution of BLA glutamatergic synapses to the antidepressant-like effect conferred by AM281.

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List of Abbreviation & Chemical Names

2-AG	2-arachidonoylglycerol
AEA	Anandamide
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide
AMPAR	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	Adenosine tri-phosphate
BCCAO	Bilateral Common Carotid Artery Occlusion
Bicuculline	(6R)-6-[(5S)-6-methyl-5,6,7,8-tetrahydro[1,3]dioxolo[4,5-g]isoquinolin-5-yl]furo[3,4-e][1,3]benzodioxol-8(6H)-one
BLA	Basolateral Nucleus of the Amygdala
CB1R	Cannabinoid Type-1 Receptor
CB2R	Cannabinoid Type-2 Receptor
CNS	Central Nervous System
CUMS	Chronic Unpredictable Mild Stress
DAG	1,2-diacylglycerol
DEA	Docosatetraenoylethanolamide
DGL	Diacylglycerol Lipase
DH	Dorsal Hippocampus
DMSO	Dimethyl Sulfoxide
eCB	Endocannabinoid
FAAH	Fatty Acid Amine Hydrolase
Fluoxetine	N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine
FST	Forced Swimming Test
GABA	γ -Aminobutyric acid
HC	Home Cage
HPA	Hypothalamic-Pituitary-Adrenal

IP	Intraperitoneal
Ketamine	(RS)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone
KO	Knock-Out
LTD	Long-term depression
LTP	Long-term potentiation
MAGL	Monoacylglycerol Lipase
MCAO	Middle Cerebral Artery Occlusion
MDD	Major Depressive Disorder
NAE	N-acylethanolamine
NMDAR	N-Methyl-D-Aspartate Receptor
NVP-AAM077	(1S)-1-(4-bromophenyl)ethyl]amino)-(2,3-dioxo-1,4-dihydroquinoxalin-5-yl)methyl)phosphonic acid
OEA	Oleolyethanolamide
PE	Phosphatidylethanolamine
PEA	Palmytoylethanolamide
PNS	Peripheral Nervous System
PLC	Phopholipase C
PID	Post-ischemia Depression
PSD	Post-Stroke Depression
RO-25-6981	(α R, β S)- α -(4-Hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidinepropanol maleate
SPT	Sucrose Preference Test
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
THC	Δ^9 -tetrahydrocannabinol
VMH	Ventral Medial Hypothalamus

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*“With ordinary talent and
extraordinary perseverance,
all things are attainable.”*

Thomas Fowell Buxton

Chapter 1: Introduction

1.1 – Defining ischemia, depression and post-ischemia depression

Ischemia is the most common cause of brain injury (Giffard & Swanson, 2005). Acute brain ischemia includes global and focal ischemia, which are produced most frequently via cardiac arrest and cerebral artery blockade, respectively. Approximately 45% of patients surviving from cardiac arrest, a major cause of morbidity and mortality worldwide, developed post-ischemia depression (PID) (Roine et al, 1993). Stroke, which affects approximately 38,000 Canadians each year (Public Health Agency of Canada, 2011), is the leading cause of adult disability worldwide (WHO, 1999). Over 750,000 strokes, both new and recurrent, happen each year (Yang et al, 2003), with the financial cost per survivor amounting to approximately \$40,000 USD annually (Schellinger et al, 2004). PID is the most frequent psychological sequela following stroke, as PID has been observed in 1/3 to 1/2 of patients during their early or late stage of stroke recovery (Dafer et al, 2008; Hackett et al, 2005; Kotila et al, 1998; Loubinoux et al, 2012; Paolucci, 2008). Despite these facts, the mechanism underlying PID continues to remain unknown (McCann et al., 2014; Paolucci, 2008; Robinson, 2002), and traditional antidepressants are producing limited efficacy in treating patients' PID (Hackett et al, 2008a, 2008b; Loubinoux et al, 2012).

1.1.1 – Ischemia (Stroke and cardiac arrest)

Stroke can be defined as the interruption of blood flow to the brain either due to a thrombus (ischemic stroke, which accounts for 85% of all strokes) or a brain bleed (hemorrhagic stroke) and usually only affects one brain hemisphere (Heart and Stroke Foundation, 2014; Schellinger et al, 2004). On the other hand, cardiac arrest can lead to global cerebral ischemia where all blood circulation to the brain is interrupted and injury will occur in the most vulnerable cerebral areas such as the CA1 region of the hippocampus (Darwin, 1995). In both cases, focal or global cerebral ischemia, the ischemic cascade is initiated where oxygen supply to the regions of the brain affected become almost non-existent, thus creating an energetic deficit. Adenosine triphosphate (ATP) levels become critically low, leading to an ionic imbalance and the release of neurotransmitters, especially glutamate. This increase in glutamate release is known as excitotoxicity and binding of this neurotransmitter to its N-Methyl-D-aspartate receptor (NMDAR) promotes calcium entry and leads to necrosis or apoptosis (Xing, 2012).

1.1.2 – *Depression*

According to the DSM-V, to be diagnosed with Major Depressive Disorder (MDD), patients need to show at least 5 of these symptoms almost every day for a period of 2-week time: depressed mood, markedly diminished interest or pleasure, significant weight loss, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthlessness or excessive or inappropriate guilt, diminished ability to think or concentrate and recurrent thoughts or death. These symptoms must

also impair the person's ability to function in everyday activities (American Psychiatric Association, 2013).

Depression can also be present in patients who are affected by another disease, predominately those with a chronic medical illness. Some chronic illnesses, especially if they restrict the patient (cannot perform everyday activities), will lead to the development of certain depressive symptoms. On the other hand, it has been demonstrated that certain disorders alter the normal biological function of the brain and will increase the odds of developing depression. These disorders usually affect the central nervous system (CNS), the vascular system or the endocrine system (Simon, 2001).

1.1.3 – *Post-Ischemia Depression*

Many neurological disorders are associated with neurodegeneration and, like any of them, loss of neuronal tissue in patients affected by brain ischemia can lead to certain deficits. These impairments can be defined as post-ischemia conditions and can be divided into different categories. The better known conditions are physical, which is comprised of paralysis, fatigue and seizures, and cognitive, which can include memory loss and aphasia. As well, more and more emphasis is being put on the emotional conditions of ischemia where PID is the most studied aspect (National Stroke Association, 2016). PID affects a large proportion of stroke and cardiac arrest survivors and has a deep impact on the quality of life of the patients. In fact, '[PID] is associated with an increased disability, increased cognitive impairment, increased mortality,

increase risk of falls and, finally, with worse rehabilitation outcome' (Paolucci, 2008). By developing new screening processes and identifying new treatment options, the quality of life of these patients could easily be improved and these discoveries could even have an impact on physical and cognitive rehabilitation (Paolucci, 2008; Dwyer Hollender, 2014).

PID has been identified as being the result of social factors (isolation of the patient), behavioral factors (difficulty performing past activities) and more importantly neurobiological factors (changes to the brain due to the ischemic injury) (Feng, Fang & Liu, 2014). Some theories behind the development of PID include the 'biogenic amine theory', where lesions to the limbic system due to the ischemia might be responsible for the development of the depressive symptoms. On the other hand, some argue that cytokines are responsible for the development of this condition since they play both an important role in the etiology of stroke and depression. Understanding these key aspects that lead to PID is an important area of research as it can lead to the development of new therapeutic treatments (Spaletta et al., 2006).

In the clinical setting, two main antidepressants are prescribed: tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). SSRIs are more popular for the treatment of PID since they are more tolerated as TCAs can have an effect on the cardiovascular system and therefore are not the best option following stroke or cardiac arrest. Previous studies have identified SSRIs as having an effect on emotionality and improving quality of life following ischemia but not directly on PID. This suggests that the serotonin system is not the key aspect responsible for the

development of PID. Further research should focus on other systems or key aspects that are altered following cerebral ischemia and identifying which one/ones could play a role in the development of PID. This might lead to the development of new therapies that would have a direct impact on the symptoms of PID (Paolucci, 2008; Dwyer Hollender, 2014).

1.2 – The endocannabinoid system

1.2.1 – Overview

The use of *Cannabis* for medical purposes goes back to three millenniums BC where it was administered to relieve a variety of symptoms including pain and anxiety (Palcher, Batkai & Kunos, 2006). The discovery of Δ^9 -tetrahydrocannabinol (THC) as the main active ingredient in cannabis led to the identification of the endocannabinoid (eCB) system. The eCB system includes two main receptors, some endogenous lipids, which can also be referred to as eCBs, as well as degradative enzymes which are important for the regulation of the system. This system acts in a retrograde mechanism by regulating the probability of neurotransmitter release at the pre-synaptic terminal (Castillo et al, 2012).

1.2.2 – Endocannabinoids, receptors and mechanism of action

The two main receptors associated with the eCB system are the cannabinoid type-1 receptor (CB1R) and the cannabinoid type-2 receptor (CB2R). CB1R can be most prominently found in the CNS, and CB2R is primarily located on immune cells and in the

peripheral nervous system (PNS); which is why CB1R is the main focus of this study. Different compounds can bind to these receptors including natural cannabinoids (THC), synthetic cannabinoids, as well as eCBs. The most studied endogenous lipids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). AEA is synthesized via two main steps: phosphatidylethanolamine (PE) converted to N-arachidonoyl-PE by N-acyltransferase and cleavage of this latter molecule by phospholipase D to yield AEA. 2-AG synthesis starts off with a different membrane lipid, phosphatidylinositol, which is converted to 1,2-diacylglycerol (DAG) by a phospholipase C. DAG is then converted to 2-AG by the enzyme diacylglycerol lipase (DGL). Synthesis of these eCBs is performed on demand where constant stimulation of the post-synaptic neuron will increase Ca^{2+} concentration and send a signal to the cell to synthesize and release eCBs. The eCBs will then travel to the pre-synaptic cell either through simple diffusion or with the help of transporter proteins (Piomelli, 2003; Palcher, Batkai & Kunos, 2006).

CB1Rs located at the pre-synaptic terminal of glutamatergic and GABAergic synapses are G_i/o protein coupled receptors. Activation of this receptor leads to a reduction in neurotransmitter release either via short-term stimulation which blocks voltage-gated Ca^{2+} channels or in the case of long-term stimulation, inhibition of adenylyl cyclase. More recent studies have identified CB1Rs present on astrocytes. The mechanism of action of this receptor in this cell type is a little different since the receptor is coupled to a $G_q/11$ protein. Activation of this receptor leads to the activation of phospholipase C (PLC) which in turn leads to increased Ca^{2+} concentration in the astrocyte and glutamate release. Therefore, activation of CB1R on astrocytes stimulates

glutamate release but activation of the same receptor on glutamatergic cells decreases glutamate release and as a result have opposite effects (Castillo et al., 2012).

As was mentioned previously, eCBs are synthesized on demand and thus requires control through specific degradative enzymes to ensure that there isn't an overload of the system. Each eCB has its specific degradative enzyme. AEA is mainly degraded by fatty acid amine hydrolase (FAAH), an enzyme mainly located at the post-synaptic cell intracellularly. On the other hand, 2-AG is degraded by monoacylglycerol lipase (MAGL) located at the presynaptic membrane (Alger & Kim, 2011).

1.2.3 – *CB1R location and functions*

CB1R is expressed in high density throughout the brain and is in fact 'the most abundant G-protein-coupled receptor in the mammalian brain'. This supports the assumption that the eCB system could easily have effects on a variety of physiological processes and could be used as a therapeutic target for a multitude of neurological diseases (Piomelli, 2003). CB1Rs are prominently located in the cortex, cerebellum, hippocampus, amygdala and basic ganglia (Pacher, Batkai & Kunos, 2006; Piomello, 2003). Taking under consideration the repartition of this receptor, it is evident that it has an important role in the process of learning and memory (hippocampus), the regulation of emotions (amygdala), the control of movement (basal ganglia) and many more physiological processes (Piomelli, 2003).

Different categories of ligands exist for CB1R, one of them being natural cannabinoids which are derived from plants and include THC as well as cannabidiol. HU-

210 is an extremely potent agonist to CB1R and can be categorized as a synthetic cannabinoid. There are also the endogenous ligands, AEA and 2-AG, which were described earlier. Finally, the category important for this study, the antagonists and inverse agonists, which include rimonabant, AM251 and AM281, that block the activity of the receptor. This wide diversity of ligands opens the door to a variety of potential pharmacological manipulations that could lead to a better understanding of the eCB system and its role in the development and treatment of many disorders (Seely et al., 2011).

1.3 – The endocannabinoid system and depression

1.3.1 – Background

Cannabis has been used for centuries for its positive effects on mood. This has led scientists to the hypothesis that the eCB system could have a role in the regulation of emotion, especially anxiety and depression. Researchers have even identified an antidepressant effect of cannabis use in individuals affected by depression (Gorzalka & Hill, 2011; Rubino, Zamberletti and Parolaro, 2015). On the other hand, studies have suggested that heavy cannabis use could actually lead to the development of depression. These contradictory results point towards the complexity of the eCB system as receptors can be found throughout the body and in many different brain regions (Lev-Ran et al, 2014).

Rimonabant, CB1R antagonist, was first examined for its potential to treat obesity and was suggested to have a great effect on controlling patients' metabolism. As

this drug progressed through clinical trials, an effect on mood was identified where some patients who received the drug reported an increase in anxiety and depressive symptoms. This unexpected discovery led to multiple studies investigating the effect of both CB1R agonists and antagonists on mood regulation (Hillard & Liu, 2014).

1.3.2 – Effect of eCB agonists on depression

Animal studies with the use of agonists have had fairly constant results. Most studies focus on the forced swimming test (FST) where more time spent immobile can be interpreted as behavioral despair, a symptom of depression. Studies performed on male rats have supported the antidepressant-like effect of HU210 and Win 55212-2, two CB1R specific agonists. Furthermore, AM404 and URB597 which block the activity of one or both degradative enzymes also reduced immobility time. These data show that increasing CB1R signaling either via administration of synthetic agonists or by increasing eCB signaling leads to significant antidepressant-like effects (Gorzalka & Hill, 2011; Patel & Hillard, 2009).

1.3.3 – Effects of eCB antagonists on depression

Compared to the effect of agonists on depressive symptoms, studies focussing on antagonists lead to contradictory results. Rimonabant is one of the most studied antagonists. As was discussed above, human studies have found a depressive effect of the drug (Hillard & Liu, 2014). On the other hand, administration of rimonabant in C57 mice had no effect (Steiner et al. 2008; Patel & Hillard, 2009) and administration of

acute high doses (10mg/kg) of the antagonist had antidepressant effects on the FST (Shearman et al. 2003; Patel & Hillard, 2009). The differences in results between these studies could be due to varying experimental protocols, differences in species or strains as well as the type of behavioral test used (Patel & Hillard, 2009). It is also important to consider the location of these receptors as they can be found on glutamatergic, GABAergic and astrocytic cells. Blocking receptors on each of these cell types has varying effects and thus can explain the varying effects of antagonists as observed in these studies (Castillo et al., 2012).

Mice lacking CB1R have been studied and the results show, in most studies, an increase in depressive-like symptoms. These mutant mice show increased activity of the hypothalamic-pituitary-adrenal (HPA) axis as well as a reduction of neural plasticity. These effects show the important role of the CB1R for the regulation of emotions (Valverde & Torrens, 2012).

Taken together, these results confirm the implication of the eCB system in the development and treatment of depression.

1.3.4 – *Effects of current antidepressants on the eCB system*

Even though modulating eCB signaling has been proven to have some potential for the treatment of depression, these drugs are not yet prescribed to patients in Canada. Current popular antidepressants include SSRIs, of which fluoxetine and citalopram are probably better known, as well as TCAs which include desipramine and imipramine. Studies lead to conflicting results where some treatments increased eCB

signaling and others decreased signaling in different regions of the brain. In a CB1R knock-out mouse, the increased serotonin efflux within the prefrontal cortex as a result of SSRI treatment was absent, highlighting the importance of CB1Rs for the effect of current antidepressants.

A more natural treatment of depression is voluntary exercise. A study found that eight to ten days of voluntary exercise was able to increase CB1R expression and signaling in the hippocampus. Exercise improves cell proliferation and treatment with a CB1R antagonist reduces this effect. Furthermore, this effect was completely blocked in CB1R mutant mice.

Taken together, these results emphasize the importance of the eCB system for the antidepressant effect. This further confirms the important role that the eCB system plays in the development of depression (Gorzalka & Hill, 2011).

1.4 – The endocannabinoid system and cerebral ischemia

1.4.1- Neuroprotection and eCBs

Most of the research studying the relationship between the eCB system and cerebral ischemia is focussing on the potential neuroprotective role of this system. Following ischemia, neuronal cell death does not happen immediately. In fact, the delay between the traumatic event and the injury varies but can usually be observed within a few days. This time frame is important as treatment with different compounds could reduce the brain damage as a result of the ischemia. These compounds are known as neuroprotective agents (Pellegrini-Giampietro, Mannaioni & Bagetta, 2008).

The first study investigating the effect of CB1R activation suggested that the administration of a CB1R agonist could reduce neuronal loss following either global or focal ischemia and that this effect could be blocked by rimonabant (Capettini, et al., 2012). This outcome is thought to be a result of a protective effect against glutamate-mediated excitotoxicity (Hillard, 2008). Knocking-out the CB1R gene in mice lead to increased mortality following focal ischemia as well as increased cell loss and neurological deficits (Parmentier-Batteur, 2002). These results confirm the important neuroprotective role of CB1R signaling following ischemia. On the other hand, studies have also identified a neuroprotective role of CB1R antagonists including rimonabant. This effect was not found to result from excitotoxicity attenuation which suggests that CB1R agonists and antagonists confer their neuroprotective effects via different mechanisms. It is also important to note that some studies have also recorded detrimental effects of CB1R agonists and antagonists on neuronal cell death (Pellegrini-Giampietro, Mannaioni & Bagetta, 2008). In any case, these results strongly support the involvement of the eCB system in the ischemic cascade.

1.4.2 – *Post-ischemic effects on the eCB system*

1.4.2.1 – Endocannabinoids level – Studies report an upregulation of eCBs in the region of the injury following cerebral ischemia. eCB levels increase exponentially throughout time. Combining these results with the neuroprotective aspect of the eCB system, this suggests that the upregulation of the eCB system might be an endogenous

mechanism developed by the body to reduce, to a certain extent, the severity of the damage following ischemia (Capettini, 2012).

Post-mortem studies can help to understand what happens in ischemia as in both cases neurons die as a result of a lack of oxygen. AEA is a member of the family of N-acyl ethanolamines (NAEs) and early studies 'demonstrated that members of this family accumulate in tissues deprived of blood flow' (Hillard, 2009). Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) both members of the NAE family, increase drastically after death in a time-dependent manner. AEA is present in lower concentrations but significantly increases; as can be measured a couple of hours' post-mortem. 2-AG is harder to study post-mortem since its concentration increases rapidly and goes back down within a few minutes following death (Hillard, 2009).

Focussing on ischemia and reperfusion models, studies have identified an even larger increase in NAEs, including PEA, AEA and docosatetraenoylethanolamide (DEA) when compared to studies which used permanent ischemia. One study even compared levels of AEA following ischemia or ischemia and reperfusion. It became apparent that reperfusion further increased AEA levels compared to rats who only received the ischemia. These results demonstrate that NAE increase is due to the ischemia and that reperfusion exacerbates this effect. On the other hand, reperfusion is responsible for the increase in AEA content. One human case of hemispheric stroke supports these results, where PEA, OEA and AEA concentrations were significantly upregulated in the tissue surrounding the injury (Hillard, 2009).

Studies performed in rats and mice demonstrated that there was either no change or a decrease in 2-AG concentration following ischemia or ischemia and reperfusion. However, an interesting discovery was made where mice who received the sham surgery had a higher 2-AG brain concentration. This suggests that the surgery itself, anesthesia or the stress of the procedure, increases 2-AG levels and that ischemia has the opposite effect (Hillard, 2009).

1.4.2.2 – CB1R expression – As was explained earlier, two main eCB receptors can be found in the body. Since this study focusses on the CB1R, only the changes in CB1R expression following ischemia will be discussed. An ischemia and reperfusion study performed on rats identified an increased expression of CB1R around the infarct but not in the core starting two hours after the surgery (Jin et al., 2000). These results were further confirmed by another study which demonstrated an increase in brain CB1R mRNA following transient ischemia. However, no change was identified following permanent ischemia suggesting that the reperfusion is responsible for the increase in CB1R receptors' expression (Zhang et al., 2008; Hillard, 2009).

Taken together, these results point towards an upregulation of the eCB system following ischemia and reperfusion with an effect on both eCB and CB1R levels.

1.5 – Rationale for the Current Study

Considering the above information, most antidepressants used for the treatment of PID target the serotonin system and might not be the best option for all patients. Furthermore, focussing the research on a different system might lead to better

treatment options (Paolucci, 2008; Dwyer Hollender, 2014). Since the eCB system has an effect on such a wide variety of brain structures, including those responsible for the development of depression, it might make a good target for the treatment of PID (Pacher, Batkai & Kunos, 2006; Piomello, 2003). Finally, studies have demonstrated the implication of the eCB system in depression (Gorzalka & Hill, 2011) and ischemia (Hillard, 2009) yet at the beginning of this study, to our knowledge, no study had looked at all three variables together: eCBs, depression and ischemia. This study will attempt to fill that gap by focussing on the role and mechanism of eCB signalling in the development of PID.

1.6 – Aims

The first aim of this study was to establish and validate a reliable PID model where depression could be measured in the ischemic group seven days later using the FST; and significant cell loss could be quantified in the CA1 region of the hippocampus. This model would then be used to test the effect of acute AM281 administration on the FST and be compared to the effect of known antidepressants (fluoxetine and ketamine). It was hypothesised that AM281 would reduce the immobility time measured. If it did indeed have an antidepressant-like effect, we would then identify, with pharmacological manipulations, the cell-type responsible for this effect: glutamatergic, GABAergic or astrocytic cells. The second aim was to confirm these results using a different behavioral test: the sucrose preference test (SPT). We hypothesized that the results obtained with the SPT would reflect what was obtained with the FST. Finally, bilateral intra-BLA

(basolateral nucleus of the amygdala) administration of AM281 would be performed to determine the importance of this brain region for the antidepressant-like effect observed. We hypothesised that intra-BLA administration of AM281 would be sufficient to significantly reduce the immobility time on the FST. This study allowed us to investigate the effect of a CB1R antagonist with two behavioral tests as well as identify its mechanism of action; identifying an important cell-type and brain region.

Chapter 2: Materials & Methods

2.1 – Experimental Subjects

The subjects of this study are male CD1 mice obtained from Charles River Laboratories (Rochefort, Québec, Canada). Mice weighed between 22 and 25 grams when they arrived at the housing facility where they were habituated for a minimum of 5 days before any kind of manipulation. They were housed four per cage under standard laboratory conditions (ad libitum food and water; 12-hour light-dark cycle [7AM-7PM]). All experiments and procedures were in accordance with the guidelines set by the Canadian Council on Animal Care and the Animal Care Committee of the Institute of Mental Health Research (Ottawa, Ontario).

2.2 – Drugs & Drug Administration

Multiple drugs were used throughout this study in order to investigate the role of eCB signaling in the development of PID and compare the effect of the CB1R antagonist with common antidepressants. These drugs include:

- AM281 (Tocris – Burlington, ON), a potent and selective CB1R antagonist/inverse agonist. AM281 was either injected intraperitoneally (i.p) at a dose of 3mg/kg (Han et al., 2012) or in the BLA via cannulas at a dose of 50ng/0.2µL per side. Thirty minutes after drug administration mice were subjected to the FST or the SPT.
- Bicuculline (Sigma, Steinheim), a GABAA antagonist was administered 5 minutes prior to AM281 administration at a dose of 0.5mg/kg i.p.

- NVP-AAM077 (Novartis Pharma, AG), a NMDA receptor antagonist that has a higher selectivity for NR2A than NR2B containing NMDA receptors (Jimenez-Sanchez et al., 2014). It was administered 5 minutes before AM281 at a dose of 1.2mg/kg i.p.
- RO-25-6981 (Sigma, St. Louis), also a NMDA receptor antagonist but is selective for NR2B containing NMDA receptors (Jimenez-Sanchez et al., 2014). 6mg/kg of RO25-6981 was administered 5 minutes prior to AM281 administration.
- TAT-GLUR2 peptide (GL Biochem, Shanghai), blocks long-term depression (LTD) by blocking AMPAR endocytosis (Dalton, G. et al, 2008). The peptide and its control were both administered 30 minutes prior to the FST at a dose of 4mg/kg i.p.
- Fluoxetine (Sigma, St. Louis), SSRI and a popular antidepressant treatment. The drug was administered at a dose of 10mg/kg i.p 30 minutes prior to the FST.
- Ketamine (Sigma, St. Louis), NMDA receptor antagonist which has recently been used as an antidepressant. 10mg/kg of ketamine was injected i.p 30 minutes prior to the FST.

AM281 was dissolved in a solution of Dimethyl sulfoxide (DMSO): Tween 80: 0.9% NaCl (1:1:18) and was compared to its DMSO: Tween 80: 0.9% NaCl vehicle. All other drugs were dissolved in 0.9% saline and tested against its control 0.9% saline.

2.3 – Surgery

2.3.1 – Global Ischemia

This protocol was derived from Onken, Berger and Kristian (2012) where global ischemia is induced by bilateral common carotid artery occlusion (BCCAO) paired with the induction of hypotension. The reduction in mean arterial blood pressure was obtained by increasing isoflurane concentration (5%). In their study, C57 mice had been used and since it is well known that different strains of mice have different sensitivities to ischemia (Barone, et al., 1993), different ischemic time frames were first tested (10, 15 and 20 minutes).

Mice were anesthetized with 3% isoflurane while maintaining 0.5-0.6 N₂O:O₂. The common carotid arteries were isolated via neck incision. Isoflurane concentration was increased to 5% and 2 minutes later, the common carotid arteries were clamped using mini-clips (keep the clips on for 10, 15 or 20 minutes). Three minutes before the release of the clamps, the isoflurane level was set to 0%. The clips were removed and the neck incision was closed with nylon sutures. The body temperature was maintained at 37.0°C throughout the surgery with the use of a heating pad. Mice were kept on the heating pad until they woke-up and started moving around. They also received 0.2mg/kg of Metacam on the day of surgery and for two days following the surgery.

Sham-operated mice were kept anesthetized with 3% of isoflurane for 10, 15 or 20 minutes after the isolation of the common carotid arteries.

2.3.2 – Cannula implantation

Mice were placed under isoflurane anesthesia and were implanted with two guide cannulae (33 Ga) into both sides of the BLA (-1.35, +3, -4.75). One week later, they

were subjected to the global ischemia surgery as previously described. One week following forebrain ischemia, intra-BLA administration of AM281 (0.5 ng/side) was performed. After FST, brains were collected from the mice by transcardial perfusion with 4% paraformaldehyde and were then cut to confirm the correct cannula placement.

2.4 – Behavioral Tests

Mice were placed in the test room one hour prior to the behavioral test to allow for habituation. All behavioral tests were performed seven days following the ischemia surgery.

2.4.1 – Forced swimming test (FST)

Mice were placed into a Plexiglas cylinder (65x30 cm) filled with 10 cm of water at a temperature of 23-24°C. An automated behavioral tracking system (View Point Life Sciences, Montreal) quantified the immobility time -making only the necessary movements to stay up float- during the 10-minute test. A higher immobility time indicated a depressive-like phenotype (Can, et al., 2012).

2.4.2 – Sucrose Preference Test (SPT)

This test lasts a total of 6 days. On the first two days, mice received 1% (w/v) sucrose for 2 days followed by 2 days with free access to one bottle of water and one bottle of 1% sucrose. Mice are then deprived of water for 24 hours before having access

to both bottles (one of water and one of sucrose solution) for 1 hour in its home cage. The percentage of sucrose consumption is calculated after the hour of testing using this formula: $\text{sucrose consumption (g)} / \text{water + sucrose consumption (g)} \times 100$. For some of the experimental groups, a 10-minute forced swimming period is added one hour prior to the SPT. Drug administration was either done 30 minutes before the FST or 30 minutes before the SPT. Healthy mice would usually show a preference for the sucrose solution and mice with a depressive-like phenotype would not show this preference (Banar & Duman, 2008).

2.4.3 – Beam Breaks

Using an open field apparatus (40x40x40 cm), locomotor activity was measured as the number of beam breaks in a 10-minute period. The mouse movement interrupts infrared photobeams which are interpreted as beam breaks and are calculated by a computer.

2.5 – Histology

Seven days after the ischemia or sham surgery and right after behavioral testing, mice were transcardially perfused with saline and 4% paraformaldehyde. The extracted brains were then stored in the solution of 4% paraformaldehyde for at least 2 hours at 4°C. Tissue was dehydrated by being stored in a 20% sucrose solution for 24 hours and then a 30% solution for another day. Brains were then cut in 20µm-thick coronal slices using a cryostat (-1.7 to -2.5 from Bregma) to view the CA1 region of the hippocampus

(Franklin & Paxinos, 1997). Slices were then stained with Cresyl Violet for quantification of CA1 neurons in the hippocampus. Slices were examined at 40X magnification using an Olympus BX51 microscope attached to a Microfire by Optonics digital camera and image analysis software Picture Frame (v2.2). Counting was done on three brains slices for each mouse on both the right and the left hemisphere. Neuronal density for a given animal represents the average of those six measures.

2.6 – Statistical analysis

Statistics were completed on SPSS (version 22) where statistical significance was set at $p \leq 0.05$. Data was analyzed either with a one- or two-way ANOVA with Dunnett's (for Fig 1. only) or Tukey's (for all other figures) post-hoc test; or with an independent T-test.

Chapter 3: Results

3.1 – Establishing and Validating the PID model

The first step consisted of establishing a reliable model of global ischemia that would lead to the development of depression as measured with the FST seven days later. Three different time frames- ten, fifteen and twenty minutes of ischemia with hypotension - were compared to the sham group.

As was previously mentioned, global ischemia leads to cell loss in the most vulnerable areas of the brain. The CA1 region of the hippocampus is one of the first brain regions affected, where cell death can be observed as early as three days following ischemia (Darwin, 1995; Onken, Berger and Kristian, 2012). A one-way ANOVA revealed a significant difference in the number of cells located in the CA1 region (as measured at 40X magnification) between groups (Fig. 3.1a, $F_{3,15} = 27.808$, $*p=0.001$). All three time frames – 10 ($*p=0.002$), 15 ($*p=0.001$) and 20 ($*p=0.001$) - were able to significantly reduce the number of viable cells in the CA1 region of the hippocampus.

On the other hand, the FST data showed no difference between the groups when analysed with a one-way ANOVA (Fig. 3.1d, $F_{3,40} = 2.365$, $p=0.087$). A statistically significant difference was identified between the mice groups that received the 15-minute ischemia + hypotension and the sham surgery ($*p=0.038$). It is important to note that the 20-minute ischemia + hypotension resulted in a high mortality rate (about 33% - data not shown). Considering these results, the 15-minute ischemia + hypotension protocol was used for all the other experiments to induce global ischemia. Fig. 3.1b

shows the CA1 region of a mouse who received the sham surgery and Fig. 3.1c of a mouse in the 15-minute group.

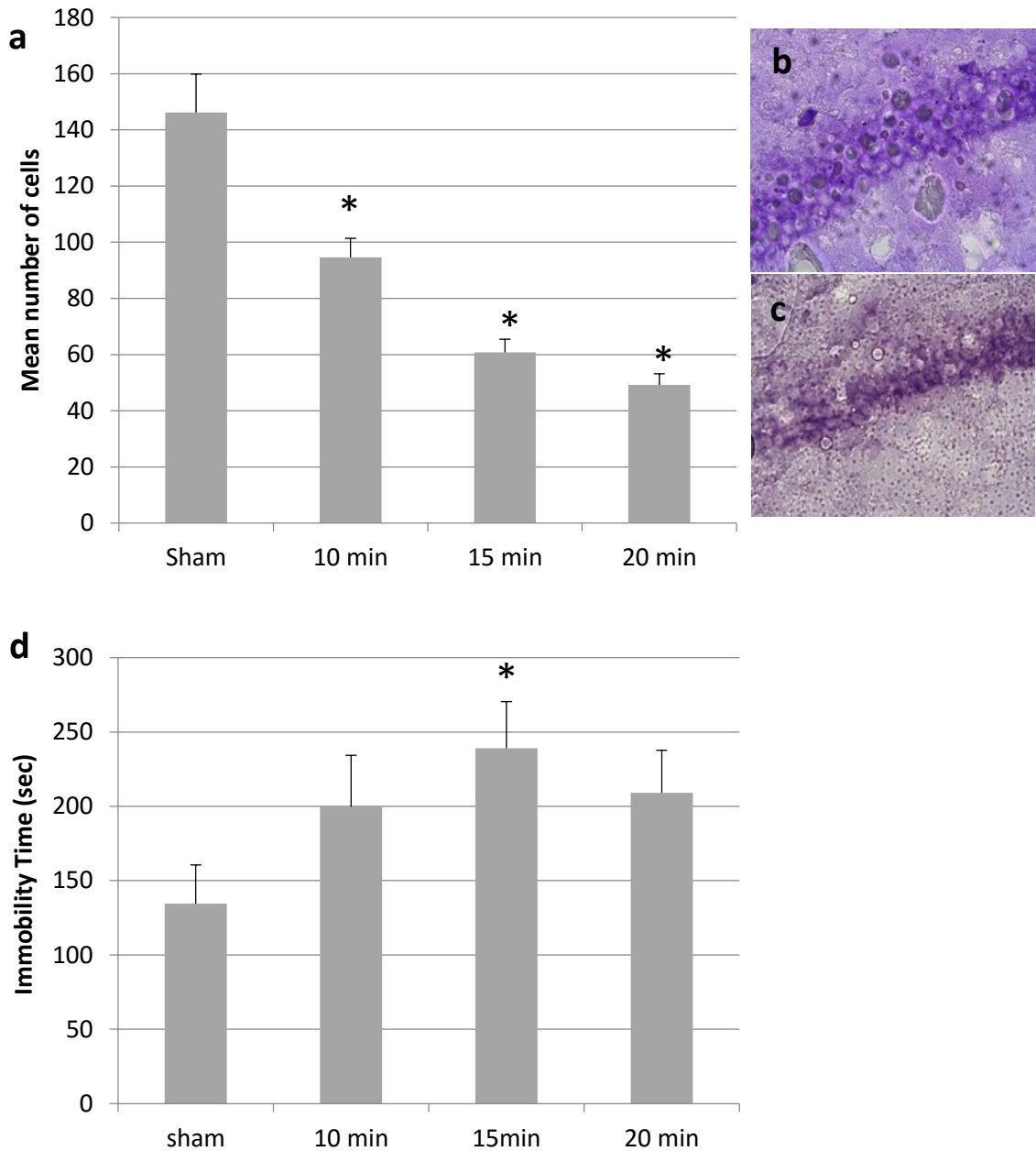


Figure 3.1. Establishing and validating the PID model. a, mean number of cells in the CA1 region of the hippocampus for different ischemic durations, measured at 40X one week after the surgery (n=4). b, CA1 region of a sham mouse. c, CA1 region of a mouse subjected to 15 minutes of global ischemia + hypotension. d, Total immobility time during a 10-minute FST measured one week after the surgery (n=11,11,10,9). Data expressed as mean + standard error of the mean (SEM). Data was analyzed with the use of a one-way ANOVA and the Dunnett's post-hoc test where the sham group was used as control. *p≤0.05

3.2 – AM281 administration and effects on the FST

The main goal of this experiment was to investigate the effect of AM281 administration on the development of depression following global ischemia. Mice were separated into three surgery groups (Ischemia, Sham and Home Cage-HC) and received either AM281 (3mg/kg) or a vehicle 30 minutes before the FST. A two-way ANOVA revealed a significant effect of surgery ($F_{2,60} = 4.940$, $*p=0.011$), AM281 treatment ($F_{1,60} = 28.965$, $*p=0.001$) and interaction – surgery*AM281 ($F_{2,60} = 13.016$, $*p=0.001$) on behavioral despair measures of the FST (Fig. 3.2a). The ischemic-vehicle mice spent significantly more time immobile than all other groups ($*p\leq 0.05$) further confirming the efficacy of our global ischemia model in triggering the development of a depressive-like phenotype one week post-ischemia. Furthermore, AM281 administration was able to reduce the immobility time of the ischemic mice ($*p=0.001$) to mirror that of the sham and HC mice.

The effect of AM281 was also compared to the effect of fluoxetine and ketamine (both 10mg/kg), two drugs currently used as antidepressant treatments. A one-way ANOVA showed a significant difference between the groups (Fig. 3.2b, $F_{3,39} = 8.326$, $*p=0.001$). However, only AM281 administration was able to significantly reduce the immobility time ($*p=0.001$) which was also significantly different than the effect of fluoxetine administration ($*p=0.050$) on the immobility time measured during the FST. These results suggest that AM281 has a better antidepressant potential in this model of PID than fluoxetine and ketamine.

Finally, a beam-break test was performed to assure that the results observed were not due to a locomotor impairment as a result of the drug or the surgery (Fig. 3.2c). A one-way ANOVA indicated that there was no difference between the four groups ($F_{3,36} = 0.587$, $p=0.628$). These results indicate that the ischemia surgery and the administration of AM281 did not affect locomotion and thus confirming that the reduction of immobility conferred by AM281 is due to an antidepressant-like effect.

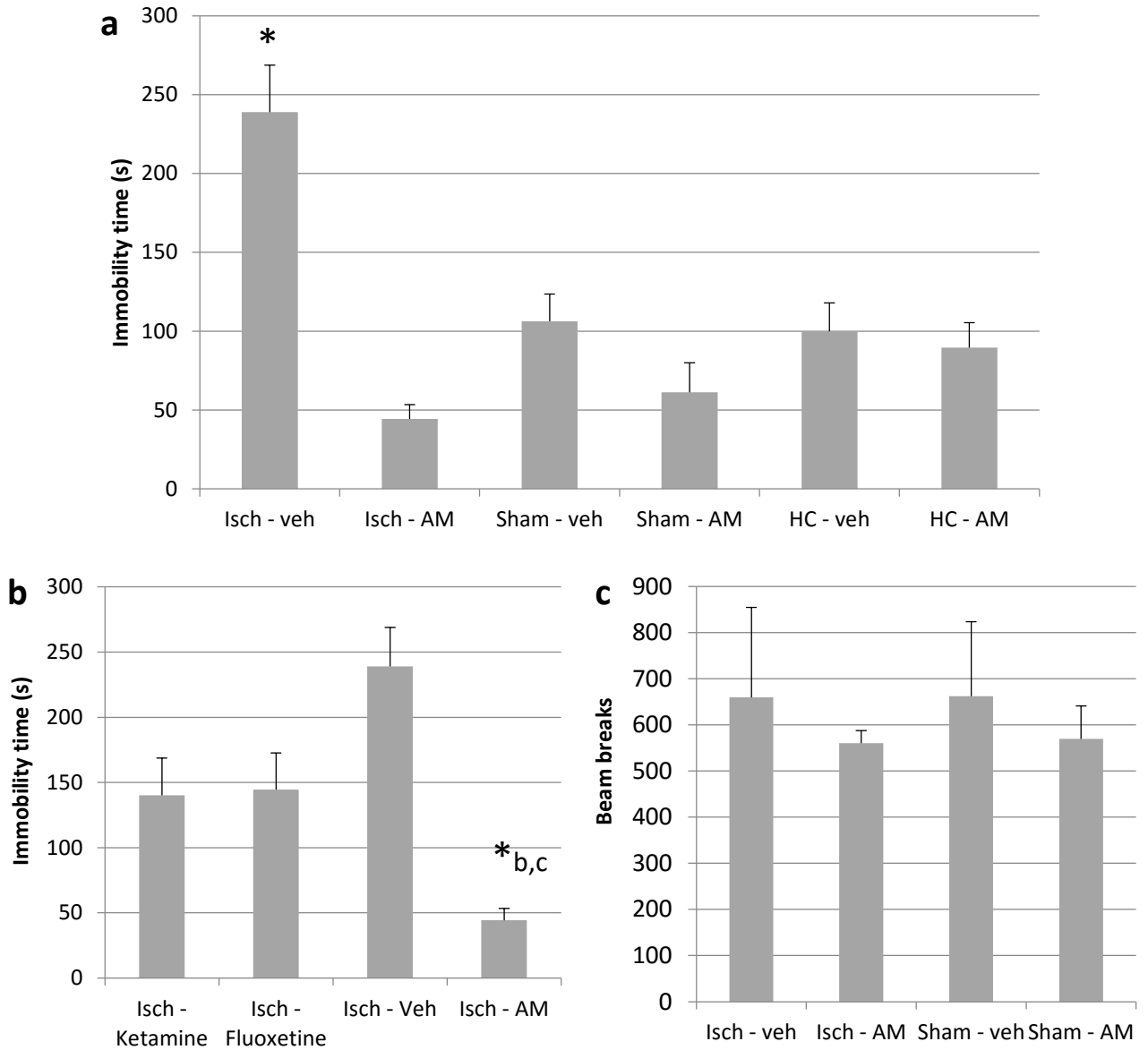


Figure 3.2. Administration of AM281 and effects on the FST. a, effect of surgery and AM281 administration on the immobility time during the FST (n=9,9,9,10,11,12). b, comparison of the effect of AM281, ketamine and fluoxetine on the immobility time during the FST (n=11,11,9,9). c, number of beam breaks in a period of 10 minutes (n=9,9,9,10). All behaviour tests performed one week post-surgery. Data expressed as mean + SEM. Data analyzed with one- or two-way ANOVA with post-hoc Tukey's test. *p≤0.05.

3.3 – Which cell-type is responsible for the effect of AM281?

As was discussed previously, CB1Rs can be predominantly found on three main types of cells: GABAergic, glutamatergic and astrocytic (McIver, Faideau & Haydon, 2013). Now that AM281 has been found to have an antidepressant-like effect in this model, this next part of the study was focussing on identifying the cell-type responsible for this effect. Different pharmacological manipulations were used to determine the relative role of each cell-type for the antidepressant effect observed.

3.3.1 – GABAergic cells

First looking at GABAergic cells, bicuculline (GABA_A antagonist) was administered five minutes prior to AM281 administration to test if it could block the effect of AM281. An independent T-test showed that 0.5 mg/kg of bicuculline had no effect on its own (Fig. 3.3a, $p=0.832$) and this dose was therefore used for the next experiment. Ischemic mice received either vehicle or bicuculline 5 minutes prior to AM281 or vehicle administration (30 minutes prior to FST). A statistically significant difference was found between the groups as revealed with a one-way ANOVA (Fig. 3.3b, $F_{3,39} = 6.992$, $*p=0.001$). As expected, the administration of bicuculline prior to vehicle administration had no effect on the immobility time ($p=0.752$). As observed previously, administration of AM281 significantly reduced the immobility time ($*p=0.024$). However, the administration of bicuculline prior to AM281 did not block its effect ($p=0.861$). This suggests that AM281 does not induce its antidepressant-like effect via GABAergic neurons.

3.3.2 – Glutamatergic cells

A similar protocol was adopted to determine the role of glutamatergic cells in the effect conferred by AM281. In this case NVP-AAM077 (NMDAR antagonist) was administered instead of bicuculline. An independent T-test confirmed that 1.2mg/kg of NVP had no effect on its own and thus this dose was used in the next experiment (Fig. 3.3c, $p=0.978$). Four groups were created: Vehicle + Vehicle, NVP + Vehicle, Vehicle + AM281 and NVP + AM281. A one-way ANOVA showed a significant difference between the groups (Fig 3.3d, $F_{4,67} = 8.293$, $*p=0.001$). Vehicle + Vehicle and NVP + Vehicle groups both showed high immobility times and AM281 was able to significantly reduce the immobility time ($*p\leq 0.05$). Furthermore, a statistically significant difference was observed between mice who received Vehicle + AM281 and NVP + AM281 ($*p=0.036$). This confirms that AM281 requires glutamatergic cells to confer its antidepressant effect. To go one step further, a different NMDAR antagonist was also tested, Ro-25-6981. As a reminder, NVP has a higher selectivity for NR2A- than for NR2B-containing NMDARs and Ro-25-6981 is selective for NR2B containing NMDAR (Jimenez-Sanchez, 2014). Ro-25-6981 was not able to block the effect of AM281 ($p=0.474$) unlike NVP which was able to block the effect. Taken together, these results suggest that AM281 induces its antidepressant-like effect via glutamatergic neurons and requires signalling via NR2A-containing NMDARs.

3.3.3 – Astrocytic cells

Since AM281 administration would reduce glutamate release from astrocytic cells, a different approach had to be used. A study published by our laboratory has shown that activation of CB1Rs on astrocytes increases glutamate release which leads to the activation of NR2B-containing NMDARs. This activation will lead to the endocytosis of AMPARs and the induction of LTD (Han et al., 2012). Considering this, if AM281 induces its antidepressant effect at astrocytic synapses, its administration should block this cascade and therefore block the expression of LTD. TAT-GLUR2 peptide is able to prevent LTD by blocking the internalization of AMPARs, thus having the same end-effect as AM281 (Dalton, G. et al, 2008).

Mice received either a treatment of TAT-GLUR2 peptide or control peptide (both 4mg/kg) 30 minutes prior to the FST. If AM281 induces its antidepressant effect via CB1Rs located on astrocytes, TAT-GLUR2 should be able to reduce the immobility time as does AM281. Comparing both peptides with an independent T-test, no difference was found between the two groups (Fig. 3.3e, $p=0.312$) which suggests that astrocytic cells are not involved in the antidepressant-like effect of AM281.

In conclusion, GABAergic and astrocytic cells do not seem to play a role in the antidepressant-like effect conferred by AM281. Glutamatergic cells are the key cell-type involved in this effect and more specifically would require a specific type of NMDAR which contains NR2A subunits.

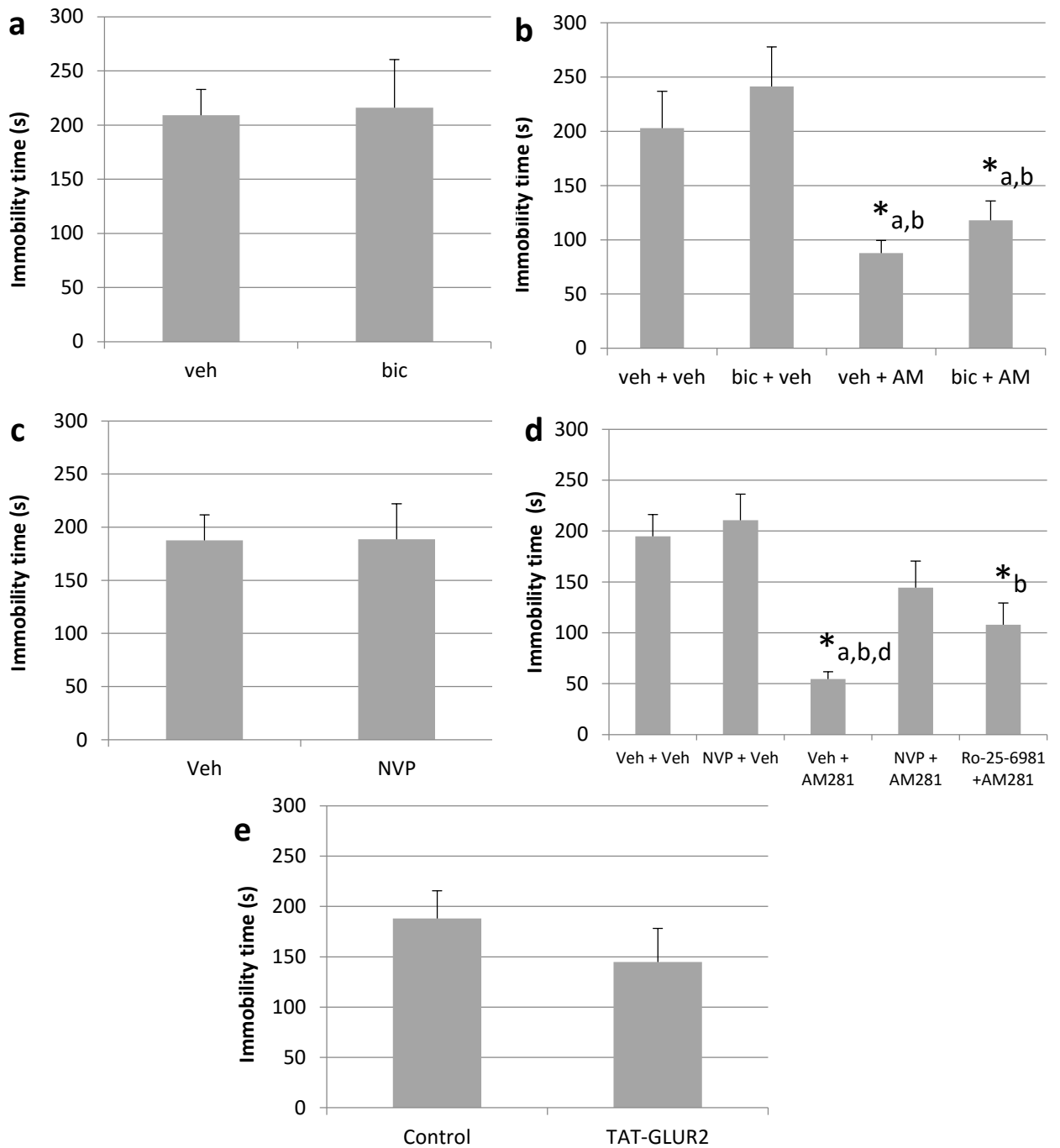


Figure 3.3. Which cell type is responsible for the effect of AM281? a, verification that 0.5mg/kg of bicuculline does not have an effect on its own (n=11). b, effect of the treatment with bicuculline 5 minutes prior to AM281 administration on the immobility time during the FST (n=10). c, verification that 1.2mg/kg of NVP-AM077 does not have an effect on its own (n=10,11). d, effect of the treatment with NVP-AM077 and Ro-25-6981 5 minutes prior to AM281 administration on the immobility time during the FST (n=15,13,13,15,12). e, Comparison of TAT-GLUR2 and control peptide treatment on the immobility time during the FST (n=9,11). Data expressed as mean + SEM. Data analyzed with independent T-test or one-way ANOVA with post-hoc Tukey's test. *p \leq 0.05.

3.4 – Can the antidepressant effect of AM281 be confirmed with the SPT?

As seen in the above experiments, AM281 was found to have antidepressant effects as tested with the FST; and NVP administration was able to reverse this effect. In this next experiment, a different depression test, the SPT, was used to confirm these results. The SPT relies on the principle that healthy mice prefer drinking a 1% sucrose solution compared to water and that mice with a depressive-like phenotype do not show this preference.

3.4.1 – Establishing baselines

The first step was to establish baselines for HC, sham and ischemic mice. A one-way ANOVA revealed a statistically significant difference between the three surgery groups (Fig. 3.4a, $F_{2,25} = 5.044$, $*p=0.015$). As suspected, ischemic mice did not show any preference for the sucrose solution but HC and sham groups did show a preference (ischemia vs sham, $*p=0.025$; ischemia vs HC, $*p=0.046$). A 10-minute forced swimming period was added one hour before the SPT as a stressful event (Fig 3.4b). Again, the one-way ANOVA showed a difference between the groups ($F_{2,33} = 9.816$, $*p=0.001$; ischemia + FST vs sham + FST, $*p=0.001$; ischemia + FST vs HC + FST, $*p=0.002$). The forced swimming period seemed to accentuate the difference between ischemic and sham mice, therefore this protocol was used for all future experiments. In fact, figure 3.4c shows an effect of stress or FST ($F_{1,60} = 11.323$, $*p=0.001$), surgery ($F_{2,60} = 13.729$, $*p=0.001$), but no interaction – stress*surgery ($F_{2,60} = 1.069$, $*p=0.351$). And a one-way ANOVA revealed a significant difference between the groups ($F_{5,59} = 8.2$, $*p=0.001$) and

showed that only the ischemic + FST group significantly reduced the percentage of sucrose consumption (* $p \leq 0.05$).

3.4.2 – Confirmation of FST results

The next part of this experiment consisted of confirming the results obtained with the FST. The procedure of this experiment was as follows: drug administration 30 minutes prior to 10 minutes of FST, followed by a one-hour break and finally mice were subjected to the SPT for one hour. The mice either received a vehicle, AM281, NVP + AM281, ketamine or fluoxetine before the FST. A statistical difference was identified between the treatment groups following a one-way ANOVA analysis (Fig. 3.4d, $F_{4,65} = 2.998$, * $p = 0.025$). As stated previously, ischemic mice did not show a preference for the sucrose solution, furthermore AM281 was able to increase sucrose consumption and thus confirming its antidepressant-like effect (* $p = 0.018$). On the other hand, fluoxetine ($p = 0.549$) and ketamine ($p = 0.993$) administration did not reverse the depressive-like phenotype and therefore further confirming the results from the FST. Finally, pre-treatment with NVP did not block the effect of AM281 ($p = 0.538$) as was observed with the FST. It is possible that NVP, since such a small dose was used, did not have an effect one hour and 30 minutes later during the SPT.

The experiments were repeated with a modified protocol where administration of the drugs was done 30 minutes before the SPT. Four treatment groups were tested: vehicle, NVP, AM281 and NVP+AM281. A significant difference between the groups was identified with the use of a one-way ANOVA (Fig. 3.4e, $F_{3,41} = 5.232$, * $p = 0.004$). The

administration of AM281 was still able to reverse the antidepressant-like phenotype (* $p=0.020$) and this time, NVP was able to block this effect and therefore confirmed the results obtained with the FST (* $p=0.013$).

These series of experiments allowed to confirm the results obtained with the FST. They also showed that AM281 has an effect on two symptoms of depression: behavioral despair tested by the FST and anhedonia tested by the SPT; and that this effect is mediated via glutamatergic neurons in both cases.

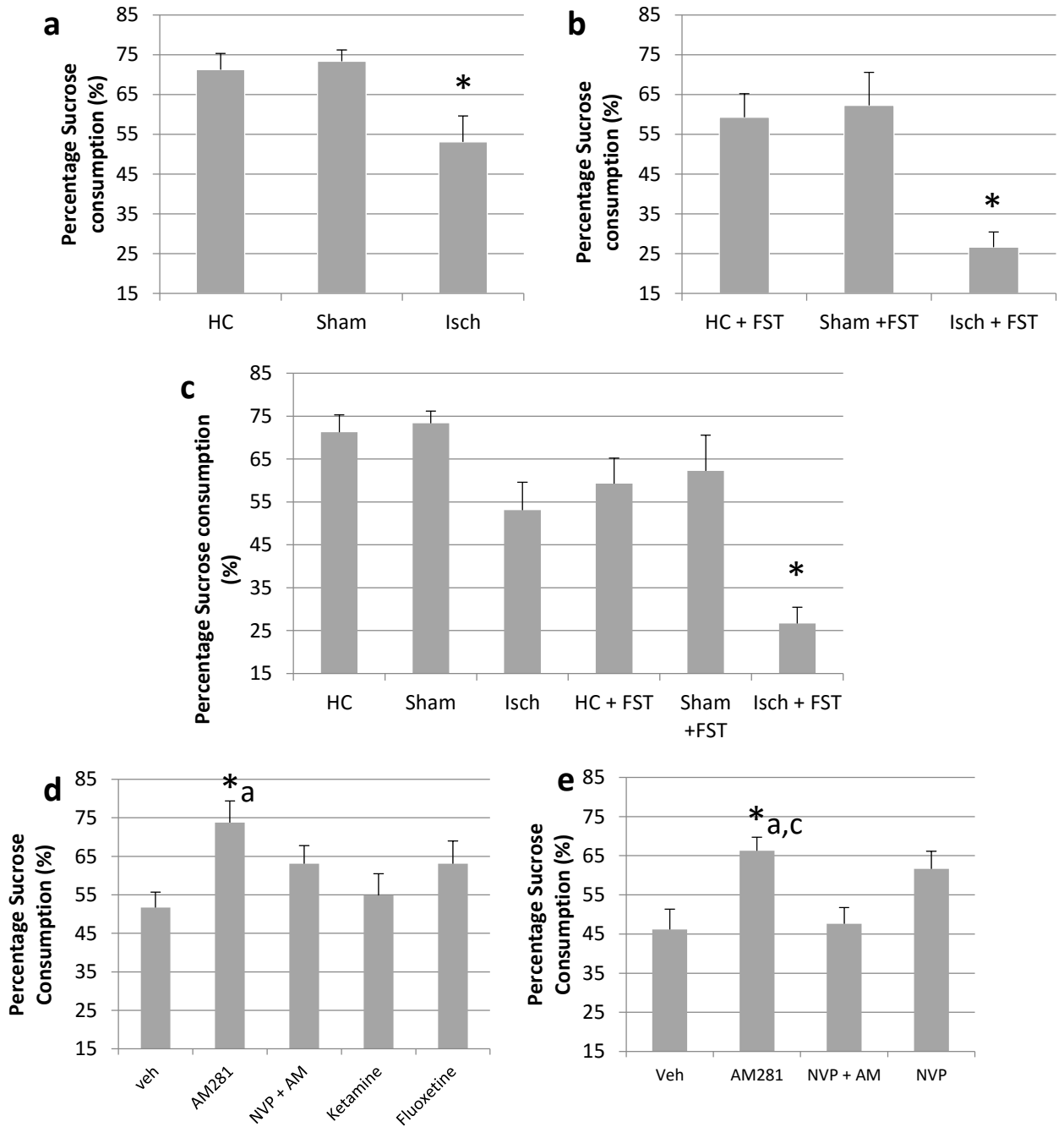


Figure 3.4. Confirmation of results with the SPT. a, difference between surgery groups on sucrose consumption during the SPT (n=8,8,10). b, difference between surgery groups on sucrose consumption during the SPT when 10 minutes of FST is added 1 hour prior to the SPT (n=12,11,11). c, graphs a and b put together. d, effect of the administration of different drugs 30 minutes before the FST (1h30min before the SPT) (n=15,16,15,12,12). e, effect of the administration of different drugs 30 minutes before the SPT (n=12,10,11,9). Data expressed as mean + SEM. Data analyzed using ANOVA and post hoc Tukey's test. *p<0.05.

3.5 – Bilateral intra-BLA administration of AM281

Different brain regions are involved in the development of depression of which hippocampus, pre-frontal cortex and amygdala are probably the most studied (Maletic, 2007). The goal of this experiment was to investigate the BLA and its importance for the antidepressant effect conferred by AM281. Cannulas are implanted in the BLA one week prior to the global ischemia surgery. Figure 3.5b shows the confirmation of the location of the cannulas in the BLA of both hemispheres. One week after the ischemia surgery, either a vehicle or AM281 is administered (50ng/0.2ul per side) in the BLA via cannulas. Thirty minutes after drug administration mice are subjected to the FST. An independent T-test revealed a significant reduction of the immobility time in the mice treated with AM281 (Fig. 3.5a, *p=0.007). These results suggest that the BLA is important for the antidepressant-like effect conferred by AM281. Nevertheless, the hippocampus and the pre-frontal cortex could also play a role in this antidepressant effect. Further studies would be required to determine if indeed these two brain regions are also important for this effect.

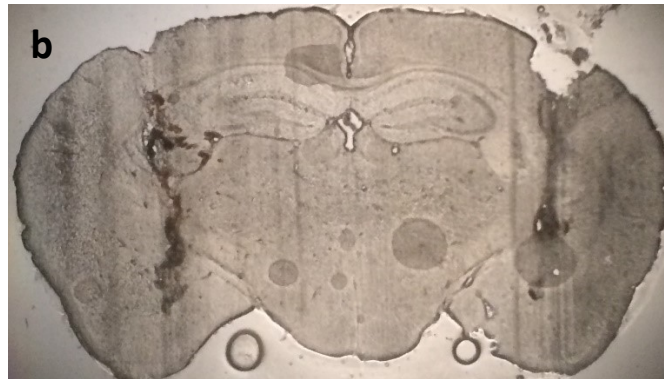
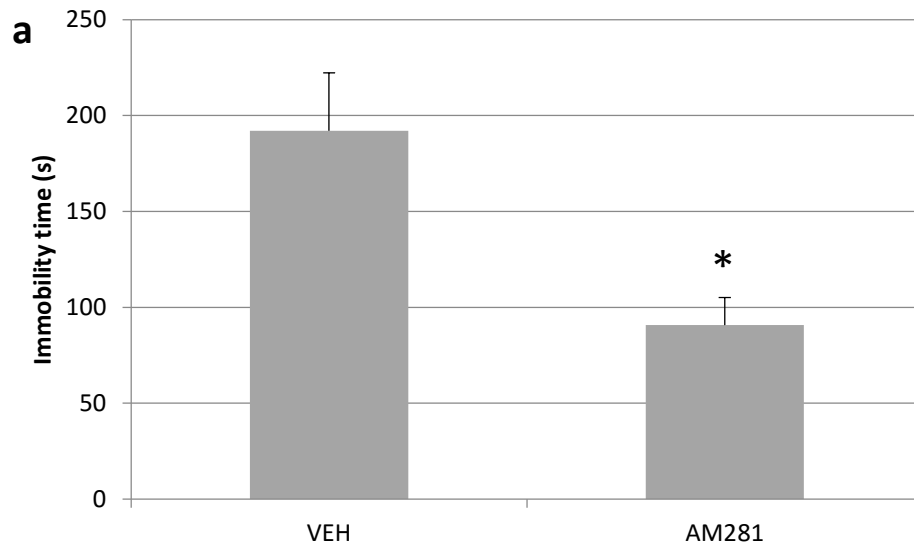


Figure 3.5. Bilateral intra-BLA administration of AM281. a, Total immobility time during the FST which was performed 30 minutes after either vehicle or AM281 administration in the BLA (n=10). b, confirmation of cannula location in the BLA of both hemispheres. Data expressed as mean + SEM. Data analyzed with independent t-test. * $p \leq 0.05$.

Chapter 4: Discussion

Depression and ischemia have both been associated with the 'dysregulation of the HPA axis, impaired neurogenesis and cellular plasticity, altered neurotrophin signalling, neuroinflammation and even downright neuronal loss' (Kronenberg et al., 2014). These facts bring further proof that the development of depression following ischemia is the result of the disturbance of biological mechanisms. Importantly, the eCB system was identified as playing a role in the regulation of most of these biological processes (McPartland, 2008). Therefore, we hypothesised that the eCB system plays a role in the development of PID and that pharmacological regulation of this system is a potential therapy for its treatment. This study focussed on the effect of acute AM281 treatment on the development of two important post-ischemic depressive symptoms: behavioral despair and anhedonia. Following this, the mechanism of antidepressant action of AM281 was explored using pharmacological manipulations as to identify the important cell type(s) and specific receptor(s) important for its effect. Finally, since all our drug administrations were performed i.p and thus had an effect on the whole brain, we investigated the effect of direct administration to the BLA to identify the involvement of this brain region in the antidepressant effect of AM281. This study highlights the important role of the eCB system in the development of post-ischemia depression and, in the future, these results could be translated to patients affected by post-stroke depression (PSD).

4.1 – Model of PID

A model of global ischemia accompanied by hypotension was used in this study to induce the development of depression (Onken, Berger and Kristian, 2012). In our CD1 mice, a 15-minute ischemic time frame was found to be ideal as 10 minutes was not enough to induce behavioral despair one week following ischemia and 20 minutes' lead to a high death rate. This model was also able to induce anhedonia as observed with the SPT. Finally, cell loss was identified in the CA1 region of the hippocampus bringing further validity to our model.

Two main models of cerebral ischemia exist: middle cerebral artery occlusion (MCAO) and global ischemia, which in mice is usually obtained via BCCAO paired with hypotension. Some articles might argue that MCAO better models what is observed in human stroke and that global ischemia leans more towards a model of cardiac arrest but both models lead to the same ischemic cascade. In both cases, oxygen and glucose supply is reduced in the brain which leads to an ionic imbalance and excitotoxicity (Traystman, 2003). Furthermore, 'recent systematic reviews argue against an association between PSD and the type (i.e., ischemic or hemorrhagic) or mechanism (i.e., thrombotic, embolic, etc.) of stroke' or even the injury location (Robinson & Jorge, 2016). Results obtained in our study could then potentially be translated to stroke patients. In addition, our laboratory is working on reproducing these results with a MCAO model. If successful, this would further validate our results indicating the strong antidepressant effect of AM281 in multiple models of PID/PSD.

4.2 – Antidepressant effect of AM281

In this study, we found an antidepressant effect of AM281 acute administration at a dose of 3mg/kg in both the FST and SPT. These results fall partially in line with the literature since as was discussed previously; CB1R antagonists can have no significant effects, antidepressant effects or pro-depressant effects. Most studies looking at CB1R antagonists and depression have focused on rimonabant, however AM281 has a very similar structure and acts both as a CB1R antagonist and inverse agonist, as does rimonabant. Therefore, results obtained with rimonabant can easily be compared to our results with AM281. In clinical trials, rimonabant was found to increase the risk of developing depression and committing suicide in healthy individuals. On the other hand, animal studies of depression have found either no significant effect or antidepressant effects of rimonabant administration in animal models of depression. This suggests that the downregulation of the eCB system in healthy individuals might have a depressive-like effect, whereas in individuals with depressive symptoms, this downregulation might be beneficial and might even attenuate the symptoms of depression (Hillard & Liu, 2014; Patel & Hillard, 2009). Our study supports this idea as AM281 administration in our post-ischemic mice reduced the immobility time on the FST and increased sucrose consumption as measured during the SPT. We also found that sham and HC mice who received AM281 or vehicle did not show a significant difference in the amount of time spent immobile.

4.2.1 – Comparison of AM281 to fluoxetine and ketamine

Since AM281 was found to have an antidepressant effect in post-ischemia mice, the next step was to compare this effect to known antidepressants. In this study, we investigated fluoxetine (SSRI) and ketamine (NMDAR antagonist). Fluoxetine has been studied extensively as an antidepressant and 10mg/kg acute administration has been shown to reduce immobility on the FST in rodents with a depressive-like phenotype (Dulawa, 2004). However, we observed here that fluoxetine did not produce significant antidepressant effects on both the FST and SPT. SSRIs usually require two to three weeks of chronic administration to have an effect in humans. The antidepressant potential of SSRIs can be measured on the FST after acute administration but other behavioral tests require chronic administration to observe an effect (Norman, 1999). In summary, the present study demonstrated that a single administration of AM281 but not fluoxetine produced rapid antidepressant effects on post-ischemia mice.

Recent studies have identified the rapid antidepressant effect of ketamine (NMDAR antagonist). Ketamine has been used at high doses as an anesthetic but recent studies have shown that low doses of this drug could have rapid antidepressant effects (within 72 hours) and more importantly could have an effect in treatment-resistant depressive patients (Réus et al., 2016). Considering the growing popularity of ketamine, we decided to study its antidepressant potential in our model of PID and compare it to the effect of AM281. In a previous study, ketamine was shown to decrease immobility time on the FST at both a dose of 10mg/kg and 15mg/kg administered acutely (Garcia et al., 2008). Our results showed no significant antidepressant-like effect of ketamine (10mg/kg) in both the FST and SPT.

These interesting results raise an important question: are the development of MDD and PID based on the same mechanisms? Our results would be in favor of a difference in mechanisms leading to the development of these two disorders/conditions as fluoxetine and ketamine seemed to have different effects in our model of PID compared to data accumulated with MDD animal models. Furthermore, a study performed by Deplanque, Venna and Bordet (2011) also supports this hypothesis as they found that the antidepressant effects of TCAs and SSRIs were changed following brain ischemia and even suggested that not all antidepressants could be prescribed following stroke. In any case, some studies definitely support the idea that the development of MDD and PID rely on some similar mechanisms but others also report some mechanistic differences which highlights the complexity of PID and the definitive lack of knowledge in this area of research (Loubinoux et al., 2012). Understanding the mechanisms leading to PID and how these resemble or differ from those leading to MDD will help us discover new therapeutic targets and develop more effective treatment options.

4.2.2 – PID and the eCB system

At the start of this study, to our knowledge, we did not find any articles examining the link between the eCB system and the development of PID. At the beginning of this year, however, two studies were published studying this link but both focussed on the administration of CB1R and CB2R agonists as opposed to our study that focusses on the administration of a CB1R antagonist/inverse agonist.

The first study by Wang et al. (2016) was based on a MCAO model of cerebral ischemia accompanied by chronic unpredictable mild stress (CUMS) to model PID. Their study focussed on the hypothalamus as the HPA axis and the eCB system are both important in the regulation of stress. The study measured, in their model of PID, the expression of both cannabinoid receptors in the hypothalamus under baseline conditions and after the administration of CB1R and CB2R agonists directly in the ventral medial hypothalamus (VMH). The behavioral test used in this study is the SPT and thus only measured anhedonia. Their results showed that the MCAO + CUMS led to decreased levels of CB1R but not CB2R in the VMH. The intra-VMH administration of only the CB2R agonist was able to increase sucrose consumption. These results suggest that the reduction in CB1R expression in the VMH might be involved in the development of PID but that only the CB2R agonist was identified as a potential treatment target for PID.

First, their model of PID is different than the one used in our study and the addition of CUMS might induce other neurobiological changes that would not be observed in our model. Also, our study used two behavioral tests and thus brings further validity to our results compared to Wang et al.'s study which used only the SPT. Importantly, their study did not investigate systemic administration of CB1R or CB2R agonist and antagonist, thus could not provide valuable information for the potential clinical treatment of PID or PSD with eCB enhancement or blockade strategy.

Zhang et al. (2016) indirectly studied the CB1R inhibition in the dorsal hippocampus. They used a transient global ischemic model where rats received a BCCAO

surgery, followed by chronic exposure of rats to sevoflurane and then measured the effect on the SPT. Sevoflurane treatment was able to increase sucrose consumption, which was blocked by the administration of a CB1R antagonist in the dorsal hippocampus (DH). Following the sevoflurane treatment, CB1R expression and eCB levels were found to be elevated in the DH. An important point is that sevoflurane exposure has been linked with increased GABAA activity (Lecker et al., 2013; Zhang et al., 2016) as well as a reduction in NMDAR activation (Brosnan & Thiesen, 2012; Zhang et al., 2016). In our study, the antidepressant effect of AM281 was found to be mediated via glutamatergic neurons and requires NMDAR activation. It is possible that the antidepressant effect of sevoflurane treatment requires NMDAR suppression in the hippocampus.

4.3 – Mechanism of action of AM281

4.3.1 – Glutamatergic neurons and NMDAR

Pharmacological manipulations were used to determine the cell-type responsible for the antidepressant effect conferred by AM281. Our results strongly suggest that the effect is mediated via glutamatergic synapses but not GABAergic synapses or astrocytic cells. Furthermore, two different NMDAR antagonists were used for these manipulations and only the one with a preference for the NR2A-containing NMDARs was able to block the effect of AM281. This highlights the importance of NMDARs with NR2A subunits for the antidepressant action of AM281.

NMDARs are composed of four or five subunits of which two are NR1 subunits and the rest are varying types of NR2 subunits. Four different NR2 subunits can be found in the brain but NR2A and NR2B are more predominantly expressed. These NR2 subunits will change the location and function of the NMDAR. NMDARs which contain NR2A subunits will be preferentially expressed in the synaptic zone and their activation is required for long-term potentiation (LTP). On the other hand, NR2B containing NMDARs are mostly located extrasynaptically and are primarily involved in LTD (Massey et al., 2004).

All the available evidence, together with our results in this study, suggests the following mechanism of action of AM281 for the treatment of PID. AM281 would block CB1R on glutamatergic synapses, therefore allowing the presynaptic release of more glutamate. Glutamate would then bind to postsynaptic NR2A-containing NMDAR to induce LTP.

The most popular theory of depression is the 'monoamine hypothesis' and is based on the fact that monoamine levels are decreased in depressed patients and that antidepressant drugs elevate these levels leading to an improved mood. Recent research has turned towards a 'glutamate hypothesis' of depression where levels of glutamate were found to be either elevated or reduced in patients depending on the subtype of mood disorder. Glutamate transmission is involved in mood and cognitive regulation and therefore has been an interesting new target for the treatment of depression (Sanacora, Treccani & Popoli, 2012). In our study AM281 was found to act on glutamatergic cells and thus would fall in line with this 'glutamate hypothesis'.

4.3.2 – Role of the amygdala

Finally, direct administration of AM281 in the BLA of both hemispheres highlighted the importance of this brain region for the antidepressant effect of systemic administration of AM281. A study by Sachdev et al. (2007), determined that some stroke patients had relatively smaller amygdalae and that this was correlated to a higher chance of developing PSD. Furthermore, a study exploring the cerebral activity in stroke compared to healthy individuals showed reduced activity in the amygdala of stroke patients when presented with a frightening stimulus (Turner, et al., 2007). This suggests that the administration of AM281 in the BLA increases glutamate transmission and therefore leads to increased amygdalar activity and an antidepressant effect. Exploring the relative activity of the BLA in our PID model would shed further light on this hypothesis. Electrophysiological measures from glutamatergic neurons in the BLA would be essential to validate our hypothesis.

Our study focussed on pharmacological manipulations of a mouse PID model. The next step would be to confirm our results with the use of a transgenic model. Using a conditional knock-out (KO) model with a specific deletion of CB1R from glutamatergic neurons in the BLA would bring further validity to our results. Furthermore, comparing the CB1R KO mice on glutamatergic cells to CB1R KO on GABAergic and astrocytic cells would confirm the importance of CB1Rs located on glutamatergic neurons for the development of PID. To confirm our results, knocking out CB1Rs on glutamatergic

neurons, but not on GABAergic neurons and astroglial cells, would prevent the development of PID.

4.4 – Conclusions

The current study has presented novel antidepressant effects of AM281 in a model of global ischemia that have not yet been reported in the literature. First, fifteen minutes of global ischemia accompanied by hypotension lead to the development of two important PID symptoms: behavioral despair and anhedonia. A systemic administration of AM281, but not fluoxetine or ketamine, was able to reduce immobility time of the FST and increase sucrose consumption on the SPT. AM281 was found to interact with CB1Rs located on glutamatergic cells and requires signalling via NR2A-containing NMDARs to confer its antidepressant effect. In addition, the BLA of the amygdala was identified as an important brain region required for the antidepressant effect of systemic AM281. Mutant mice are required to further confirm these results and determine if CB1Rs on glutamatergic neurons are required for the development of PID. Furthermore, electrophysiological experiments studying the BLA activity would be important to support our hypothesized mechanism behind the antidepressant-like effect of AM281. Finally, repeating these experiments on a MCAO-induced PID model would further confirm the translatability of our results to stroke patients affected by PSD. This study may lead to the development of new therapeutic strategies for the treatment of PID and in turn improve rehabilitation outcomes.

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