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The effects of the ketamine derivative N-ethyldeschloroketamine (2-oxo-PCE) on synaptic transmission and plasticity.

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Scientific abstract

Ketamine's psychotomimetic effects (e.g. hallucination, delusion) and its short acting effects have made it a popular recreational drug. Despite misuse, both classical psychoactive substances and research chemicals have shown clinical relevance in therapeutic treatments for various neurological disorders and neurogenerative diseases. Interestingly, ketamine has had worldwide clinical interest for its fast-acting antidepressant effects, which differ to conventional antidepressants that exhibit a delayed onset of action. Recently, the use of N-ethyldeschloroketamine (2-oxo-PCE), a ketamine derivative, has become a recent phenomenon in the streets. However, the effects of '2-oxo-PCE' on the brain are currently unknown. Thus, the aim of the project is to characterise the pharmacology of '2-oxo-PCE' by investigating its effects on N-methyl-D-aspartate receptor (NMDAR) mediated synaptic transmission and plasticity. Using in vitro electrophysiological field recordings in the CA1 region of rat hippocampal slices, the data shows that 2-oxo-PCE is a fast-acting, potent, and selective NMDAR antagonist. Therefore, the effects of 2-oxo-PCE presented here serves as a foundation on which further investigations can be build and provide the base for the development of new strategy for the treatment of depression.

Lay abstract

Classical psychoactive substances (e.g. ketamine) have many therapeutic implications in the treatment for epilepsy, neurological disorders and neurogenerative diseases. Particularly, ketamine has seen worldwide clinical interest for its fast-acting antidepressant effects compared to conventional antidepressants that exhibit a delayed onset of action. However, ketamine's psychomimetic effects (e.g. hallucination, delusion) in humans and its short acting effect have made it a popular recreational drug over the years. This abuse liability has resulted in ketamine and other classical psychoactive drugs becoming illegal in the UK and other countries. Hence, an influx in research chemicals, which are drugs that are designed intentionally to mimic classical psychoactive substances whilst avoiding legislative restrictions. However, research chemicals have also demonstrated therapeutic potential in the treatment of various neurological disorders, despite their misuse. Recently, a research chemical '2-oxo-PCE', which has a similar structure to ketamine, has become a recent phenomenon for its use on the streets. However, the direct effects of '2-oxo-PCE' on the brain are currently unknown. This was investigated in this work through the use of electrophysiological recordings in rat brain slices. 2-oxo-PCE rapidly and potently reduced synaptic transmission (i.e. a mechanism by which brain cells communicate with each other) by blocking a neurotransmitter, glutamate in the brain. Also, 2-oxo-PCE affected synaptic plasticity (i.e. a mechanism involved in memory formation in the brain). In conclusion, 2-oxo-PCE is fast-acting and more potent acting than ketamine. Further experiments are necessary to advance the understanding of the effects of 2-oxo-PCE in the brain and it's therapeutic potential in the treatment of depression.

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Finally, I would like to express my deepest appreciation to my mother. Thank you for your unconditional love during the completion of my thesis. I could not have done it without you.

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Covid-19 statement

This is a Covid-19 statement to acknowledge the impact on my master's completion. Covid-19 played a role since the very beginning of my masters. For instance, I did not begin my masters a bit later than I anticipated. Initially my masters began in October 2020 instead of September on Zoom and remained that way until mid-December 2020. My supervisor had no access to the animal facilities between September- mid December 2020. Therefore, we could not order the animals needed for my research and thus delayed the start of my masters. Between October 2020 – mid December 2020, I only account for attending the lab four times. The rest of my time was used for independent reading and meetings via Zoom/teams meeting with my supervisor.

Furthermore, COVID-19 second lockdown in December 2020 meant that I did not fully begin my lab research until the end of January 2021. I only had 6 months in the lab instead of the official 9 months for a MScR student because of the impact of COVID-19 second lockdown. I had no choice but to ensure I learned the techniques in the lab as quickly as possible because I've already lost out on very valuable time in the lab between September 2020-January 2021. Also, my peer in the lab tested positive for Covid-19 in July 2021 and that also meant I missed out on 2 weeks' worth of experiments. This also affected my schedule of experiments I anticipated during that time period. Hence, the n number for some experiments were slightly compromised.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....

Table of contents

List of abbreviations

(2R,6R)-HNK - (2R,6R)- 6-hydroxynorketamine

(2S,6S)-HNK - (2S,6S)- 6-hydroxynorketamine

2-oxo-PCE - N-ethyldeschloroketamine; deschloro-N-ethyl-ketamine; 2-phenyl-2-(ethylamino)-cyclohexanone

AP5 - (2R)-amino-5-phosphonovaleric acid

aCSF - Artificial cerebrospinal fluid

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMPAR- fEPSP - AMPA receptor mediated field excitatory post-synaptic potential

CA1- Cornu ammonis area 1

CA3 - Cornu ammonis area 3

CGP55845 - (2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride

CNS - Central nervous system

D-AP5 - D-2-amino-5-phosphonopentanoate

IC_{50} - Half maximal inhibitory concentration

eGFP - Enhanced Green Fluorescent Protein

Ephenidine - N-ethyl-1,2-diphenylethanamine

fEPSP - Field excitatory post-synaptic potential

Fluorolintane - 1-(1-(2-fluorophenyl)-2-phenylethyl)pyrrolidine

GABA - Gamma-Aminobutyric acid

HEK-293 - Human embryonic cells (293)

HFS - High frequency stimulation

iGluR - Ionotropic receptors

KARs - Kainate receptors

KO – Knockout

LFS - Low frequency stimulation

LTD - Long term depression

LTP - Long term potentiation

MAOIs - Monoamine oxidase inhibitors

MDD - Major depressive disorder

mGluR - Metabotropic glutamate receptors

MK-801 - Dizocilpine; [5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

NAM - Negative allosteric modulator

NBQX - 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NIMH PDSP - National institute of mental health psychoactive drug screening program

NMDA - N-methyl- D-aspartate

NMDAR-EPSCs - NMDAR mediated excitatory postsynaptic currents

NMDAR-fEPSP - NMDA receptor mediated field excitatory post-synaptic potential

NMDARs - N-methyl- D-aspartate receptors

PCP - Phencyclidine; 1-(1-Phenylcyclohexyl)piperidine)

PPI - Pre-pulse inhibition

PTSD – Post-traumatic stress disorder

PTX - Picrotoxin

R^2 - Coefficient of determination

S0 - Stimulation electrode 0

S1 - Stimulation electrode 1

SCCP - Schaffer collateral-commissural pathway

SSRIs - Selective serotonin reuptake inhibitors

TBS – Theta burst stimulation

TRD - Treatment resistant depression

1 Introduction

1.1 Glutamate receptors

The discovery of glutamate receptors began in 1950s by Berl and Waelsch (1958) confirming the existence of L-glutamate and L-aspartate being ubiquitously expressed throughout the whole brain. Glutamate is a vital fast excitatory neurotransmitter that is believed to predominately activate the glutamate receptors in the human brain and is responsible in mediating the fast-postsynaptic excitatory neurotransmission in the mammalian central nervous system (Pinheiro & Mulle, 2008). Early research has confirmed the significance of glutamate receptors in synaptic plasticity and in learning and memory (Collingridge et al., 1983; Morris et al., 1986)

The glutamate receptors are differentially expressed throughout the CNS and have been implicated in various neurological disorders such as schizophrenia, epilepsy, depression and neurodegenerative diseases. These receptors have gained widespread acceptance as pivotal therapeutic targets for the diseases described above and others (Kornhuber and Weller, 1997)

1.1.1 Types of glutamate receptors

Based on pharmacological, electrophysiological and biochemical studies, glutamate receptors are composed of two main families: ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). mGluRs are G-protein coupled receptors that contribute to longer lasting effects in synaptic transmission (Sugiyama et al., 1987) by staying in an activated state longer than iGluRs (i.e. milliseconds to seconds) (Monaghan et al.,1989; Sugiyama et al., 1987). mGluRs are responsible in modifying neuronal excitability at the postsynaptic site and regulating neurotransmitter release (Pinheiro and Mulle, 2008).

iGluR are ligand gated cation- specific ion channels (Monaghan et al.,1989), that facilitates faster excitatory synaptic transmission (Sugiyama et al., 1987). iGluRs are categorized into three subfamilies: α-amino-3-hydroxy-5-methyl-4- isoxazolepropionate receptors (AMPARs), kainate receptors (KARs) and N-methyl- D-aspartate receptors (NMDARs) (Watkins and Evans, 1981; Kehl and McLennan, 1983). The subfamilies of ionotropic receptors were first determined based on their pharmacology and followed by their molecular biology in the early 1980s (McLennan and Lodge, 1979; Davies et al., 1981; Watkins and Evans, 1981; Meldrum, 2000; Monaghan et al.,1989).

Interestingly, NMDARs are suggested to play a pivotal role in the dissociative effects of classical dissociative drugs e.g. ketamine and phencyclidine (PCP) in humans (Morris and Wallach, 2014; Lodge and Mercier, 2015; Ingram et al., 2018). Consequently, this project will be focusing on NMDARs principally.

1.1.2 NMDARS

1.1.2.1 Structure of NMDARs & sites of drug action

In the 60s, Professor Jeff Watkins and his colleagues synthesized N-methyl- D-aspartate (NMDA) which led to the discovery of NMDARs (Watkins and Jane, 2009). NMDARs are heterotetrameric complexes that are composed of different combinations of four subunits from three known subgroups: GluN1, GluN2(A-D) and GluN3(A-B) subunits (Fig 1.1) (Vyklicky et al., 2014). The GluN3 family is the least studied subgroup. Each subunit is encoded by a different gene. NMDAR complexes involve two obligatory GluN1 subunits combined with two GluN2 subunits or combinations of GluN2 and GluN3 subunits.

Consequently, NMDARs exhibit diverse subunit arrangements including di-heteromeric and tri-heteromeric NMDARS, that form varied functional receptors and ultimately demonstrate different functional and pharmacological properties (Fig 1.1) (Traynelis et

al., 2010; Vyklicky et al., 2014). For instance, GluN2C subunit containing NMDARs exhibit low conductance upon opening which consequently affects calcium (Ca^{2+}) influx during NMDAR activation (Momiyama et al., 1996). There are also many forms of di-heteromeric and tri-heteromeric NMDARs subunit compositions, demonstrating the complexity of the receptor that contributes to the wide-ranging actions across different brain regions (Paoletti and Neyton, 2007).

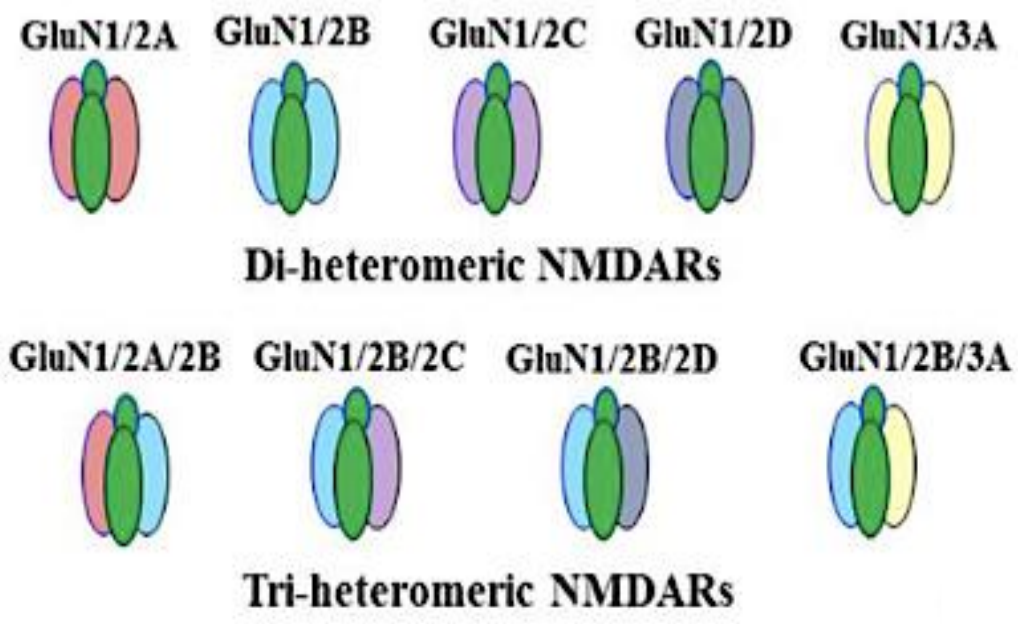


Figure 1.1. Examples of different NMDAR subunit compositions that form di-heteromeric and tri-heteromeric NMDARs in the mammalian brain. *The NMDAR typically consists of two GluN1 subunit and two of the same GluN2/GluN3 subunits subtypes to form di-heteromeric NMDARs. Conversely, two GluN1 subunit and combinations of one GluN2 and one GluN3 subunits or two different GluN2/GluN3 subunits subtypes to form tri-heteromeric NMDARs in the brain. However, GluN1/GluN3 are known as glycine channels. (Adapted from: Kumar, 2015).*

The membrane topology is consistent for all NMDAR subunits. This includes the extracellular amino-terminal domain, intracellular cytoplasmic C terminal domain, M2 re-entrant pore loop and transmembrane segments that include M1, M3 and M4. It is believed that the M2 is linked to the channel pore that is responsible in regulating the magnesium (Mg^{2+}) blockade and calcium permeability (Fig 1.2) (Kumar, 2015).

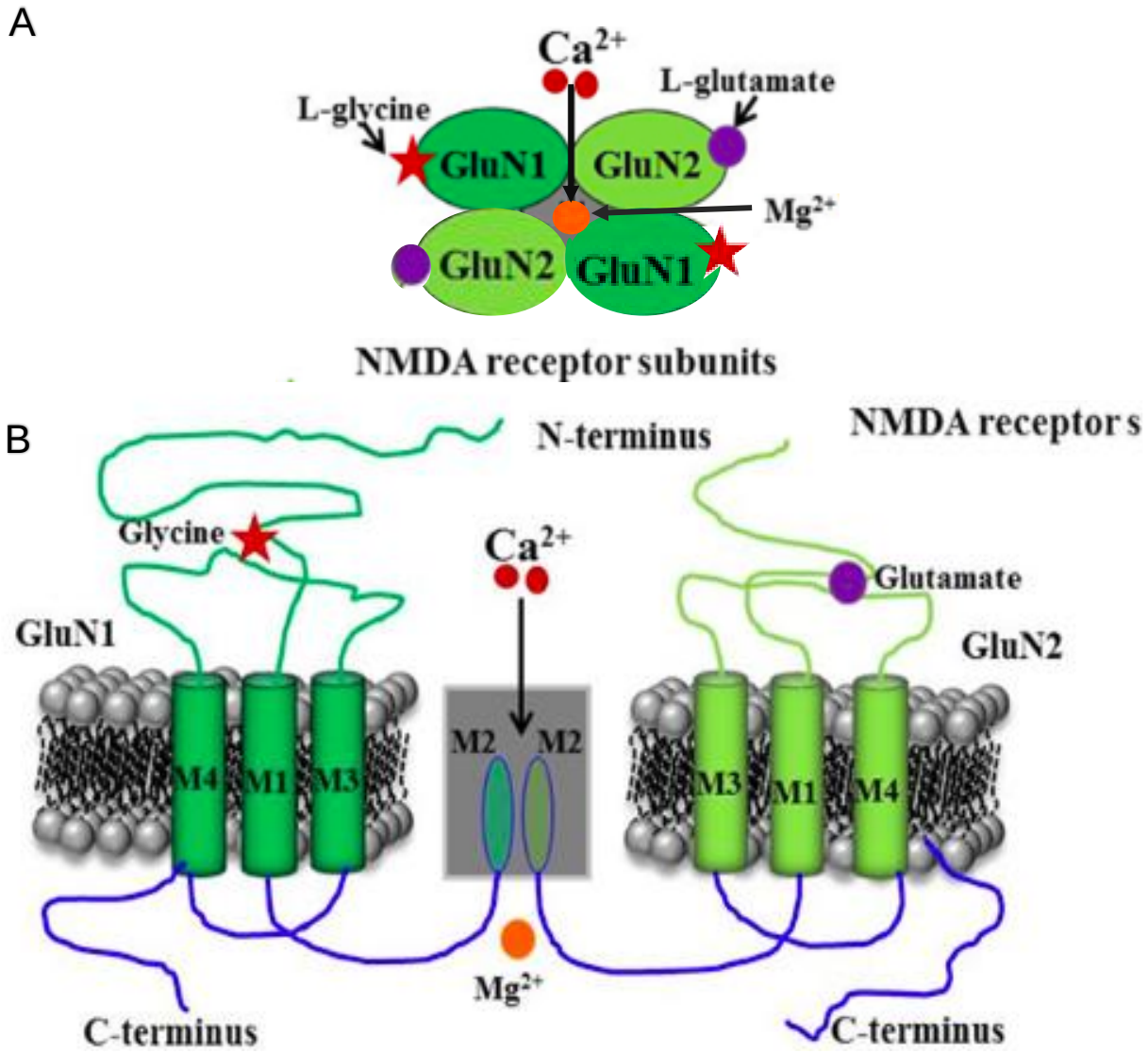


Figure 1.2. Membrane topology of the NMDAR subunits. (A) *Illustrates the top view of the di-heteromeric NMDAR.* ***(B)*** *NMDAR subunit membrane topology. GluN1 subunit membrane topology is consistent with GluN2 subunit. NMDAR activation requires the binding of two L-glutamate ligands on the GluN2 subunits and two co-agonist ligands L-glycine on the GluN1 subunits concomitantly. The M2 re-entrant pore loop is linked to the channel pore and this is believed to be where Mg2+ binds. (Adapted from: Kumar, 2015).*

Although the close resemblance in the NMDAR subunit structure to KAR and AMPARs subunits, there are many differences between the subclasses based on ion permeability and sites of drug action. For instance, NMDARs exhibit many different binding sites for Zinc (Zn^{2+}), polyamines, glycine/D-serine and Mg^{2+} ions (Fig 1.3).The Mg^{2+} ion is a critical ion that binds to the channel pore and restricts ion flow through the NMDAR channel at the resting membrane potential (Mayer et al., 1984; Nowak et al., 1984; Mori and Mishina, 1995). NMDAR activation requires the binding of both glycine and glutamate to GluN1 and GluN2 subunits, respectively and are considered co-agonists (Johnson & Ascher, 1987; Kleckner and Dingledine, 1988; Hirai et al.,1996; Anson et al.,1998).Figure 1.3 shows the NMDAR complex binding sites for various ions, endogenous ligands and drugs. For instance, D-2-amino-5-phosphonopentanoate (D-AP5), is a known NMDAR competitive antagonist that competes with glutamate at the GluN2 subunit binding site. Kynurenic acid is an endogenous ligand is another example of a competitive antagonist, but it only binds to the GluN1 subunit binding site and is competitive with glycine. Interestingly the concentration of zinc determines different NMDAR subunit affinity (Ghasemi and Schachter, 2011). For example, low Zn^{2+} concentration (nM) expresses affinity for GluN2B containing subunit, similar to ifenprodil, a negative allosteric modulator (NAM) that binds to the same subunit and inhibits channel opening (Mony et al., 2009; Kew and Kemp, 1998 Lodge and Mercier, 2015). However very high concentrations, Zn^{2+} can function as a pore NMDAR channel blocker (Ghasemi and Schachter, 2011).

PCP and ketamine are dissociative drugs and are believed to bind near the asparagine residue of the pore lining M2 loops located inside the channel pore (Burnashev et al.,1992; Kashiwagi et al., 2002; Kotermanski and Johnson, 2009; Retchless et al., 2012), consequently blocking the NMDAR noncompetitively (Khors and Durieux, 1998). NMDAR antagonists have been beneficial in pharmacological studies in better understanding the structure and function of NMDARs and sites of drug action. NMDAR antagonists in studies have provided knowledge and understanding to develop high efficacy NMDAR modulating drugs for use in treating NMDAR related neurological disorders.

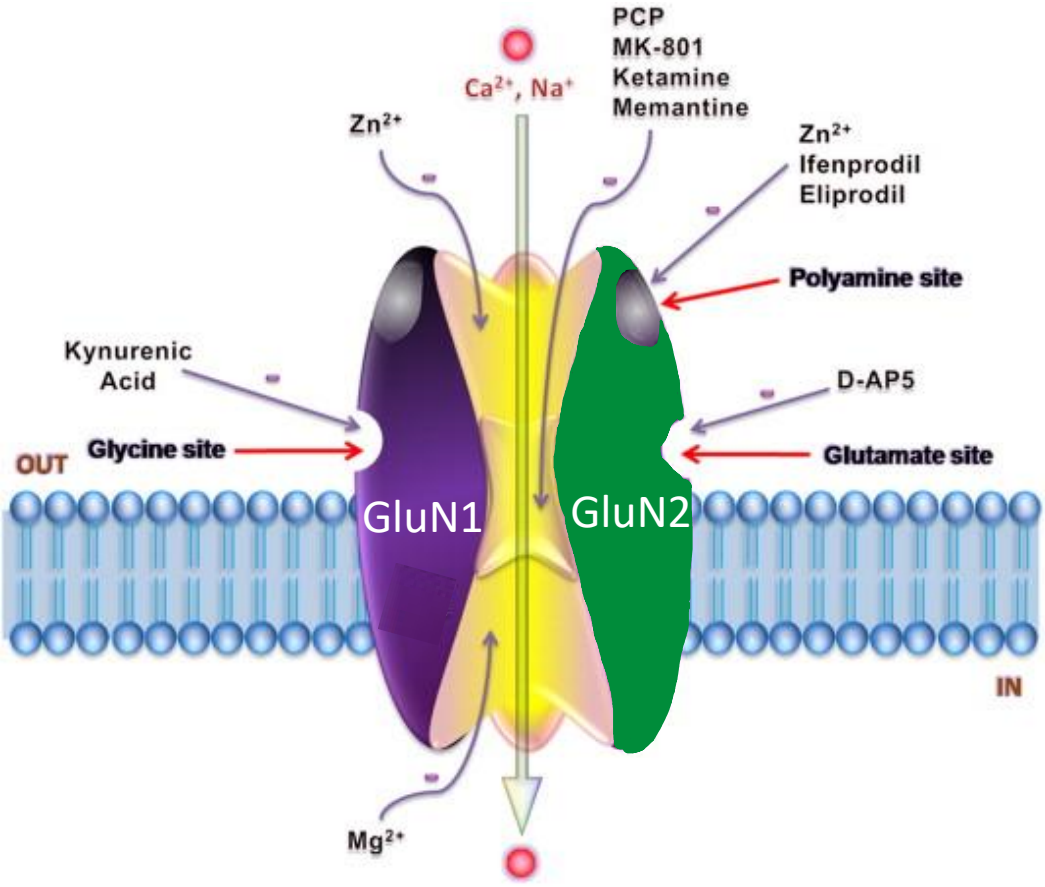


Figure 1.3. Representation of the NMDAR complex illustrating different binding sites*. Glycine binds to the NR1 subunit binding site (i.e. glycine site; GluN1). Kynurenic acid also competes with glycine to bind to the same site. Glutamate binds to the NR2 subunit binding site (I.e. glutamate site; GluN2). D-AP5, a well-recognised competitive antagonist, binds to the same site as glutamate. Other ions, endogenous ligands and drugs also bind to various sites. Specifically, ketamine, PCP, MK-801 and memantine are non-competitive antagonists that bind to the pore lining M2 loops located inside the channel pore, thereby acting to block it. Mg2+ also binds to the channel pore and subsequently restricts the ion flow through the NMDAR channel at resting membrane potential. (Adapted from: Ghasemi and Schachter, 2011).*

1.1.2.2 Localisation of NMDARs

1.1.2.2.1 Presynaptic: locations and functions of NMDARs

Although NMDARs are normally found in postsynaptic sites in the majority of excitatory synapses, they are also found at presynaptic sites in many brain regions. For instance, in the cortex, hippocampus, amygdala, and cerebellum (Corlew et al., 2007; Aoki et al., 1994; Jourdain et al., 2007; Farb et al., 1995; Rossi et al., 2012).

Furthermore, increase spontaneous release at glutamatergic synapses in regions including the hippocampus, amygdala, and cerebral is mediated by activation of presynaptic NMDARs (Brasier and Feldman, 2008; Corlew et al., 2007; Jourdain et al., 2007; Humeau et al., 2003). Also, the same effects are observed at GABAergic synapses in the hippocampus and cerebellum (Xue et al., 2011; Rossi et al., 2012; Glitsch and Marty, 1999). These studies have shown that indeed NMDARs at GABAergic synapses can regulate neurotransmitter release. Thus, spontaneous GABA release have been observed. Therefore, this suggests that NMDARs may regulate the inhibitory neurotransmitter release and possibly impact the equilibrium between excitatory and inhibitory signalling in neural circuits.

Xue et al., 2011; Rossi et al., 2012; Glitsch and Marty, 1999 are example of studies that have provided evidence that NMDARs affects GABA release presynaptically. These studies have shown that increase release of GABA is mediated by activation of NMDARs at GABAergic terminals. Thus, suggests that NMDARs affects GABA release and further concludes impacting the equilibrium between excitation and inhibition in neural circuits.

1.1.2.2.2 Postsynaptic: locations and functions of NMDARs

NMDARs are widely expressed across the CNS. However, many NMDAR subunits are expressed differently in the brain. For example, GluN2A and GluN2B subunits are found predominately expressed in the hippocampus and cerebral cortex and it can be speculated that these subunits have an important role in synaptic plasticity (Monyer et al., 1994; Laurie et al., 1997; Takai et al., 2003).

Interestingly, the expression of NMDAR subunits varies at different developmental phases (Paoletti and Neyton, 2007). For example, GluN2B subunits are predominately expressed during the initial stages of development. This slowly declines during the start of puberty and GluN2A containing NMDAR increase during the course of a lifetime (Liu et al., 2004; Monyer et al.,1994; Laurie et al.,1997; Laurie and Seeburg,1994; Law et al., 2003; 2003).

NMDARs are present in both synaptic and extra-synaptic regions. The actions of synaptic and extra-synaptic NMDARs can collectively influence and modulate the fast-excitatory synaptic transmission (Papouin et al., 2012). However, both synaptic and extra-synaptic NMDARs exhibit unique functions and are speculated to have different intracellular signalling pathways and signalling roles in the brain and different co-agonists to activate them respectively (Léveillé et al., 2008; Hardingham and Bading, 2010; Papouin et al., 2012). Synaptic NMDARs have many important roles however, the primary role is to trigger intracellular Ca^{2+} influx upon glutamate binding. Synaptic NMDARs also have a modulating effect on long term potentiation (LTP) induction. This is because when learning occurs, synaptic NMDARs plays a significant role in strengthening the synaptic connections in the brain. This modulation is critical for the memories formed in the brain to be accurate and adaptable. On the other hand, long term depression (LTD) requires the activation of both synaptic and extra-synaptic NMDARs.

Simon et al., 1984 concluded that overstimulation of NMDARs contributes to neuronal death in the brain. Specifically, it was suggested that extra-synaptic NMDARs have a neurotoxic role and can trigger neuronal death. This has been supported by other research papers, suggesting overstimulation most likely leads to excitotoxicity (Hardingham and Bading, 2010) and could possibly underlie the pathophysiology of neurodegenerative disorders e.g. Alzheimer's disease (Wallace, 2014).

1.1.2.3 Role of NMDARs in synaptic transmission and synaptic plasticity

NMDAR was discovered in the 1960s (Watkins and Jane, 2009). Subsequently, many researchers started investigating the functional and pharmacological properties of these receptors. NMDAR's involvement in modulating synaptic transmission, synaptic plasticity, and neurodegeneration was later discovered and confirmed by research studies (Yamakura and Shimoji, 1999; Davies and Watkins, 1979).

NMDARs exhibit a unique activation profile that differs from both AMPARs and KARs. For instance, NMDAR activation is dependent on the binding of the excitatory neurotransmitter glutamate as well as glycine/D-serine as a co-agonist, alongside voltage dependent removal of the Mg^{2+} block in the pore channel during AMPAR mediated membrane depolarization in the postsynaptic neuron. Thus, NMDARs function as coincidence detectors and cause an influx of Ca^{2+} and sodium ions (Na^{+}) through the NMDAR channel into the post-synaptic neuron (Mayer et al., 1984). Thus, NMDAR voltage dependence and high Ca^{2+} permeability causes a more sustained synaptic depolarization and can induce long-term synaptic changes (Schoepp et al., 1995; Karmarkar and Buonomano, 2002; Tabone and Ramaswami, 2012).

Influx of Ca^{2+} trigger a downstream signalling cascades of events (Kandel, 2001; Lau et al., 2009). This signalling is necessary for the induction of long-term potentiation (LTP). NMDARs regulate LTP alongside long term depression (LTD), two phenomenon that are known to be involved in learning, memory acquisition, memory storage, and can also influence spatial learning memory (Collingridge et al., 1983; Morris et al., 1986). The pivotal role of NMDARs in synaptic plasticity in the hippocampus was established in 1983 by Collingridge and his fellow peers using DL-2-amino-5-phosphono-pentanoate (AP5), a selective and potent NMDAR antagonist blocked LTP induction. From this research, it was concluded that NMDAR activation is vital in LTP induction in Schaffer collateral-commissural pathway (SCCP) in the hippocampus and that LTP induction in this pathway is NMDAR dependant. However, this is not the case for all pathways in the hippocampus. For instance, D-AP5, a competitive NMDAR antagonist had no effect on LTP induction in the mossy fibers pathway in the hippocampus (Harris and Cotman, 1986).

Earlier studies have also concluded the critical role of NMDARs in modulating synaptic plasticity by influencing spatial learning memory (Morris et al., 1986). The study demonstrated that rats performed poorly on the water maze spatial memory test after an intraventricular injection of AP5 in the hippocampus. Therefore, antagonism at NMDARs can impair synaptic plasticity and lead to memory impairments (Kavalali and Monteggia, 2012). In vitro and in vivo studies demonstrated that ketamine, PCP, and MK-801 (NMDAR antagonists) also blocked the induction of LTP (Stringer and Guyenet, 1983; Stringer et al., 1983; Wöhrl et al., 2007; Graef et al., 2015).

The induction of LTP and its counterpart LTD require different protocol of stimulation. For example, high frequency stimulation (HFS) is usually used for the induction of LTP (Bortolotto et al., 2011). Theta burst stimulations (TBS) is the preferred protocol to induce LTP in the hippocampus because it is believed to more precisely reproduce the hippocampal neurons physiological firing patterns (theta activity) compared to other high frequency protocols (e.g. 100 shocks at 100Hz).Conversely, LTD is usually induced by low frequency stimulation (LFS such as 300 – 900 shocks at 1Hz (Bortolotto et al., 2011).

1.1.2.4 NMDAR related neurological disorders

NMDAR dysfunction is implicated in many neurological disorders. The involvement of the NMDAR in modulating synaptic transmission and plasticity has made this receptor a key interest in many drug developments (Dingledine et al., 1999; Lodge et al., 2009). Dysfunctional NMDARs are thought to be linked to several disorders such as schizophrenia, Alzheimer's disease, epilepsy, depression, stroke and Huntington's disease. Literature papers have also speculated that different NMDAR subtypes and localisations in the brain could possibly be involved in different pathological conditions (Zhou and Sheng, 2013).

Overstimulation of NMDARs are considered to play a role in Alzheimer's disease, stroke and epilepsy, resulting in excitotoxicity in the brain (Cull-Candy et al., 2001). Therefore, the NMDAR is a crucial therapeutic target to treat these particular disorders by targeting the overstimulation of NMDARS. Hence, reducing neuronal death in the brain due to the overstimulation of NMDARs related to these disorders. For instance, memantine is a known NMDAR antagonist, shown to have neuroprotective properties. This drug is clinically used to treat the cognitive deficits in patients with moderate Alzheimer's disease. Only medicinal drug used to treat severe cases of Alzheimer's disease. (Tariot, 2006; Reisberg et al., 2017; Olivares et al., 2012). Léveillé et al suggested that memantine preferably works on extra-synaptic NMDARs (Léveillé et al., 2008). This aligns with the idea previously mentioned that extra-synaptic NMDARs have a neurotoxic role and can trigger neuronal death.

Furthermore, schizophrenia is another psychiatric disorder that is believed to be linked to NMDAR dysfunction. There is confluence of evidence to suggest this. For instance, ketamine and PCP have demonstrated in the past to exhibit dissociate effects and closely mimic the positive, negative and cognitive symptoms of schizophrenia in both preclinical and clinical studies (Lodge and Mercier, 2015; Becker and Grecksch, 2004). Both ketamine and PCP are often used in animal studies to produce animal models of schizophrenia (Kahn et al., 2015; Buccasfusco and Terry, 2009). Similarly, when MK-801,

a very potent NMDAR antagonist was used in an animal study, observed schizophrenia like symptoms, particularly negative and positive symptoms e.g. social isolation and hyper-locomotion (Rung et al., 2005). Furthermore, research studies found that schizophrenic like symptoms were displayed in transgenic animals that lack NMDAR related genes (Balu and Coyle, 2015; Yamamoto et al., 2013; Sato et al., 2008).

Depression is a prevalent mental disorder, affecting approximately 322 million people worldwide (Friedrich, 2017). Depression notably affects 5% of adults (i.e. 4% among men and 6% among women) and 5.7% of adults over the age of 60 (World Health Organisation, 2021). Symptoms include sadness, hopelessness, fatigue, and in some cases leads to ideation of suicide (Kennedy, 2008). Unfortunately, one of the severe consequences of depression is suicide. Each year, over 700,000 individuals lose their lives to suicide, ranking as the fourth highest cause of mortality in individuals aged 15-19-year olds (World Health Organisation, 2021). Hence, a global need to develop an effective treatment for individuals diagnosed with depression.

Traditional antidepressants often target the monoamine systems in the brain e.g. serotonin (Duman & Monteggia, 2006). However, conventional antidepressants display a delayed onset of action. Often taking 2-3 weeks for patients to respond to these medications. The majority of patients do not respond and thus develop treatment resistant depression (Zarate et al., 2006) Consequently, new high efficacy fast acting antidepressant drugs are needed on the market. Previous literature has shown NMDAR involvement in the physiopathology of depression. In the early 2000s, Berman et al. demonstrated ketamine's fast acting antidepressant effects in major depressive disorder patients and the same results were found in subsequent experiments (Berman et al., 2000; Zarate et al., 2006). Ketamine was also effective in the treatment of treatment resistant depression (TRD), producing rapid antidepressant actions (Caddy et al., 2014). The effects of ketamine in clinical studies have lasted up to several weeks longer after administration compared to traditional antidepressants (Berman et al., 2000). Ketamine has also shown to cause rapid onset antidepressant effects in mice in experiments using the forced swim test (Autry et al., 2011). However, although forced swim test is a widely

used assay for depression, other behavioural tests such as learned helplessness is thought to be a better model of depression.

Ketamine's antidepressant mechanism is still an ongoing discussion. Zanos et al and other research literatures have shown that ketamine metabolites, (2R,6R)- 6-hydroxynorketamine ((2R,6R)-HNK), also exhibits fast acting antidepressant effects. However, the effects are observed at concentrations below that have an effect on NMDARs (Lumsden et al., 2019; Zanos et al., 2016; Kavalali and Monteggia, 2018). Zanos group proposes that ketamine metabolites antidepressant mechanism of action is AMPAR-dependent rather than NMDAR-dependent. Possibly, antidepressant mechanism of action is not because of NMDAR blockade but instead increases AMPA function via LTP process downstream of NMDARs. (Zanos et al., 2016). However, Kang et al contradicted this proposed idea and showed that both ketamine metabolites (2R,6R)-6-hydroxynorketamine ((2R,6R)-HNK) and (2S,6S)- 6-hydroxynorketamine ((2*S*,6*S*)-HNK) blocked NMDAR-LTP in the SCCP in the hippocampus, similar to ketamine (Kang et al., 2020). These findings support the idea that NMDAR mediated synaptic plasticity may possibly be involved in ketamine, and ketamine metabolites fasting acting antidepressants effects. However, the antidepressant mechanism of ketamine's and its metabolites is still under question.

1.2 Ketamine

1.2.1 History, synthesis and chemical structure of ketamine

Arylcyclohexylamines are compounds that include a cyclohexamine unit, that usually contains a phenyl ring as the aryl moiety and are both attached to an amine group (Ho and Dargan, 2016). 1-(1-phenylcyclohexyl) amine was the first discovered arylcyclohexylamine in the early 1900s (Kursanov, 1907). However, the most well-known arylcyclohexylamines are ketamine and PCP.

PCP was synthesised by Maddox, a chemist at the Parke-Davis company in the early 1950s (Ho and Dargan, 2016). It was found that PCP demonstrated strong anaesthetic effects in clinical trial without exhibiting respiratory depression. Subsequently, it was approved by the FDA in the 1957 (Greifenstein et al., 1958; Morris and Wallach, 2014). However, shortly after it was withdrawn due to the severe side effects reported in humans e.g. psychosis and delirium (Domino, 2010). Consequently, PCP was then only sold for veterinary use (Rappolt et al., 1979).

Ketamine was first synthesised in the early 1960s by Calvin L. Stevens who also worked at the Parke-Davis company (Morris and Wallach, 2014; Domino et al., 1965). The first time ketamine was administered to humans was in 1964, involving 20 prison volunteers (Domino, 2010). From this, ketamine was demonstrated to have shorter acting anaesthetic effects and fewer side effects than PCP (Domino, 2010). Hence, ketamine is primarily used for general anaesthetics for veterinary and human use, providing pain relief and sedation (Greifenstein et al., 1958; McCarthy et al., 1965). Subsequently, ketamine was deemed a safe anaesthetic for surgery purposes (White et al., 1980).

In the early 1980s, Professor David Lodge and his colleagues were the first to demonstrate that ketamine is a non-competitive NMDAR antagonist (Lodge et al.,1982). Subsequent studies established that ketamine is a use- and voltage-dependent open channel blocker, similar to MK-801, a potent non-competitive selective NMDAR antagonist. (MacDonald et al., 1987; Huettner & Bean, 1988). Many studies confirmed non-competitive NMDAR antagonists binding near the asparagine residue of the pore lining M2 loops located inside the channel pore (Burnashev et al.,1992; Kashiwagi et al., 2002; Kotermanski and Johnson, 2009).

N-ethyldeschloroketamine also called deschloro-*N*-ethyl-ketamine or 2-phenyl-2-(ethylamino)-cyclohexanone (2-oxo-PCE), on which this research project will focus on, is a ketamine derivative. 2-oxo-PCE belongs to the arylcyclohexylamine class and shares similar chemical structure to ketamine (Fig 1.4). For instance, both include 2-phenyl-2-aminocyclohexanone in their structures (Cheng and Dao, 2020). The only key difference is the ethyl group on the nitrogen atom of the amino group for 2-oxo-PCE and methyl

group on the nitrogen and chloride attached on the phenyl ring for ketamine (Cheng and Dao, 2020). 2-oxo-PCE was first synthesized in the early 1960s in the effort of developing a new potential PCP derivative used for human anaesthesia with shorter acting properties and less severe side effects e.g. delirium. (Stevens et al.,1966; Morris and Wallach, 2014). 1-(1-(2-fluorophenyl)-2-phenylethyl)pyrrolidine (fluorolintane), a research chemical and a ketamine derivative, has also been shown to have a similar chemical structure to ketamine. Fluorolintane has also demonstrated similar pharmacodynamic properties (Wallach et al., 2019). From this, I can speculate that 2-oxo-PCE could share similar pharmacodynamic properties to ketamine.

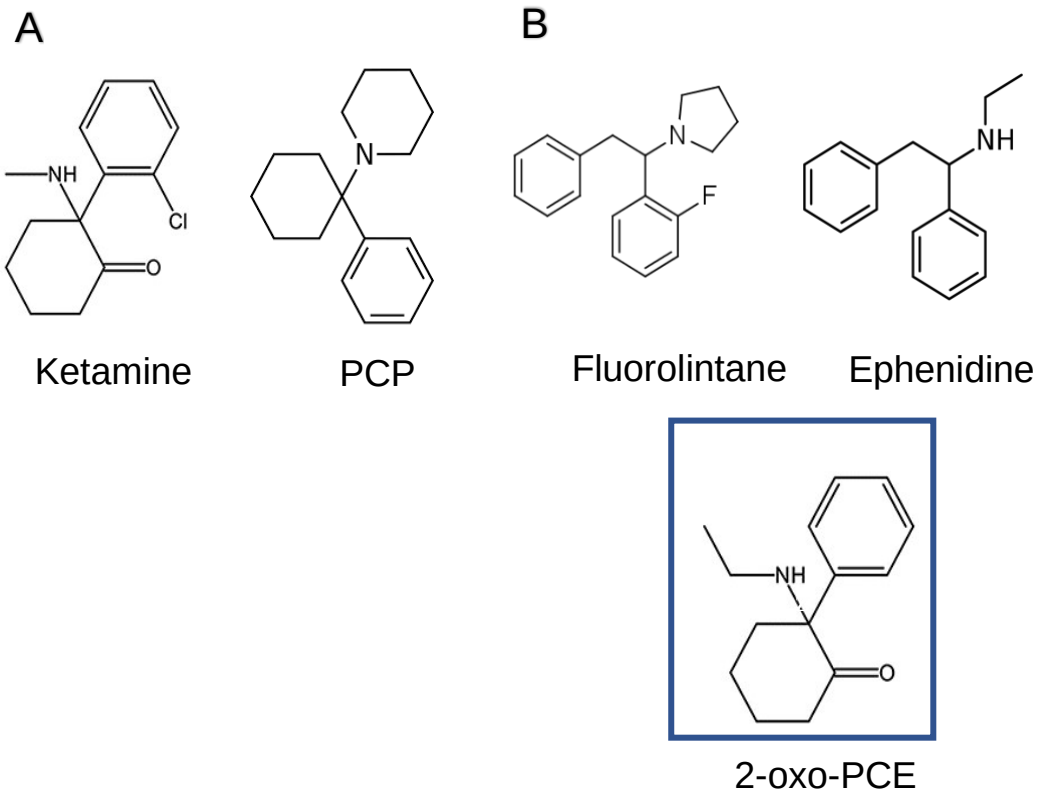


Figure 1.4. Chemical structure of dissociative drugs. (A) *Ketamine and PCP are classical psychoactive substances that are known as 'dissociative anaesthetics'.* ***(B)*** *Examples of research chemicals produced to mimic the effects found in the classical psychoactive substances e.g. ketamine. The drug in the blue box is 2-oxo-PCE, the drug of interest for this research project. (Adapted from: Wallach et al, 2019, Kang et al., 2017; Cheng and Dao, 2020).*

1.2.2 Recreational use and abuse of ketamine

The synthesis of ketamine was primarily intended for the purpose of general anaesthetics. However, has recently sparked a worldwide clinical interest for its faster-acting antidepressant effects compared to conventional antidepressants (Berman et al.,2000). Despite ketamine showing clinical promise in the treatment of depression and exhibiting anaesthetic properties, it has been shown that ketamine also exhibits dissociative effects. Speculated that these dissociative effects are due to antagonism of NMDARs (Morris and Wallach, 2014; Lodge and Mercier, 2015). Thus, it is often referred to as a 'dissociative anaesthetic'. The fundamental features of dissociative effects include hallucinations, euphoria, tactile distortions, and depersonalization (Morris and Wallach, 2014). Ketamine, a classical psychoactive substance, displays a lower potency and shorter acting (~10minutes) profile compared to PCP and the dissociative effects last up to 60 minutes when injected (Lodge and Mercier, 2015). However, it is much safer than PCP with fewer documented overdoses. Thus, it has become a popular recreational drug and often referred as "Special K" on the streets (Morris and Wallach, 2014). However, there is no established antidote for ketamine overdose (Lotfy et al., 1970). In 2005, ketamine was classed as a class C drug (Morris and Wallach, 2014). Interestingly, recreational use of ketamine is thought to be around 100,000 yearly in the UK (Lodge and Mercier, 2015; Morgan and Curran, 2012) and is among the top 6 drugs that causes immediate social and bodily harm (Lodge and Mercier, 2015; Nutt et al., 2007). For example, continuous usage of ketamine can lead to painful long-term severe urinary tract toxicity. (Lodge and Mercier, 2015; Chu et al., 2008; Morgan and Curran, 2012). Based on this, was crucial to put in place stronger legislation for ketamine in the UK and in 2014 it was classified as a class B drug. (Lodge and Mercier, 2015). Stronger legislative restrictions have resulted in explosions of research chemicals on the market for recreational purposes whilst avoiding legislation. 'New psychoactive substances'/ 'research chemicals' are produced to mimic the effects found in the classical psychoactive substances. These research chemicals mimic different classes of classical psychoactive substances. Particularly, dissociative anesthetics e.g. ketamine for its dissociative effects.

However, despite the misuse and controversies around “research chemicals”, these chemicals have also demonstrated therapeutic potential in the treatment of various neurological disorders.

1.3 New legal high drugs: 2-oxo-PCE

Although 2-oxo-PCE was reported to be synthesized in the early 1960s in the effort of developing a new potential PCP derivative that is shorter acting with less severe side effects e.g. delirium (Stevens et al., 1966; Morris and Wallach, 2014), online reports of usage has become a more recent phenomenon.

Although ketamine was first synthesised in the 1960s, it was not until 2016, the European monitoring centre for drugs and drug addiction Europol 2016 annual report first reported 2-oxo-PCE as a new psychoactive substance (Emcdda.europa.eu, 2016). The following year, reports found 2-oxo-PCE in urine samples of several patients with a history of ketamine abuse in Hong Kong (Chong et al., 2017; Tang et al., 2018). Reports online suggests that misuse of 2-oxo-PCE produces potent dissociative, anaesthetic and hallucinogenic effects. (https://psychonautwiki.org/w/index.php?title=O-PCE&_=; Theofel et al., 2018 https://www.eve-rave.ch/Forum/viewtopic.php?t=44120). Despite reported cases, little is known about the pharmacology of 2-oxo-PCE. Online user anecdotal reports have alluded 2-oxo-PCE displays comparable effects to ketamine but is deemed more potent and often sold as a substitute for ketamine to evade legislative restriction (NeuePsychoaktiveSubstanzen.de, 2016; Cheng and Dao, 2020).

Currently, pharmacological characterisations of 2-oxo-PCE have been based on the chemical structure and anecdotal reports online about the similarities to ketamine. No scientific studies have been published exploring the pharmacodynamics of 2-oxo-PCE. Ketamine has been confirmed to affect glutamate mediated synaptic transmission in the brain. Therefore, it is important to investigate the functional role of 2-oxo-PCE in synaptic

transmission, it's selectivity, and the potential capacity to influence synaptic functions in the brain, for instance synaptic plasticity.

Fluorolintane is a ketamine derivative (Wallach et al., 2019) and ephenidine have both shown to exhibit ketamine like properties (Kang et al., 2017). Both 1, 3, and 10μM fluorolintane and ephenidine have been shown to reduce NMDAR-mediated synaptic transmission in a dose dependant manner respectively. 1, 3, and 10μM fluorolintane, reduced NMDAR-fEPSP peak amplitude by 41.8 ± 4.3% (n=5), 60.4 ± 3% (n=5), and 85.9 ± 1% (n=4) respectively (Wallach et al., 2019). 1 and 10μM ephenidine reduced NMDAR-fEPSP peak amplitude approximately 25% and 78% correspondingly (Kang et al., 2017). Also, both drugs at 10μM blocked NMDAR-dependent LTP CA1 region of rat hippocampal slices respectively. The pharmacological effects these 2 drugs were comparable to those of ketamine (Wallach et al., 2016; Kang et al., 2017). Since research chemicals flurolintane, ephenidine and 2-oxo-PCE share similar chemical structures, the effects of 2-oxo-PCE should be explored and investigated including methodological techniques in a manner consistent with the research approaches demonstrated in the fluorolintane and ephenidine studies respectively. This will also enable direct comparisons between these research chemicals. Furthermore, the pharmacological actions of 2-oxo-PCE at NMDARs is likely to share similarity with ketamine because 2-oxo-PCE is a derivative of ketamine and ketamine is an established NMDAR antagonist.

1.4 Aims & hypothesis

The hypothesis proposes that 2-oxo-PCE will have a concentration-dependant inhibitory effects on NMDAR-fEPSP, similar to ketamine in CA1 region of the hippocampus. Additionally, it is hypothesised that 2-oxo-PCE will impair the induction of NMDAR-LTP and NMDAR-LTD in CA1 hippocampal slices respectively.

The aim of this project is to characterise the pharmacology of '2-oxo-PCE' by investigating the effects and pharmacological actions of '2-oxo-PCE' on NMDAR mediated synaptic transmission including at various concentrations. Moreover, this project aims to investigate the effects of NMDAR mediated induction of LTP (NMDAR-LTP) and LTD (NMDAR-LTD) respectively at the CA1 synapses in the hippocampus.

2 Methods and Materials

2.1 Animals

Male Wistar rats (Charles River, UK) aged 5-10 weeks old were used in this study. All animals were kept in standard cages (12- hour light/dark cycle) at room temperature. Food and water were accessible ad libitum following the Animals (Scientific Procedures) Act (1986).

2.2 Hippocampal slice preparations

Male Wistar rats were placed in an anaesthesia chamber and killed by terminal anaesthesia with 100% isoflurane in O_2 (with a flow rate 1.7 ml per minute) (Schedule 1 under the Animals (Scientific Procedures) Act (1986)), and swiftly decapitated using a guillotine (Animal handling rotated between myself, Dr Zuner Bortolotto and Dr Mathew Claydon throughout this project). The brain was removed quickly and placed in ice cold artificial cerebrospinal fluid (aCSF) comprised of (in mM): 124 NaCl, 3 KCL, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 1 $MgSO_4$, 2 CaCl2, 10 D-Glucose saturated at 95% O_2 and 5% CO_2 (pH value ~7.0-7.5). The brain was blocked, hemisected, and fixed (using cyanoacrylate adhesive) to the vibratome stage, a silicone block was then glued directly supporting the brain. The vibratome stage was placed inside the buffer tray which was then filled with ice cold oxygenated aCSF. Parasagittal brain slices (400µM thick) were then cut using the vibratome (World Precision Instruments). The hippocampus was then dissected out of the slice and placed in fresh oxygenated aCSF. The CA3 subregion was then carefully removed in order to reduce possible epileptic activity. Slices were left to incubate at room temperature (21±1°C) for at least 60 minutes before being transferred to the recording chamber. Stock solutions preparations alternated between my lab partner and myself on a weekly basis. However, Hippocampal slice preparations were shared daily.

2.3 Electrodes

Recording electrodes (1.2-mm O.D. x 0.69-mm I.D.) made from borosilicate glass (Harvard apparatus, USA) were pulled using an electrode puller (Sutter Instruments CO.; model p-97, USA) and filled with aCSF. The resistance of the recording electrode was between 3-6 MΩ when positioned in the stratum radiatum of the hippocampal CA1 area. Two concentric (FHC, USA) or twisted bipolar stimulating electrodes (S0 and S1) were positioned one each side of the recording electrode (see Fig 2.2A & Fig 2.2B) to stimulate 2 independent inputs, alternating 15 seconds interval at 0.03Hz.

2.4 In vitro fEPSPs recording

Slices were placed in a modified Haas style recording chamber (Fig 2.1). The slices in the center well were constantly perfused with oxygenated aCSF at a rate of 2ml per minute (2ml/min) by peristaltic pump (MasterFlex, Germany). The slices were maintained at (29 ± 2°C) in the recording chamber and only one slice was used for each experiment. The recording chamber was surrounded by purified oxygenated water constantly heated to create a non-drying and humidified atmosphere for the slices and reduce the bubble formations inside the recording chamber.

The recording electrode and stimulating electrodes were placed ~500μM from each other (shown in Fig 2.2B). Peak amplitude of fEPSP responses was recorded and analysed on WinLTP software on-line and off-line (Anderson and Collingridge, 2007) (Fig 2.3). Recordings were amplified using an AxoClamp 2B (Molecular Devices, USA) and filtered at 1.0kHz. Stimulus sweeps were signal averaged such that each data point represents 4 consecutive fEPSPs recorded over a 2-minute time frame. Both inputs alternated every 15 seconds. 30 minutes of stable baseline was recorded before NMDAR-mediated fEPSPs experiments and NMDAR dependent synaptic plasticity experiments were conducted respectively.

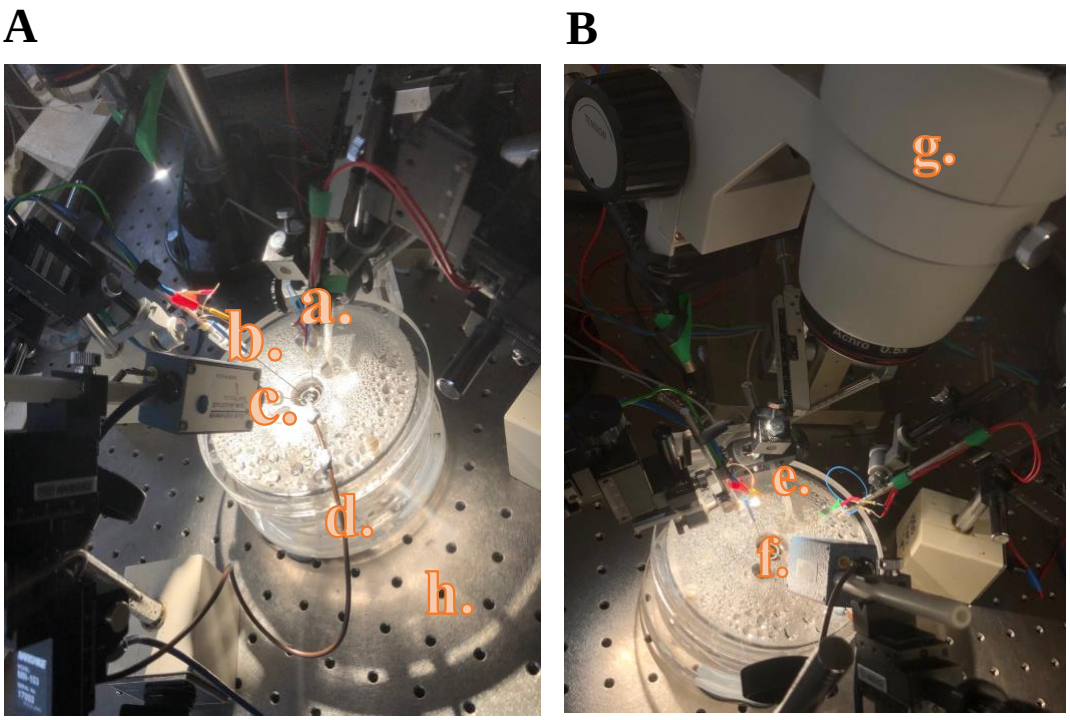


Figure 2.1. Electrophysiological recording chamber set up for in vitro fEPSPs on hippocampal slices. (A) *Front view demonstrates* ***a. & b.*** *stimulating electrodes (S0 and S1 respectively) attached to a micromanipulator (Narshishige, MN-153 model, Japan).* ***c.*** *recording electrode also connected to a micromanipulator.* ***d.*** *reference electrode is coated with silver chloride and placed underneath the net in the center well in the recording chamber. Reference electrode was connected to the headstage attached to a micromanipulator.* ***(B)*** *Side view comprising of* ***e.*** *suction system that comprises of the hypodermic needle that recycles aCSF from the third well back the oxygenated aCSF in the water bath.* ***f.*** *two slices on a net in the center well. Another net placed on top of the slices, to avoid floating when submerged (29 ±2 °C). Both stimulating electrodes and recording electrodes are positioned in the slice.* ***g.*** *stereomicroscope (Leica) to view slices.* ***h.*** *anti-vibration table and was surrounded by a faraday cage to help reduce external electrical noises. The recording chamber was placed on top of the anti-vibration table.*

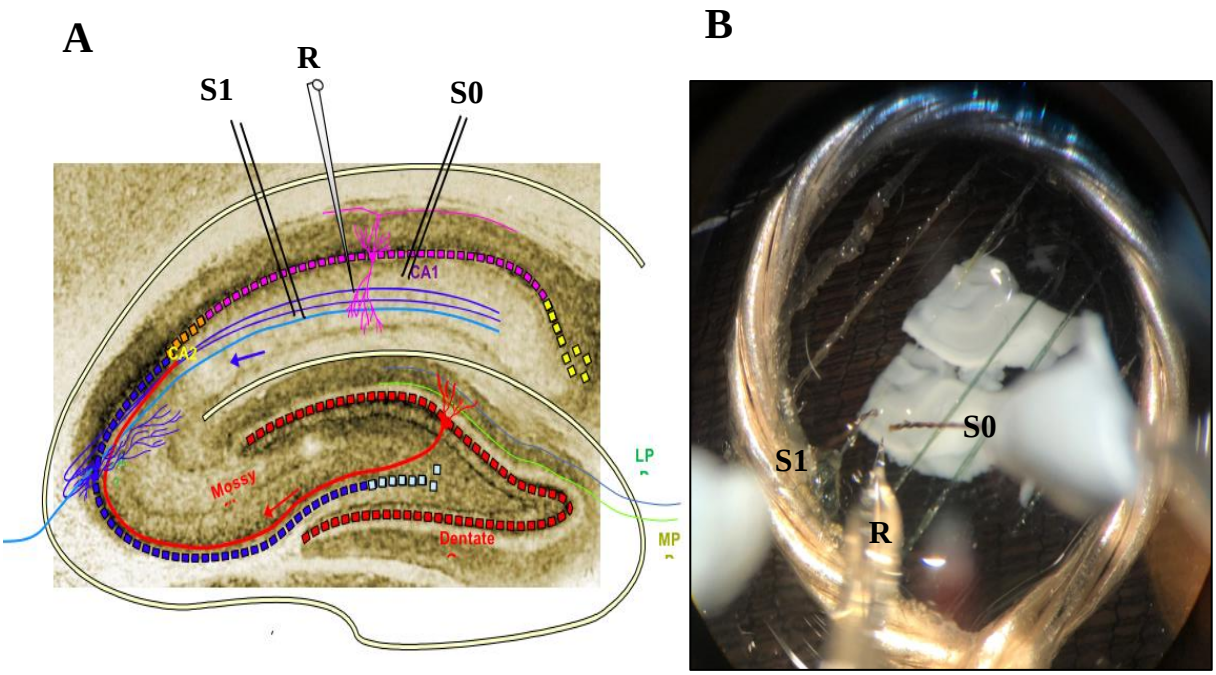


Figure 2.2. Recordings of the fEPSP responses in CA1 region of the hippocampus. (A) *Schematic representation and* ***(B)*** *visual representation (picture taken under a microscope) of the CA1 region of the hippocampus. S0 and S1 represents the bipolar stimulating electrodes positioned to stimulate the Schaffer collateral-commissural pathways to evoke field excitatory synaptic potentials (fEPSPs) recorded from a recording electrode* ***(R)*** *in the stratum radiatum of area CA1. (****A****) Adapted from (Bortolotto et al. 2011).* ***(B)*** *Illustrates two hippocampal slices from a 6-week-old wistar rat (however only one slice has been used for this experiment) placed on a net in the center well in interphase with another net on top to prevent the slices from floating when submerged. CA3 was removed to reduce the risks of possible epileptic activity.*

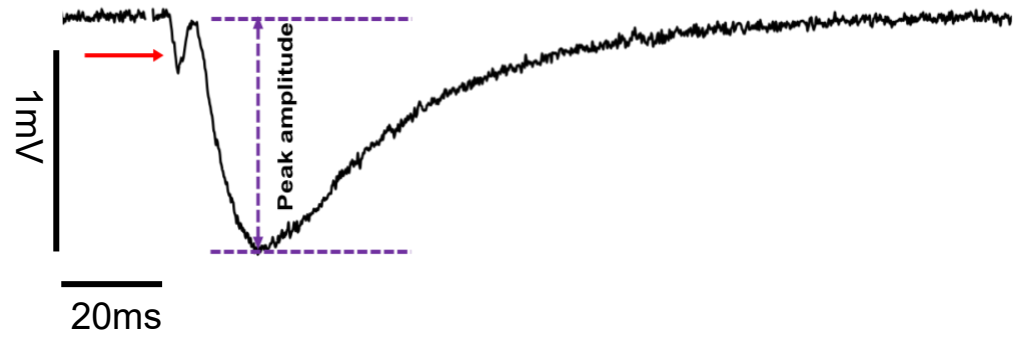


Figure 2.3. Peak amplitude measurement of fEPSP in CA1 region of the hippocampus. *Example trace fEPSP measuring peak amplitude recorded and analysed using winLTP software on-line. The trace represents averaged 4 consecutive fEPSPs recorded over a 2-minute time frame. The stimulus artefact has been removed for clear presentation of the EPSP trace. The red arrow indicates the fiber volley. Fiber volley represents the pre-synaptic component of the EPSP. This f-EPSP waveform shown above is an AMPAR mediated response. The f-EPSP waveform represents two components: fiber volley and EPSP in an extracellular recording. The fiber volley correlates to the presynaptic action potential, which demonstrates synchronous firing of neurons. Furthermore, EPSP component of the waveform, demonstrates depolarisation due to synaptic activation in the CA1 pyramidal neurons. The intital rapid phase is due to the rapid influx of sodium ions through the AMPA receptors followed by a slower phase due to calcium influx and other mechanisums involved. This f-EPSP waveform reflects the AMPAR-mediated resposne.*

2.5 Compounds

All compounds used in the experiments are stated in table 2.1. A mM concentration stock of each drug was prepared and kept at -20°C. During experiments, the drugs were defrosted and added into perfusing aCSF.

Table 2.1. All drugs used throughout this research project. *Represents the solvents used to dissolve each drug and the final concentration before adding to the aCSF perfusion*

Name	Mechanism of action	Stock concentration (mM)	Final concentration (μM)	Solvent	Supplier
CGP55845	$GABA_B$ antagonist	10	1	Water	Hello Bio., UK
Picrotoxin	$GABA_A$ antagonist	100	50	Water	Hello Bio., UK
NBQX	AMPAR antagonist	50	10	Water	Hello Bio., UK
D-AP5	Competitive NMDAR antagonist	50	100	water	Hello Bio., UK
Ketamine (Racemic form)	Non-competitive NMDAR antagonist	10	10	Water	Hello Bio., UK
2-oxo-PCE	Non-competitive NMDAR antagonist	10	0.1, 1, 3, 10, 30	Water	Dr Jason Wallach (university of Philadelphia)

2.6 Isolated NMDAR mediated fEPSPs

In experiments which required isolation of the NMDAR-mediated fEPSP, 10μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), 50μM picrotoxin (PTX), and 1μM (2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride (CGP 55845), were added to the perfusate in order to block AMPAR, $GABA_A$ and $GABA_B$ mediated transmission, respectively. The stimulus strength was then adjusted to obtain NMDAR-mediated fEPSPs at a suitable amplitude (between 0.5mV and 0.8mV) and remained consistent for all experiments (Fig 2.4). 30 minutes stable NMDAR-fEPSP baseline was obtained before adding 2-oxo-PCE in the aCSF perfusion system for 3 hours. 2-oxo-PCE concentrations

in this research included: 0.1,1,3,10,30μM. After 3 hours of 2-oxo-PCE in the aCSF perfusion system, D-2-amino-5-phosphonopentanoate (D-AP5), a competitive NMDAR antagonist was added to the system for 16 minutes as a confirmation that the recorded response was truly NMDAR-mediated.

Figure 2.4. NMDAR mediated fEPSP amplitude response. *This illustration is an example measurement of NMDAR response amplitude. The NMDAR-mediated component was obtained after 10μM NBQX, 50 μM PTX, and 1 μM CGP55845, were added to the perfusate in order to block AMPAR, $GABA_A$ and $GABA_B$ mediated transmission, respectively. Subsequently, the stimulus intensity was increased by 100-150% to obtain the measurement of NMDAR-fEPSP response amplitude.*

2.7 NMDAR dependant synaptic plasticity (LTP and LTD)

Following the same protocol discussed in '2.4 In vitro fEPSPs recordings', the hippocampal slices were subsequently perfused in (10μM) 2-oxo-PCE for at least 2 hours before inducing LTP by theta burst stimulation (TBS, 5 trains of 5 pulses at 100 Hz, 200ms inter-train interval (Fig 2.5) or LTD by low frequency stimulation (LFS, 900 pulses at 1Hz for 15 minutes) (Bortolotto et al., 2011). 30 minutes fEPSP stable baseline was obtained before delivery of TBS or LFS to the Schaffer collateral-commissural pathway and followed for 60 minutes. Control experiments LTP and LTD were performed in the presence of the vehicle (aCSF).

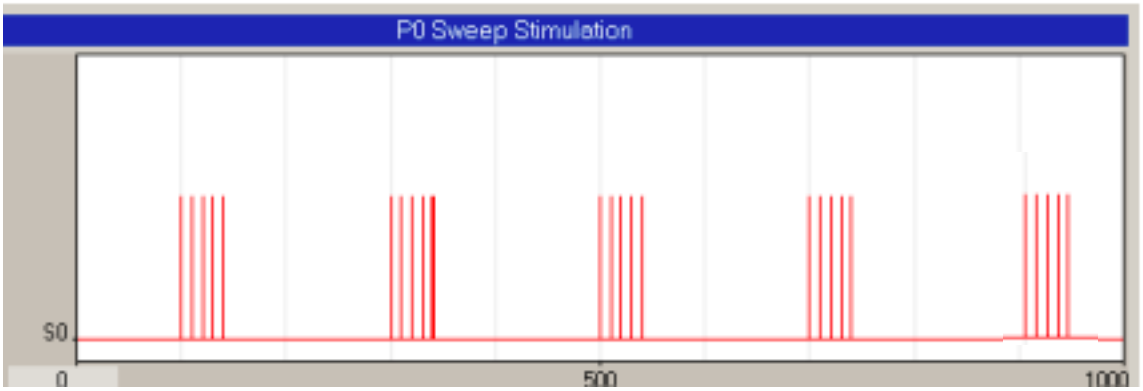


Figure 2.5. Screenshot showing the configuration of the Theta burst stimulation (TBS) protocol*. 5 trains of 5 pulses at 100 Hz, 200ms inter-train interval. (Adapted from Anderson, 2022).*

2.8 Data analysis

2.8.1 Statistical analysis

All statistical analysis in this project was performed on Prism software (version 9, GraphPad, USA). Statistical analysis was performed on the last 10 minutes of the pooled baseline (i.e. 10 minutes equivalent to 5 points) and the last 10 minutes of 3 hours pooled 2-oxo-PCE aCSF perfusion, for each concentration. Saphiro-wilks test was used to test for normality in the data. Thus, paired T-test (two-tailed) was used to determine significant differences between the two variables: baselines values vs 3 hours 2-oxo-PCE aCSF perfusion for each concentration. Unpaired T-test was also used to investigate different significant differences between two independent variables e.g. control LTP peak amplitude vs LTP peak amplitude in the presence of 2-oxo-PCE.

The level of depressions for each concentration were calculated by subtracting the average last 10 minutes of the pooled baseline (i.e. 10 minutes equivalent to 5 points) by the average last 10 minutes of 3 hours pooled 2-oxo-PCE in the aCSF perfusion. The level of depression was reported as mean ± SEM in text. One-way ANOVA with multiple

comparisons was performed to test the overall differences between level of depression for the overall concentration groups (i.e. >2 variables; multiple group comparisons). Significant ANOVA followed up with a post hoc Tukey test, to evaluate the individual differences between the level of depression for each concentration. A significant difference was observed when $p<0.05$ for all statistical analysis.

2.8.2 IC_{50} calculations

Log 2-oxo-PCE concentrations (M) against level of depression responses (%) were plotted using a three-parameter logistic equation. This particular equation allows the sigmoidal curve to have a standard Hill slope of 1. Subsequently, a sigmoidal curve was fitted and half maximal inhibitory concentration (IC_{50}) was calculated. IC_{50} (M) was calculated in order to determine the 2-oxo-PCE concentration required to produce 50% level of depression in the CA1 region of the hippocampus after 3 hours in aCSF perfusion. Coefficient of determination (R^2 value) was a statistical measure to determine how good the fit was.

3 Results

3.1 Effects of ketamine on NMDAR Mediated fEPSPs

An initial set of experiments were performed to validate the full-time course protocol for isolating NMDAR-fEPSPs in the CA1 region of the hippocampus and to assess whether our current experimental conditions was as sensitive as the system used in previous work (Wallach et al., 2016; Kang et al., 2017) As shown in figure 3.1, 10μM ketamine inhibited the NMDAR-fEPSPs in a very similar amount and profile as described in the literature (Wallach et al., 2016; Kang et al., 2017). Therefore, the results obtained served as a validation for the experimental conditions. Since the results resembled those from the literature, only three ketamine experiments were performed in order to reduce the use of animals.

A stable baseline was obtained before isolating NMDAR-fEPSPs by pharmacological blockage of AMPAR and GABAR mediated transmission. This resulted in a significant reduction in fEPSP amplitude (Fig 3.1). New stable NMDAR-fEPSP baseline was obtained before 10μM ketamine was applied. A slow reduction of NMDAR-fEPSP was recorded following ketamine application, similar to the results reported in the literature (Kang et al., 2017, Wallach et al., 2016). Before the end of the experiment the applications of 100μM D-AP5 blocked the remaining NMDAR-mediated response which was not blocked by ketamine. D-AP5 acts as a positive control and further confirms the response was NMDAR-mediated.

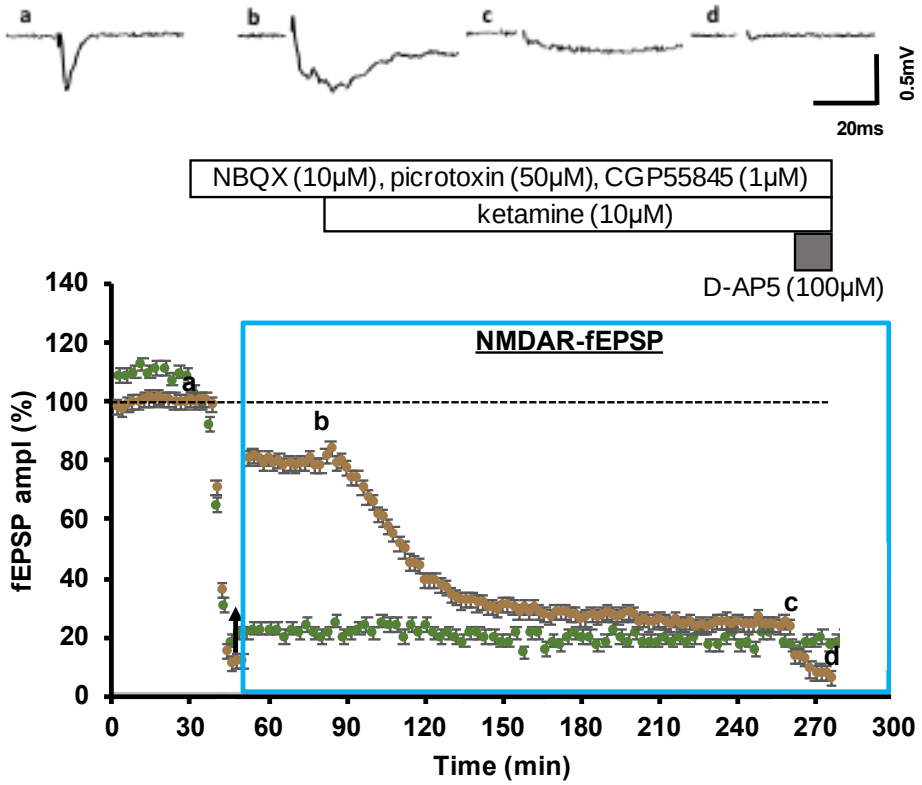


Figure 3.1. Full-time course protocol to investigate the NMDAR-fEPSPs challenged by ketamine. *In the graph above, the control conditions are depicted in green and the effects of NMDAR-fEPSPs is shown in brown. The protocol to isolate the NMDAR-mediated component of fEPSPs in the CA1 region of the hippocampus. 30 minutes stable baseline was obtained before 10μM NBQX, 50 μM PTX, and 1 μM CGP55845, were added to the perfusate in order to block AMPAR, $GABA_A$ and $GABA_B$ mediated transmission, respectively. This resulted in a significant reduction in fEPSP amplitude approximately 20 minutes after perfusion of drugs. The stimulus strength was increased by 100-150% of the initial value (black arrow) to obtain NMDAR- mediated fEPSPs at a suitable amplitude. After a new stable 30 minutes NMDAR-fEPSP baseline was obtained, 10μM ketamine was added to the perfusion system for 3 hours and subsequently 100μM D-AP5 for 20 minutes. The blue box in the graph represents the period of the experiment that will be shown in all subsequent figures to describe the effects of different doses of 2-oxo-PCE on NMDAR-mediated fEPSP. The letters* ***(a, b, c and d)*** *represent the time the traces were obtained in the graph below. Data presented as mean ± SEM (n=3). In this and following figures each point represents the average of 4 consecutives evoked fEPSP peak amplitude in WinLTP on-line.*

3.2 Effects of 2-oxo-PCE on NMDAR-fEPSPs in CA1 hippocampal slices

Following the validation of our experimental protocol described above (section 3.1), the effects of 2-oxo-PCE on NMDAR-fEPSPs was investigated. As described, all the experiments were performed at the CA1 stratum radiatum of hippocampal slices. From this point onwards, graphs illustrate only the isolated NMDAR-fEPSP portion of the experiments, in which NMDAR-fEPSP amplitude were normalised to the NMDAR-fEPSP baseline peak amplitude. The 3 hours 2-oxo-PCE perfusion reflects the time period: 30 – 210 minutes on the graphs.

To assess whether the effects of 2-oxo-PCE on NMDAR-fEPSPs were concentration dependent, a range of concentrations of the drug were tested (in µM; 0.1, 1, 3, 10, 30).

3.2.1 Effects of 0.1µM 2-oxo-PCE on NMDAR-fEPSPs

The results obtained with this experiment are shown in figure 3.2. The data shows that 3 hours perfusion of 0.1µM 2-oxo-PCE did not significantly reduce the NMDAR-fEPSP amplitude. As shown in pooled data figure 3.2B there was a small, but not significant, reduction of the NMDAR-fEPSP in the presence of the drug (8.8 ± 4.8%; of baseline; measured from the final average 10 minutes of the 3 hour drug perfusion) (n=4, t_3= 2.8, p>0.05, paired t-test vs baseline, Fig 3.2C). After 3 hours of 2-oxo-PCE aCSF perfusion, the last 10 minutes of NMDAR-fEPSP peak amplitude look very parallel (i.e.91.0 ± 6.7%) compared with the last 10 minutes of baseline (i.e. 99.8 ± 1.9%).The application of 100µM DAP-5 after 3 hours of 2-oxo-PCE completely blocked the remaining NMDAR-mediated fEPSPs.

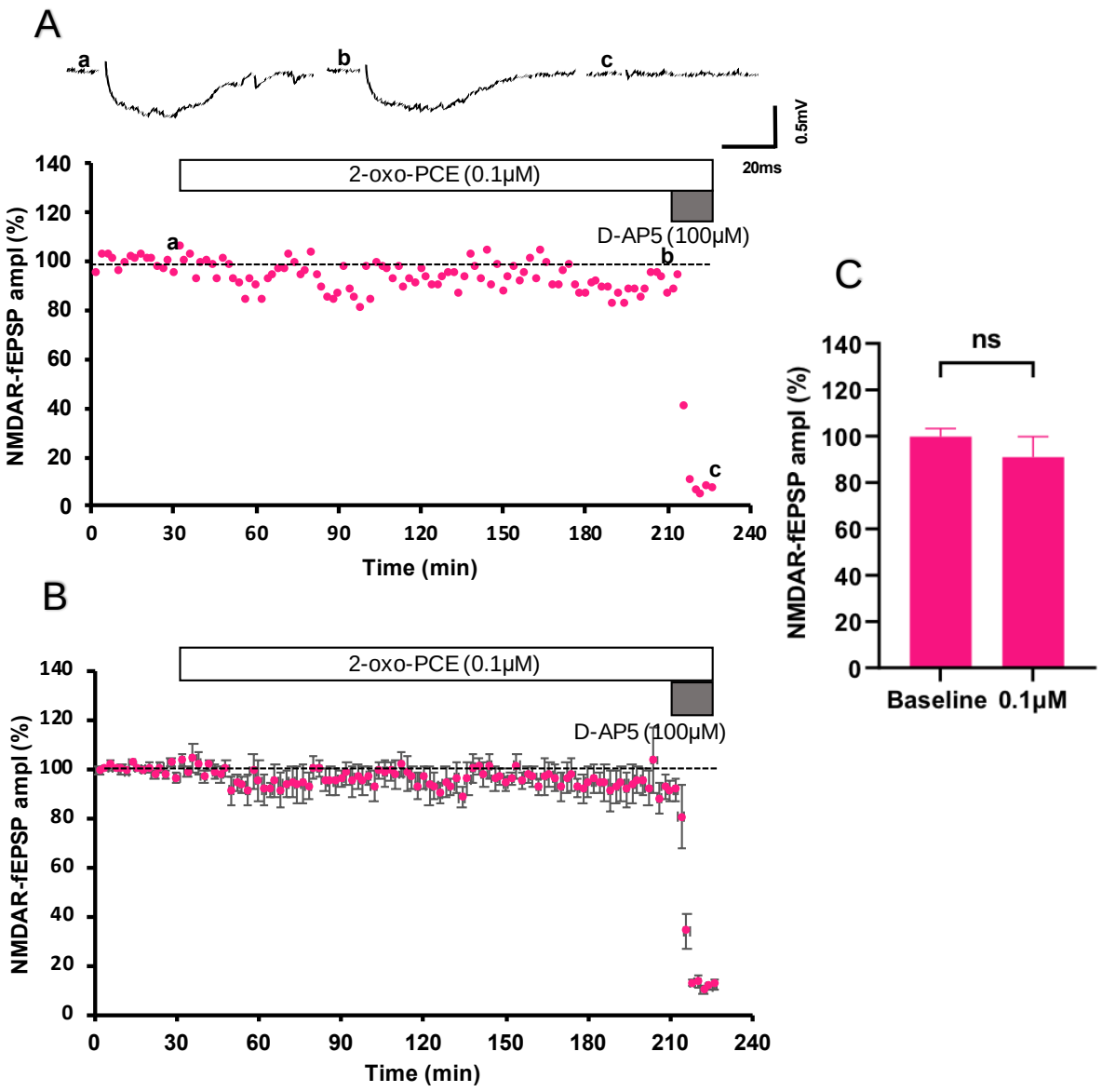


Figure 3.2. The effects of 0.1µM 2-oxo-PCE on NMDAR-fEPSPs in CA1. (A) *Shows a single representative experiment. After obtaining a stable NMDAR-fEPSP baseline of 30 minutes, 0.1µM 2-oxo-PCE and subsequently, 100µM D-AP5 were added to the perfusion system at the time indicated by the bars on the top of the plot. The insets identified by the letters* ***(a, b and c)*** *are representative averaged traces obtained at the time indicated on the graphs.* ***(B)*** *Illustrates the pooled data comprising of 4 experiments (plotted as mean ± SEM). Plots (****A****) and* ***(B)*** *show that the perfusion of 100µM D-AP5 at the final 16 minutes of the experiment completely blocked the NMDAR-fEPSP.* ***(C)*** *Bar chart used to determine the level of significant difference on NMDAR-fEPSP peak amplitude during the last 10 minutes of baseline versus during the last 10 minutes of 3 hours of 0.1µM 2-oxo-PCE perfusion from the pooled data (plotted as mean ± SEM). No significance difference between variables was identified using a paired t-test. (ns = not significant)*

3.2.2 Effects of 1μM 2-oxo-PCE on NMDAR-fEPSPs

The effects of 1μM of 2-oxo-PCE on NMDAR-fEPSPs was investigated next. The concentration of 1μM is 10 times higher than 0.1μM and the effects are shown in figure 3.3. As it can be observed, this dose slowly reduced the NMDAR-fEPSP amplitude. Figure 3.3A shows a single example experiment. Figure 3.3B shows the pooled data of 1μM 2-oxo-PCE effects on the NMDAR-fEPSP amplitude (mean ± SEM). Although it did not reach plateau, (within the 3 hours of drug perfusion), it can be observed that 1μM 2-oxo-PCE significantly reduced the amplitude of the evoked NMDAR-fEPSP. The reduction of the NMDAR-fEPSP amplitude was significant and exhibited a depression of 55.5 ± 1.8% of baseline; final average 10 minutes of the 3 hours drug perfusion ($n = 6$, $t_5 = 22.1$, $P < 0.05$, paired t-test vs baseline, Fig 3.3C).

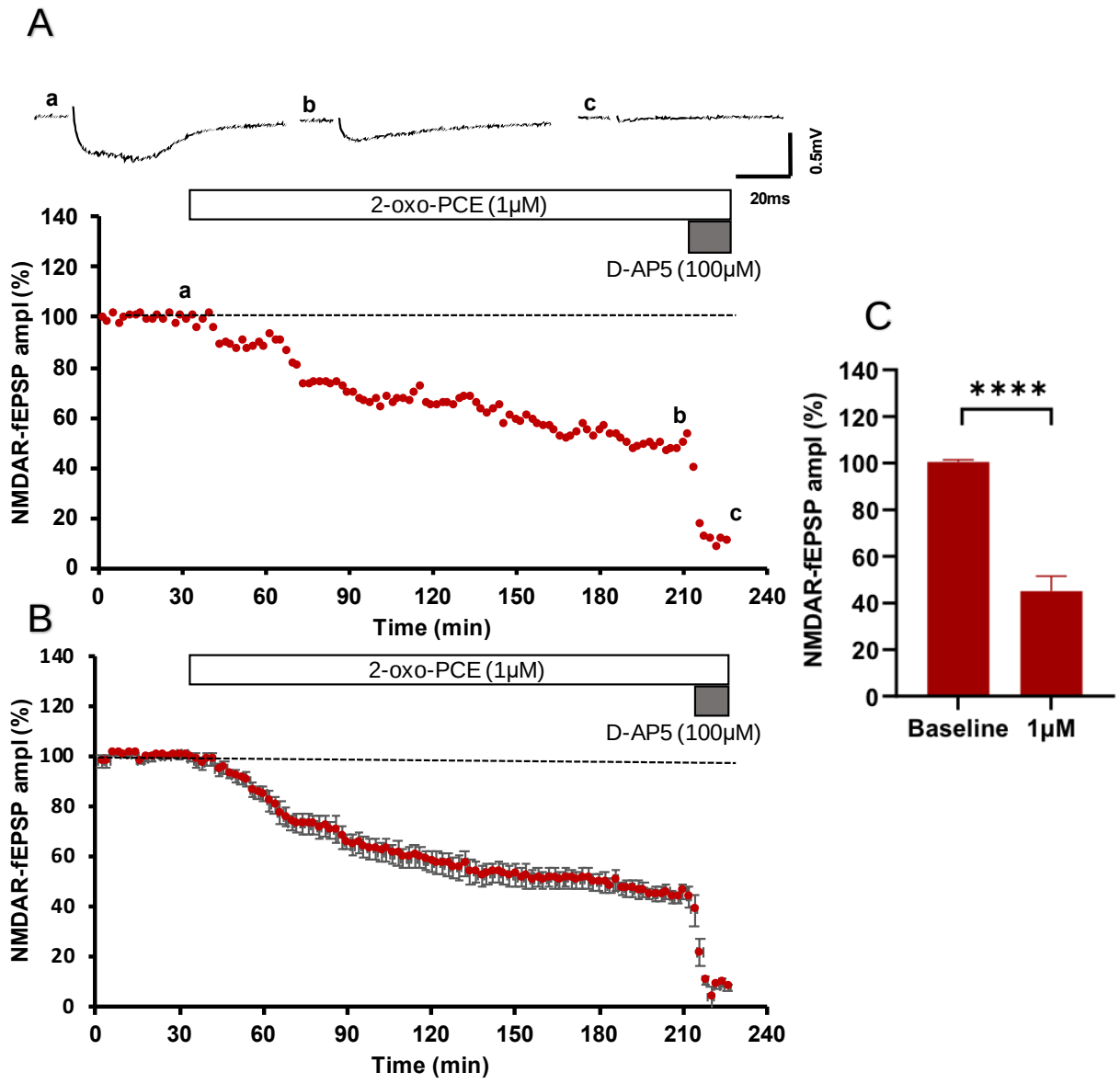


Figure 3.3. The effects of 1µM 2-oxo-PCE on NMDAR-fEPSPs in CA1. ***(A)*** *Shows a single representative experiment. After obtaining a stable NMDAR-fEPSP baseline, 1µM 2-oxo-PCE and then later, 100µM D-AP5 were added to the perfusion system. The insets identified by the letters* ***(a, b and c)*** *represent the averaged traces recorded at the correspondent time on the graph.* ***(B)*** *Show the pooled data comprising of 6 experiments (expressed as mean ± SEM).* ***(C)*** *Bar chart displaying the level of significant difference on NMDAR-fEPSP peak amplitude during the last 10 minutes of baseline versus during the last 10 minutes of 3 hours of 1µM 2-oxo-PCE perfusion from the pooled data (plotted as mean ± SEM). Statistical significance was tested using a paired t-test and is indicated as **** = p<0.0001.*

3.2.3 Effects of 3μM 2-oxo-PCE on NMDAR-fEPSPs

Progressing in the pharmacological studies on the effects of 2-oxo-PCE on the NMDAR fEPSP, 3μM of 2-oxo-PCE was applied and the results of this experiments are shown in Figure 3.4. Similar to the dose of 1μM, the 3μM dose produced a slow but larger reduction of the NMDAR-fEPSP amplitude reaching a plateau effect during the last 40 minutes of the 3 hours application (Fig 3.4B). Figure 3.4A shows the result of a single experiment. The pooled data is shown in Figure 3.4B (mean ± SEM) and revealed a significant depression of the evoked NMDAR-fEPSP amplitude induced by 3μM 2-oxo-PCE perfusion. The peak amplitude was reduced by 64.5 ± 2.9% of baseline; final average 10 minutes of the 3 hours drug perfusion ($n = 6$, $t_5 = 22.1$, $P < 0.05$, paired t-test vs baseline, Fig 3.4C).

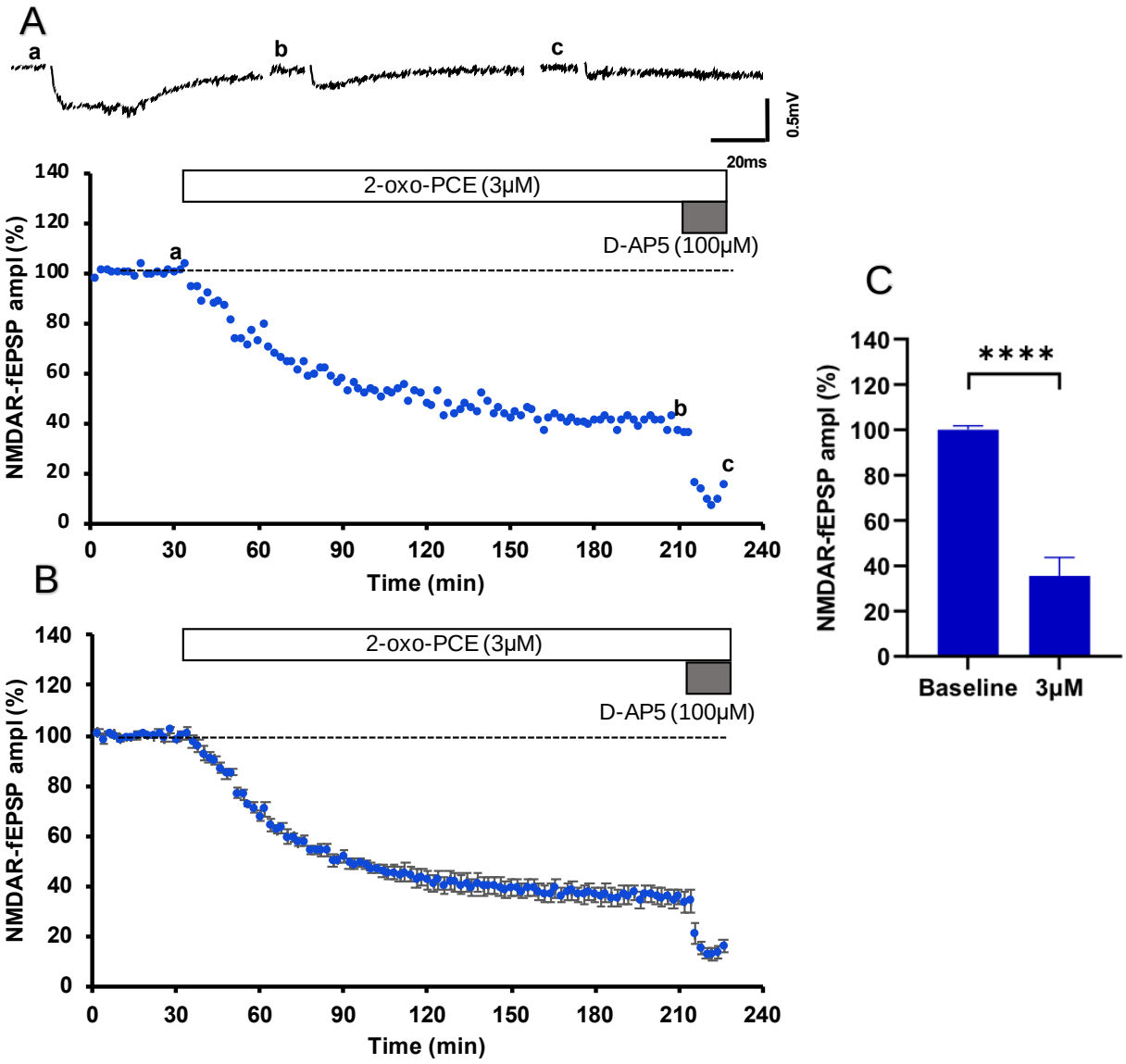


Figure 3.4. The effects of 3μM 2-oxo-PCE on NMDAR-fEPSPs in CA1. (A) *Shows a single representative experiment. After obtaining a stable NMDAR-fEPSP baseline, 3μM 2-oxo-PCE and subsequently, 100μM D-AP5 were added to the perfusion system at the time indicated by the bars on the top of the plot. The insets identified by the letters* ***(a, b and c)*** *represent averaged traces taken at the correspondent time on the graph.* ***(B)*** *Illustrates the pooled data of 6 experiments (plotted as mean ± SEM).* ***(C)*** *Bar chart shows the level of significant difference on NMDAR-fEPSP peak amplitude during the last 10 minutes of baseline versus during last 10 minutes of 3 hours of 3μM 2-oxo-PCE perfusion from the pooled data (data plotted as mean ± SEM).Level of significance revealed by application of the paired t-test: **** = p<0.0001*

3.2.4 Effects of 10μM 2-oxo-PCE on NMDAR-fEPSPs

The effects of 10μM 2-oxo-PCE on NMDAR-fEPSPs are shown in figure 3.5. Compared to previous concentrations, 10μM 2-oxo-PCE exhibited a faster inhibition effect on the NMDAR-fEPSP peak amplitude, reaching plateau about 110 min after drug perfusion (Fig 3.5B). Figure 3.5A shows a single representative experiment and figure 3.5B represents the pooled data (mean ± SEM).10μM 2-oxo-PCE significantly reduced amplitude of the evoked NMDAR-fEPSP by 78.8 ± 5.0% of baseline; final average 10 minutes of the 3 hours drug perfusion ($n = 6$, $t_5 = 18.8$, $P < 0.05$, paired t-test vs. baseline, Fig 3.5C).

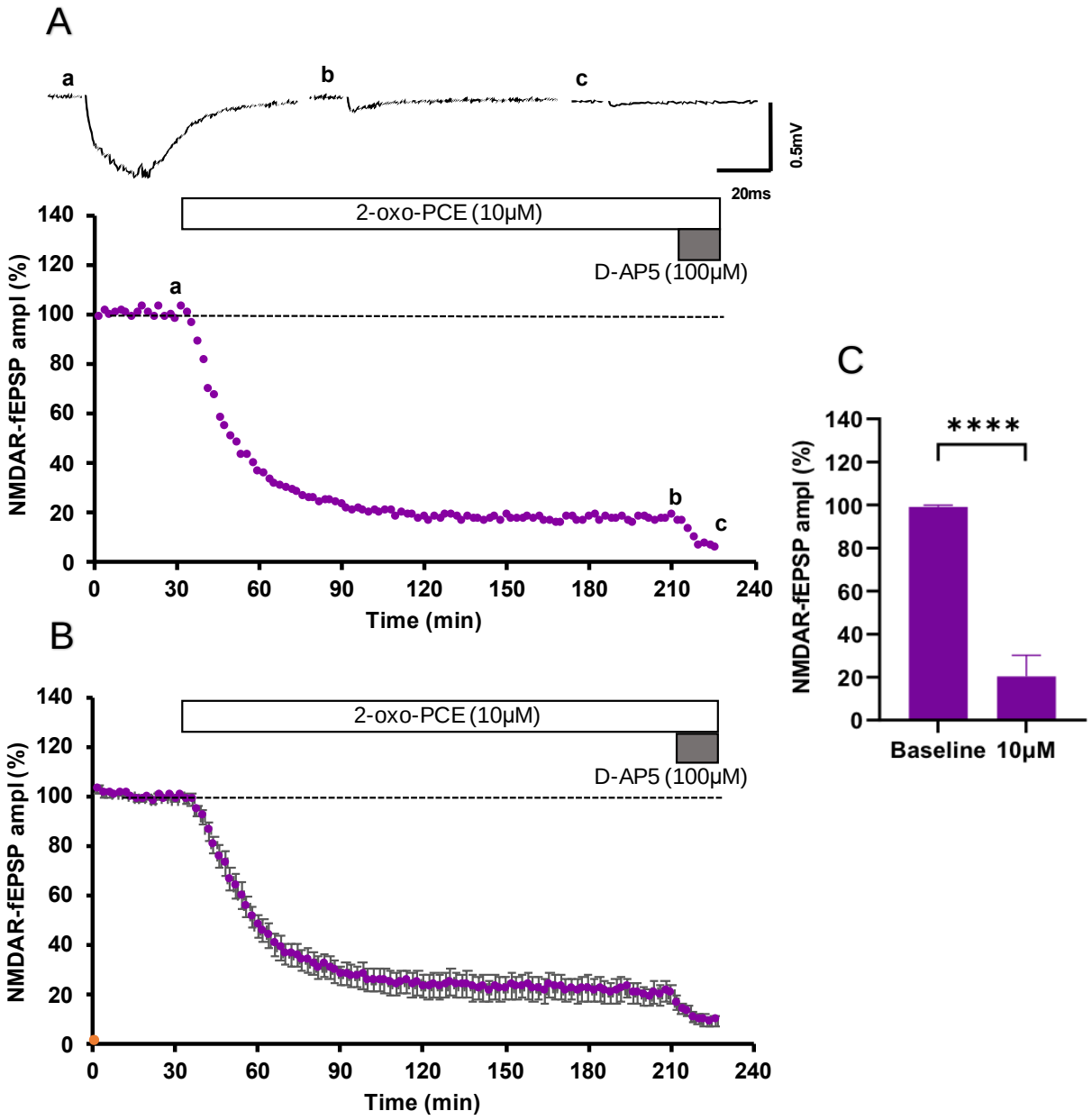


Figure 3.5. The effects of 10μM 2-oxo-PCE on NMDAR-fEPSPs in CA1. (A) *Represents a single representative experiment. After obtaining a stable 30 minute NMDAR-fEPSP baseline, 10μM 2-oxo-PCE and subsequently, 100μM D-AP5 were added to the perfusion system to measure the NMDAR-fEPSP peak amplitude. The letters* ***(a, b and c)*** *represent the time the averaged traces were obtained on the graph correspondingly.* ***(B)*** *The pooled data comprising of 6 single experiments (plotted as mean ± SEM).* ***(C)*** *Bar chart demonstrating the level of significant difference on NMDAR-fEPSP peak amplitude during the last 10 minutes of baseline versus during last 10 minutes of 3 hours of 10μM 2-oxo-PCE perfusion from the pooled data (plotted as mean ± SEM). Level of significance was determined using paired t-test and is revealed as **** = p<0.0001.*

3.2.5 Effects of 30μM 2-oxo-PCE on NMDAR-fEPSPs

Similarly, a faster reduction of NMDAR-fEPSP can be observed in the presence of 30μM 2-oxo-PCE, reaching a near maximal plateau inhibition approximately 70 minutes after drug perfusion (see Fig 3.6B). A single experiment is shown in figure 3.6A and the pooled data in figure 3.6B. Notice the small variation expressed as mean ± SEM. Figure 3.6B shows that 30μM 2-oxo-PCE significantly depressed the NMDAR-fEPSP peak amplitude by 85.4 ± 0.27% of baseline; final average 10 minutes of the 3 hours drug perfusion ($n = 4$, $t_3 = 36.2$, $P < 0.05$, paired t-test vs. baseline, Fig 3.6C).

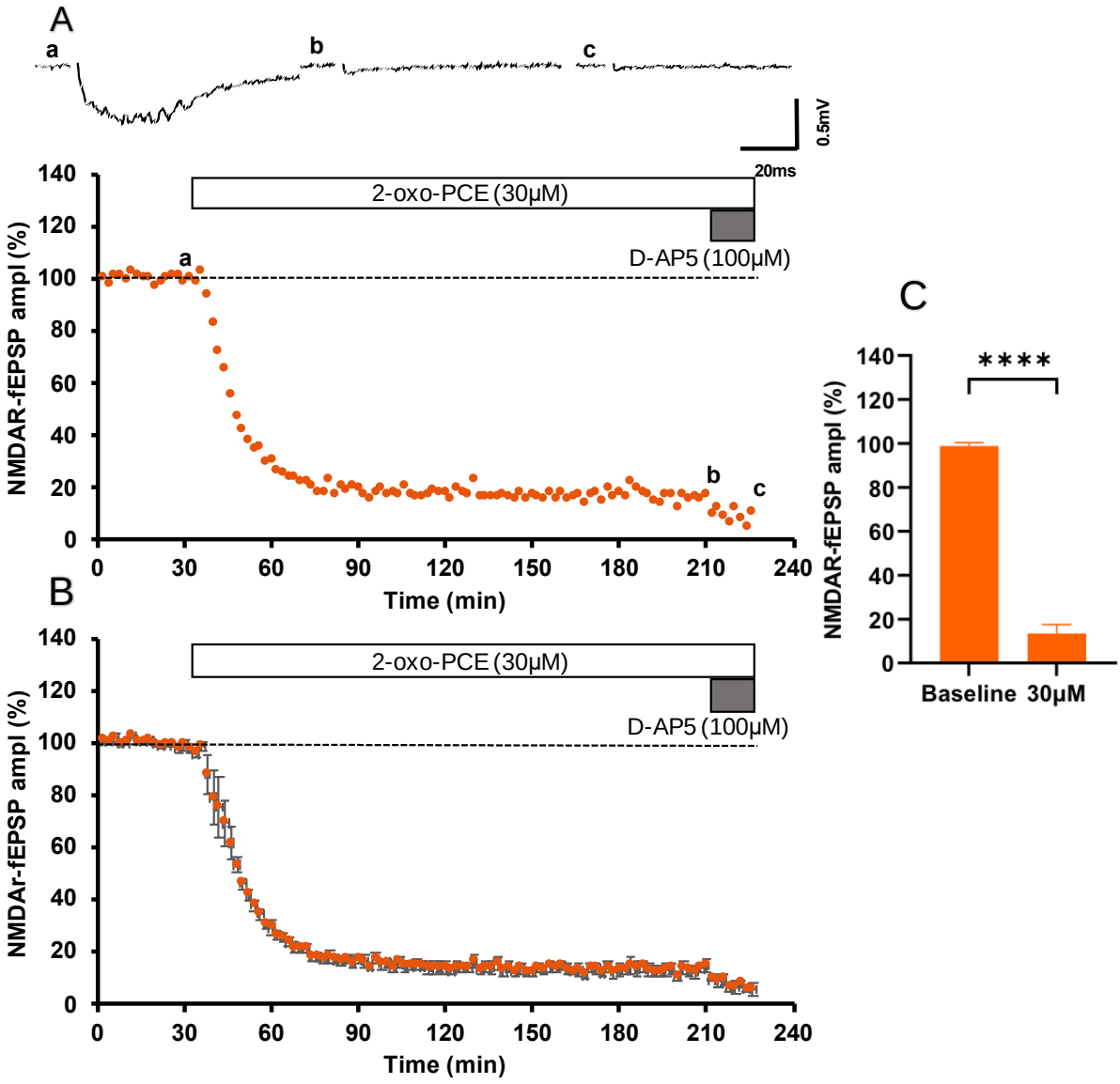


Figure 3.6. The effects of 30μM 2-oxo-PCE on NMDAR-fEPSPs in CA1. (A) Represents a single representative experiment. After obtaining a 30 minute stable NMDAR-fEPSP baseline, 30μM 2-oxo-PCE and subsequently, 100μM D-AP5 were added to the perfusion system at the time indicated by the bars on the top of the plot. The insets identified by the letters **(a, b and c)** are representative averaged traces obtained at the time indicated on the graphs. **(B)** Illustrates the pooled data comprising of 4 single experiments (data plotted as mean ± SEM). **(C)** Bar chart showcasing the level of significant difference on NMDAR-fEPSP peak amplitude during the last 10 minutes of baseline versus during the last 10 minutes of 3 hours of 30μM 2-oxo-PCE perfusion from the pooled data. Statistical significance was tested using a paired t-test and indicated as **** = p<0.0001

3.2.6 Concentration dependent effects of 2-oxo-PCE on NMDAR-fEPSPs

A summary the effects of all doses of 2-oxo-PCE studied is in figure 3.7. The time-course profile of the inhibitory effects of the different concentrations (0.1μM, 1μM, 3μM, 10μM and 30μM) of 2-oxo-PCE on the NMDA mediated fEPSP can be observed. The 2-oxo-PCE reduced the NMDAR-fEPSP in a concentration-dependant manner.

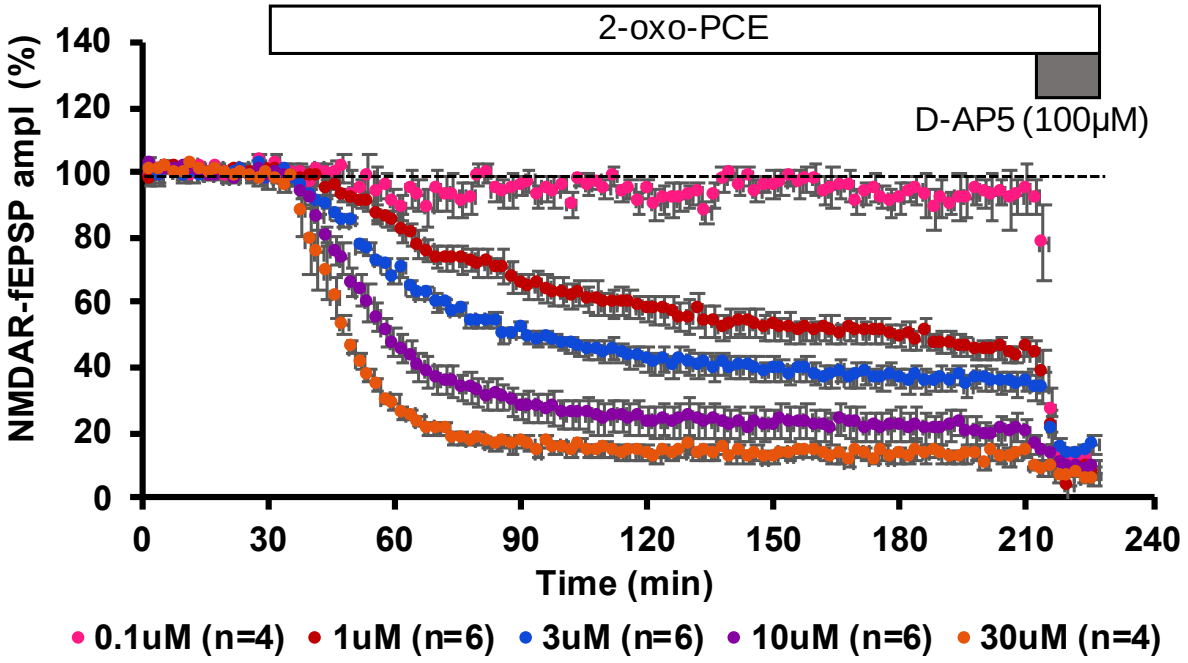


Figure 3.7. Concentration-dependant inhibitory effects of 2-oxo-PCE on NMDAR-fEPSPs. *The figure shows pooled data of all individual concentrations of 2-oxo-PCE studied: 0.1μM (pink), 1μM (red), 3μM (blue), 10μM (purple) and 30μM (orange) (plotted as mean ± SEM). After obtaining a stable NMDAR-fEPSP baseline, the hippocampal slices were perfused with various concentrations of 2-oxo-PCE and subsequently, 100μM D-AP5, at the time indicated by the bars.*

The calculated percentages of the total effect of the different dose of 2-oxo-PCE on NMDAR-fEPSP shown in the figures above, are displayed in figure 3.8. The level of depression for each concentration was calculated by subtracting the average NMDAR-fEPSP amplitude during the last 10 minutes of the pooled baseline versus during the last 10 minutes of the 3 hours of 2-oxo-PCE perfusion. Small SEM size are observed across all concentrations.

All individual data points display similar level of depression for each concentration respectively. Thus, small variations are observed within individual data points in the graph. The level of depression for 0.1; 1; 3; 10; 30μM 2-oxo-PCE are: 8.8 ± 4.8%, 55.5 ± 1.8%, 64.5 ± 2.9%, 78.8 ± 5.0%, 85.4 ± 0.27% respectively. The bar chart shows that higher the concentrations, lead to a greater level of depression of NMDAR-fEPSP. This further confirms a concentration-dependant effect of 2-oxo-PCE on NMDAR-fEPSP. A one-way ANOVA multiple comparison showed a significant effect of concentration was found on NMDAR-fEPSP amplitude ($F_{4,21} = 70.1$, $P < [0.0001]$, One-way ANOVA). Tukey's post-hoc analysis then revealed a significant difference between 0.1 and 1μM ($P < [0.0001]$), 0.1 and 3μM ($P < [0.0001]$), 0.1 and 10μM ($P < [0.0001]$), 0.1 and 30μM ($P < [0.0001]$), 1 and 10μM ($P = 0.0002$), 1 and 30μM ($P < [0.0001]$), 3 and 10μM ($P = 0.0232$), 3 and 30μM ($P = 0.0022$). All concentrations show a level of significant difference between concentrations except between 1 and 3μM and 10 and 30μM.

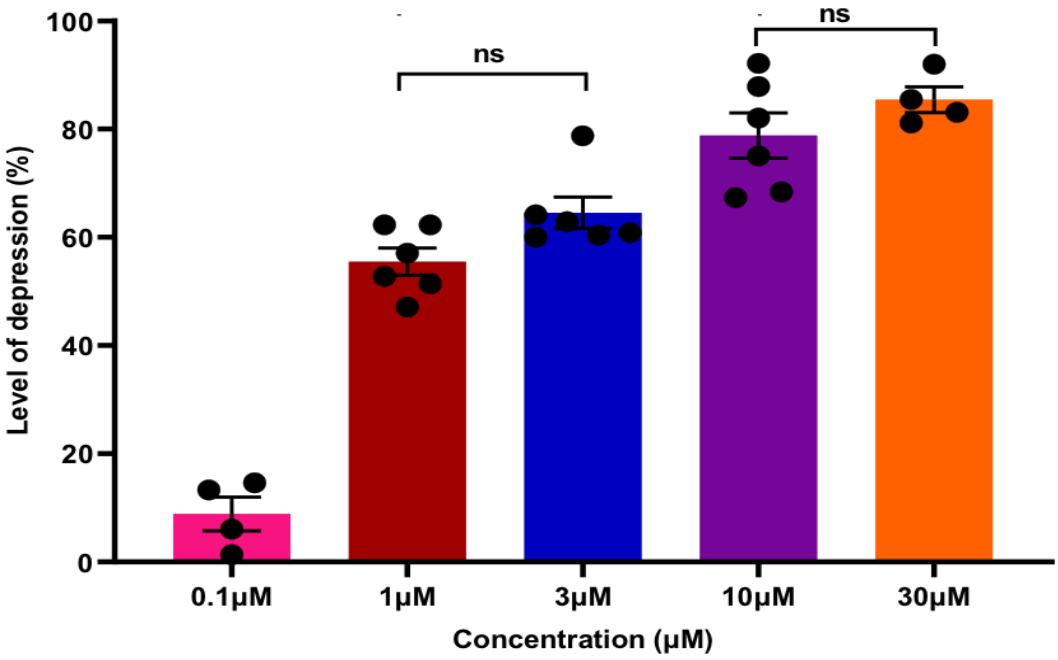


Figure 3.8. Percentual profile of the effects of increasing doses 2-oxo-PCE on evoked NMDAR mediated fEPSP at CA1. *The level of depressions for each concentration were calculated by subtracting the last 10 minutes of the pooled baseline versus the last 10 minutes of 3 hours of the 2-oxo-PCE perfusion. Each point, on each bar, indicates the variability of depression for each individual experiment contributing to the pooled data for that individual concentration. The level of depression between each concentration was compared using one-way ANOVA multiple comparisons followed with Tukey test. All concentrations show a level of significant difference between concentrations except between 1 and 3µM and 10 and 30µM as indicated (ns= not significant).*

3.3 Effects of ketamine vs 2-oxo-PCE on NMDAR-fEPSPs

As 2-oxo-PCE is derived from ketamine, the effects of 10μM concentration of these two drugs on the NMDR-fEPSP was compared. The result of this comparison analysis is shown on figure 3.9. Both drugs depressed the evoked NMDAR-fEPSP. However, the 2-oxo-PCE appears to be more potent and faster to depress the evoked NMDAR-fEPSP since the depression achieved plateau faster and was larger (78.8 ± 5.0%) compared to ketamine (63.7 ± 3.2%). 2-oxo-PCE depressed the NMDAR-fEPSP 15.1% more than ketamine at 10μM. Interestingly, the depression induced by 3μM 2-oxo-PCE closely resemble the effects of 10μM ketamine than 10μM 2-oxo-PCE, which reduced the NMDAR-fEPSP peak amplitude by 64.5 ± 2.9% in CA1 hippocampal slices (see Fig 3.4). Recent published works also describe larger levels of NMDAR-fEPSP depression induced by other ketamine derivatives: ephenidine (Kang et al., 2017) and fluorolintane (Wallach et al., 2019).

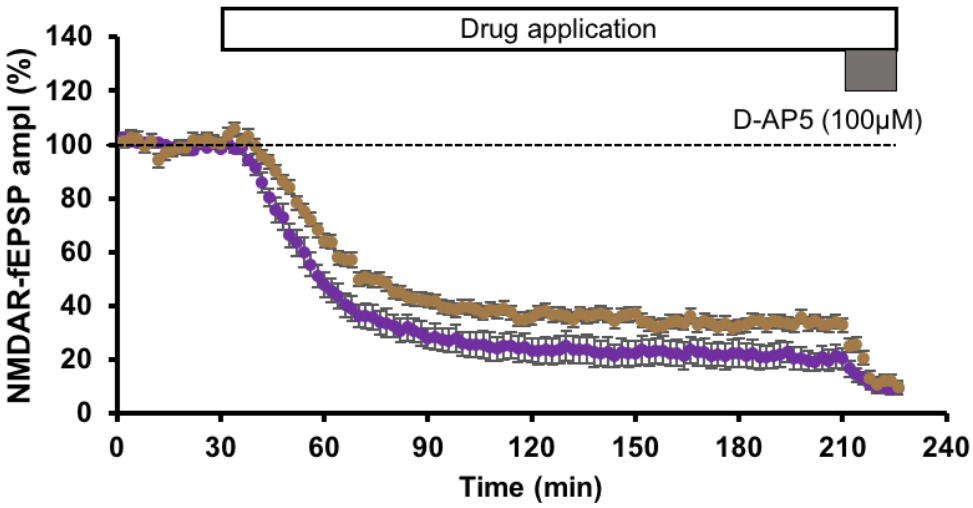
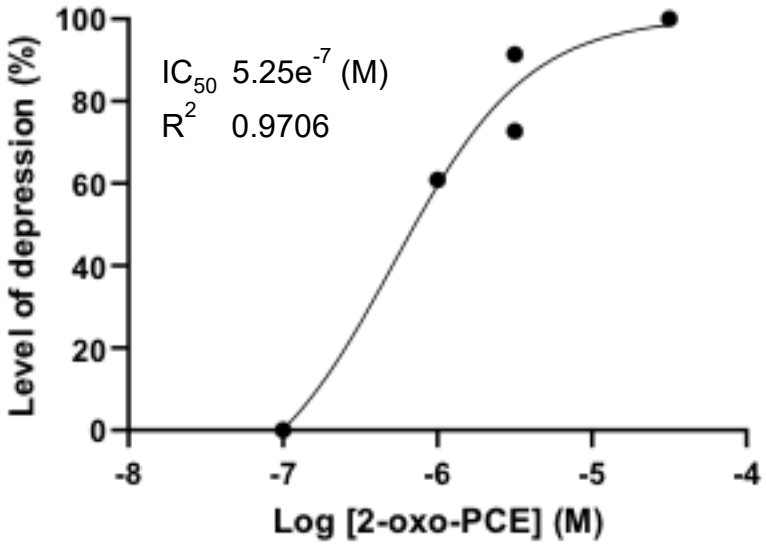


Figure 3.9. Comparison of the effects of 10μM 2-oxo-PCE and 10μM ketamine on NMDAR-fEPSPs*. The figure shows the pool data obtained with 10μM 2-oxo-PCE and 10μM ketamine as displayed in previous correspondent figures (plotted as mean ±SEM). Both drugs demonstrated different inhibitory effects on the NMDAR-fEPSPs peak amplitude. 2-oxo-PCE induced 78.8 ± 5.0% (n = 6) compared to ketamine 63.7 ± 3.2% (n = 3) depression as measured during the last 10 minutes of baseline versus during the last 10 minutes of 3 hours of drug perfusion respectively. Notice that 2-oxo-PCE induced 15.1% more depression compared to ketamine. Subsequently, perfusion of 100μM D-AP5 further reduced the NMDAR-fEPSPs which were not affected by the two drugs.*

3.4 2-oxo-PCE IC50 calculation

Figure 3.10A represents a fitted sigmoidal curve, used to determine 2-oxo-PCE $IC_{50,}$ level of NMDAR-fEPSP depression. Each log 2-oxo-PCE concentration (M) was plotted against the level of depression (%) respectively. Figure 3.10B denotes the corresponding 2-oxo-PCE micromolar concentration (μM) in molars (M) for reference and the level of depression. R^2 value was calculated as a statistical measure to determine how good the fit was. R^2 value is 0.9806 on the fitted sigmoidal curve, ensuring that the curve is a good fit. 10μM and 30μM 2-oxo-PCE reflects the minimum and maximum level of depression on NMDAR-fEPSP shown in the graph respectively (Fig 3.10B). The IC_{50} of 2-oxo-PCE is $5.25e^{-7}$M. This is equivalent to 0.525μM. IC_{50} value calculated for 2-oxo-PCE is very small.



Concentration (µM)	Log concentration (M)	Level of depression (%)
0.1	-7	8.8
1	-6	55.5
3	-5.5	64.5
10	-5	78.8
30	-4.5	85.4

***Figure 3.10. Concentration response curves of 2-oxo-PCE**. **(A)** A concentration response curve used to determine 2-oxo-PCE IC_{50} level of NMDAR-fEPSP depression. Each log 2-oxo-PCE concentration (M) was plotted against the level of depression (%) respectively. A concentration dependent depression is observed with 2-oxo-PCE. The IC_{50} of 2-oxo-PCE is $5.25e^{-7}$ M. This is equivalent to 0.525 µM. R^2 value was a measure to determine how good the fit was and R^2 value is 0.9706 on the fitted sigmoidal curve. **(B)** Corresponding 2-oxo-PCE micromolar concentration (µM) in molars (M) for reference and the level of depression.*

3.5 Effects of 2-oxo-PCE on AMPAR-mediated fEPSPs

To better understand the effects of 2-oxo-PCE on synaptic transmission and its selectivity to NMDAR, 2-oxo-PCE effects on AMPA receptor mediated fEPSP was analysed. The result of these experiments is shown in figure 3.11. No effects of 2-oxo-PCE were observed on AMPAR-mediated fEPSP as evaluated 2 hours after its perfusion (time point when the drug already achieved it plateau effect).

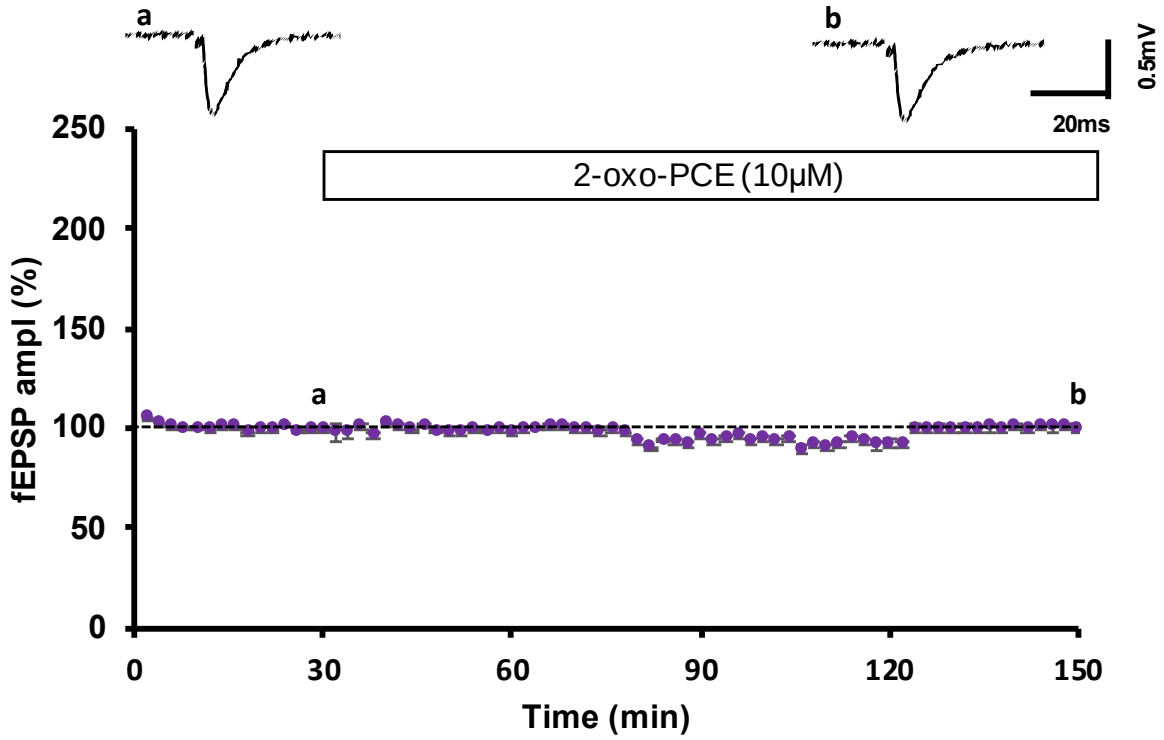


Figure 3.11. The effects of 2-oxo-PCE on AMPAR-mediated fEPSPs. *The figure shows 2 hours of 10μM 2-oxo-PCE perfusion did not affect the AMPAR-mediated fEPSP at the CA1 Schaffer collateral commissural synapses. The insets are representative traces of the average fEPSP obtained from a single experiment at the time points indicated on the plot by the low case letters* ***a & b.***

3.6 The effects of 2-oxo-PCE on NMDAR dependent synaptic plasticity

One of the most prominent effects of NMDAR antagonists is their ability to block synaptic plasticity. This has relevant consequences for the brain functionality. For example, it has been demonstrated that blocking LTP and LTD induction disrupts cognitive processes including learning and memory (Bliss and Collingridge, 1993). Ketamine and PCP are NMDAR antagonists and have both been shown to block LTP. (Stringer and Guyenet, 1983; Stringer et al., 1983). Thus, the effects of 2-oxo-PCE on NMDAR dependant LTP and LTD were assessed as described in section: '2.7 NMDAR dependant synaptic plasticity (LTP and LTD). Based on the concentration-dependant effects results, 10μM was the chosen concentration to investigate the effects of 2-oxo-PCE on synaptic plasticity. This dose was chosen because it induced a significant effect and could compare its effects on plasticity with data available in the literature. The effects of 2-oxo-PCE were investigated in the CA1 region of the hippocampus and statistical analyses were performed by comparing the average peak amplitudes of the final 10 minutes of baseline and post-TBS conditions and post-LFS conditions respectively.

3.6.1 Induction of NMDAR-dependent LTP under control conditions.

The results from the induction of LTP under control conditions are shown in figure 3.12. The delivery of theta burst stimulation (TBS) in the presence of vehicle induced LTP and was followed for 1 hour after stimulation. A single experiment is shown in figure 3.11A and figure 3.11B represents the result of the pooled control data (mean ± SEM). After 30 minutes of stable baseline, TBS was delivered and LTP was induced. fEPSP amplitude increased to 165.5 ± 10.9% of baseline; measured during the last 10 minutes of average fEPSP peak amplitude following LTP induction via TBS ($n = 6$, $t_4 = 61.0$, $P < 0.05$, paired t-test vs. baseline, Fig 3.12C). A significant level of robust LTP of 65.2 ± 1.1% was induced by TBS.

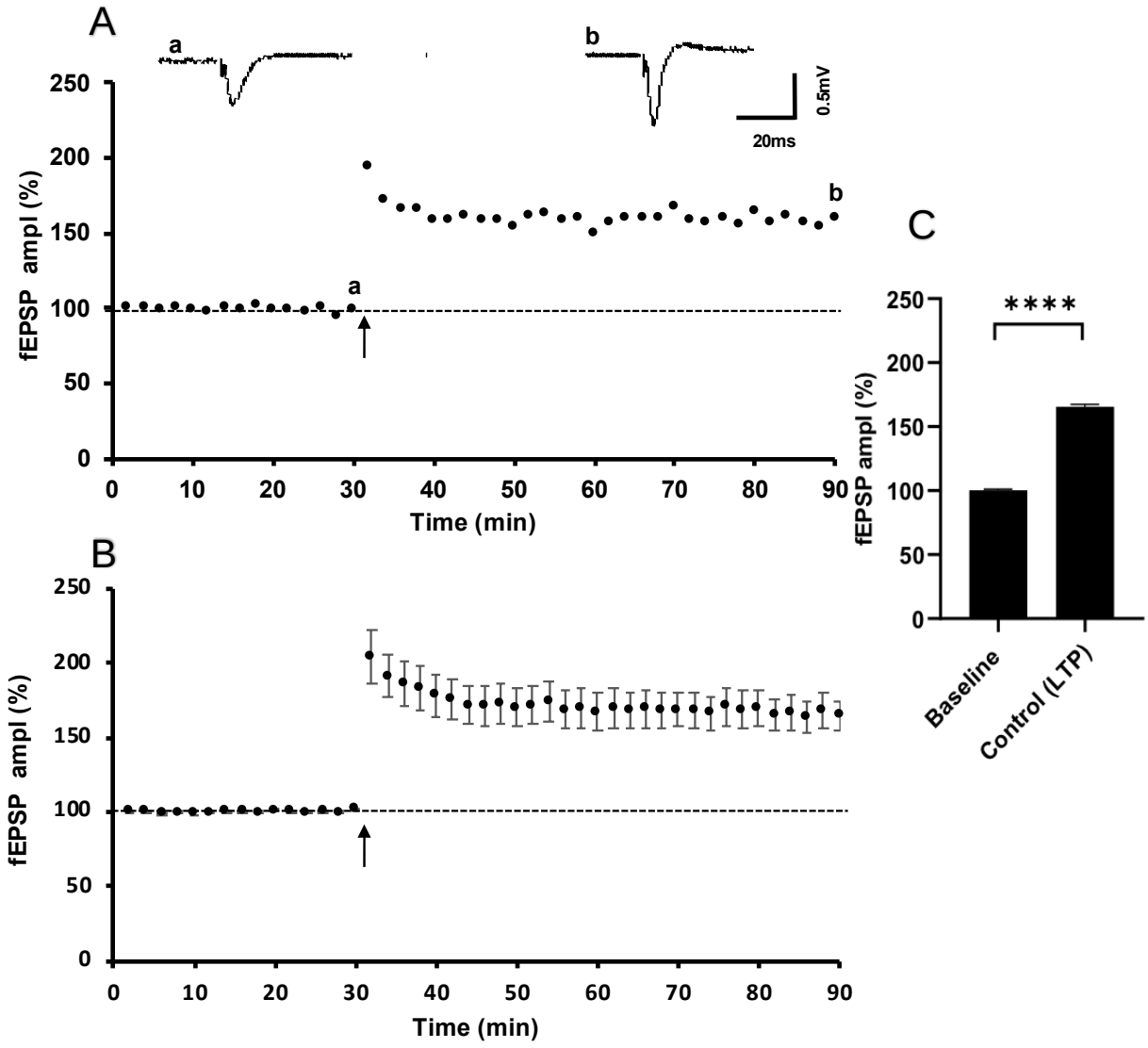


Figure 3.12. Induction of NMDAR-dependent LTP under control conditions. ***(A)*** *Shows a single experiment. After 2 hours of drug perfusion, the delivery of TBS (indicated by an arrow) induced LTP. Notice that the plots show only the last 30 minutes of drug perfusion and 60 minutes following the delivery of the TBS. The letters* ***(a)*** *and* ***(b)*** *represent the time the traces were obtained in the graph.* ***(a)*** *Displays the trace before LTP* ***(b)*** *after LTP induction.* ***(B)*** *Illustrates the pooled data comprising of 6 experiments (data presented as mean ±SEM).* ***(C)*** *Bar chart Illustrates the level of significant difference on fEPSP peak amplitude calculated during the last 10 minutes of baseline versus during the last 10 minutes of the fEPSP peak amplitude following LTP induction via TBS. Statistical significance was tested using a paired t-test and is indicated as **** = p<0.0001.*

3.6.2 Induction of NMDAR-dependent LTP in presence of 2-oxo-PCE

The result of the investigation of the effects of 10μM 2-oxo-PCE on the induction of LTP is shown in figure 3.13. 10μM 2-oxo-PCE completely blocked the induction of LTP induced by TBS. fEPSP peak amplitude measured as 101.2 ± 1.3% of baseline; during the last 10 minutes of average fEPSP peak amplitude following LTP induction in the presence of 10μM 2-oxo-PCE (that is between 50 and 60 minutes after stimulation) (n=6, t_4= 1.9, p>0.05, paired t-test vs baseline, Fig 3.13C).The data obtained in this work with 10μM 2-oxo-PCE shows similar inhibitory effects on LTP as previously observed with other NMDAR antagonists, such as ephenidine (Kang et al., 2017) fluorolintane (Wallach et al., 2019). Notice the last 10 minutes fEPSP peak amplitude following LTP induction in the presence of 10μM 2-oxo-PCE look very parallel to the last 10 minutes of baseline NMDAR-fEPSP peak amplitude, with a mean difference of 1.2 ± 0.4%.

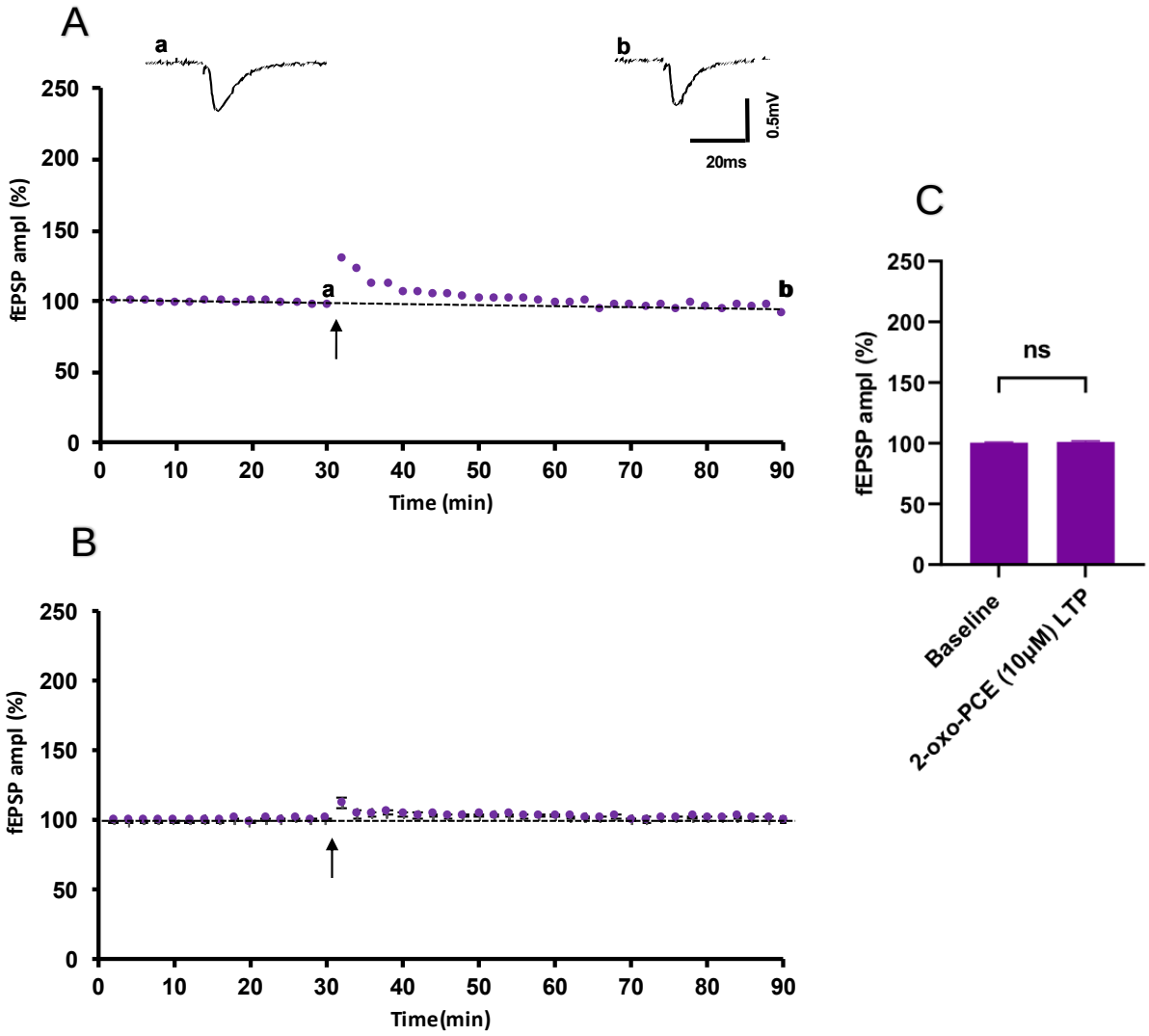


Figure 3.13. Effects of 2-oxo-PCE on the induction of LTP. (A) *Shows a single experiment. After a stable baseline of 30 minutes, the delivery of TBS (indicated by an arrow) was unable to induce LTP. 10μM 2-oxo-PCE blocked LTP induction completely. In* ***(A)*** *the insets represent typical EPSPs and the letters represent the time the traces were obtained in the graph.* ***(a)*** *Represents the trace before LTP induction* ***(b)*** *after LTP induction.* ***(B)*** *Shows pooled data comprising of 7 experiments (data plotted as mean ± SEM).* ***(C)*** *Bar chart demonstrates no significant difference between the last 10 minutes of baseline average fEPSP peak amplitude and the last 10 minutes following LTP induction in the presence of 10μM 2-oxo-PCE. Statistical significance between variables was tested using a paired t-test. (ns = not significant).*

The summary of the results about the effects of 10μM 2-oxo-PCE on NMDAR-dependent LTP versus control is shown in figure 3.14, with a mean difference of 64.3 ± 0.9% (t_8 = 73.6, P < 0.05, unpaired t-test, Fig 3.14).

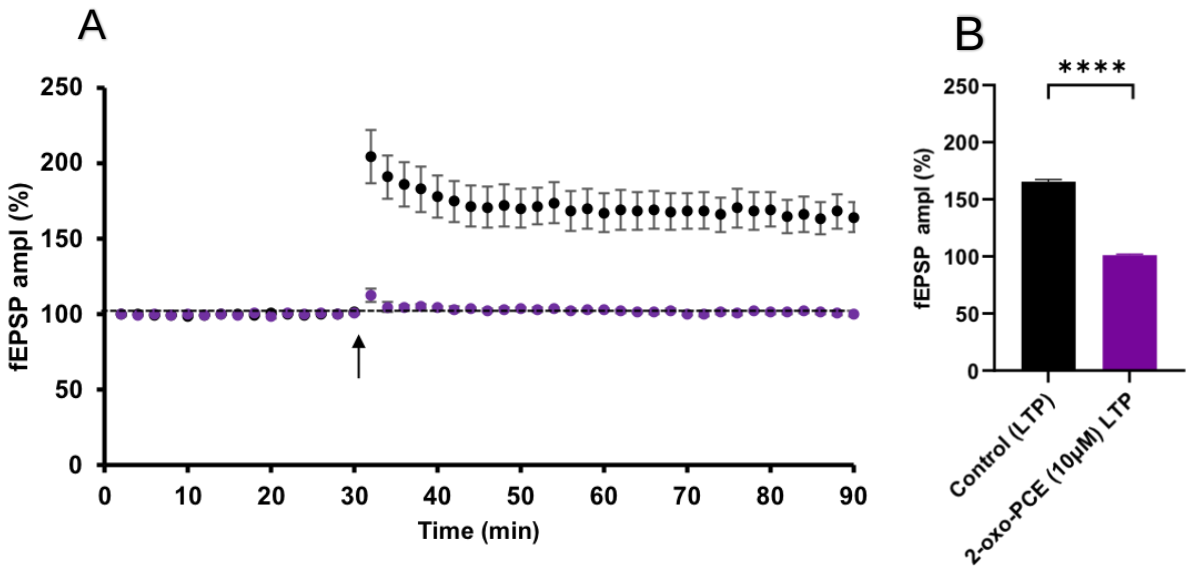


Figure 3.14. Summary of the effects of 10μM 2-oxo-PCE on NMDAR-LTP versus control conditions. (A) *Overlapping plots of the pooled LTP experiments under control conditions (black) (n=6) versus LTP in the presence of 10μM 2-oxo-PCE (purple) (n=7). (data plotted as mean ± SEM). The arrow represents when LTP was induced via TBS.* ***(B)*** *Bar chart measuring the significant difference between the last 10 minutes of average fEPSP peak amplitude following LTP induction via TBS in the presence of vehicle (aCSF) versus the last 10 minutes of average fEPSP peak amplitude following LTP induction in the presence of drug. The level of significance was tested using unpaired t-test and **** = p<0.0001.*

3.6.3 Induction of NMDAR-dependent LTD under control conditions

The results of the control LTD experiments are shown in the figure 3.15. LTD was readily induced at CA1 Schaffer collateral pathway in the presence of the vehicle by a low frequency stimulations (LFS) (900 pulses at 1Hz) applied after 30 minutes of stable baseline. The average fEPSP peak amplitude significantly decreased to 70.5 ± 4.0% of baseline; during last 10 minutes of average fEPSP peak amplitude following LTD *induction via LFS*) (n = 6, t4 = 76.1, P < 0.05, paired t-test vs. baseline, Fig 3.15C). Under control conditions, the level of LTD induced was 29.5 ± 0.4%.

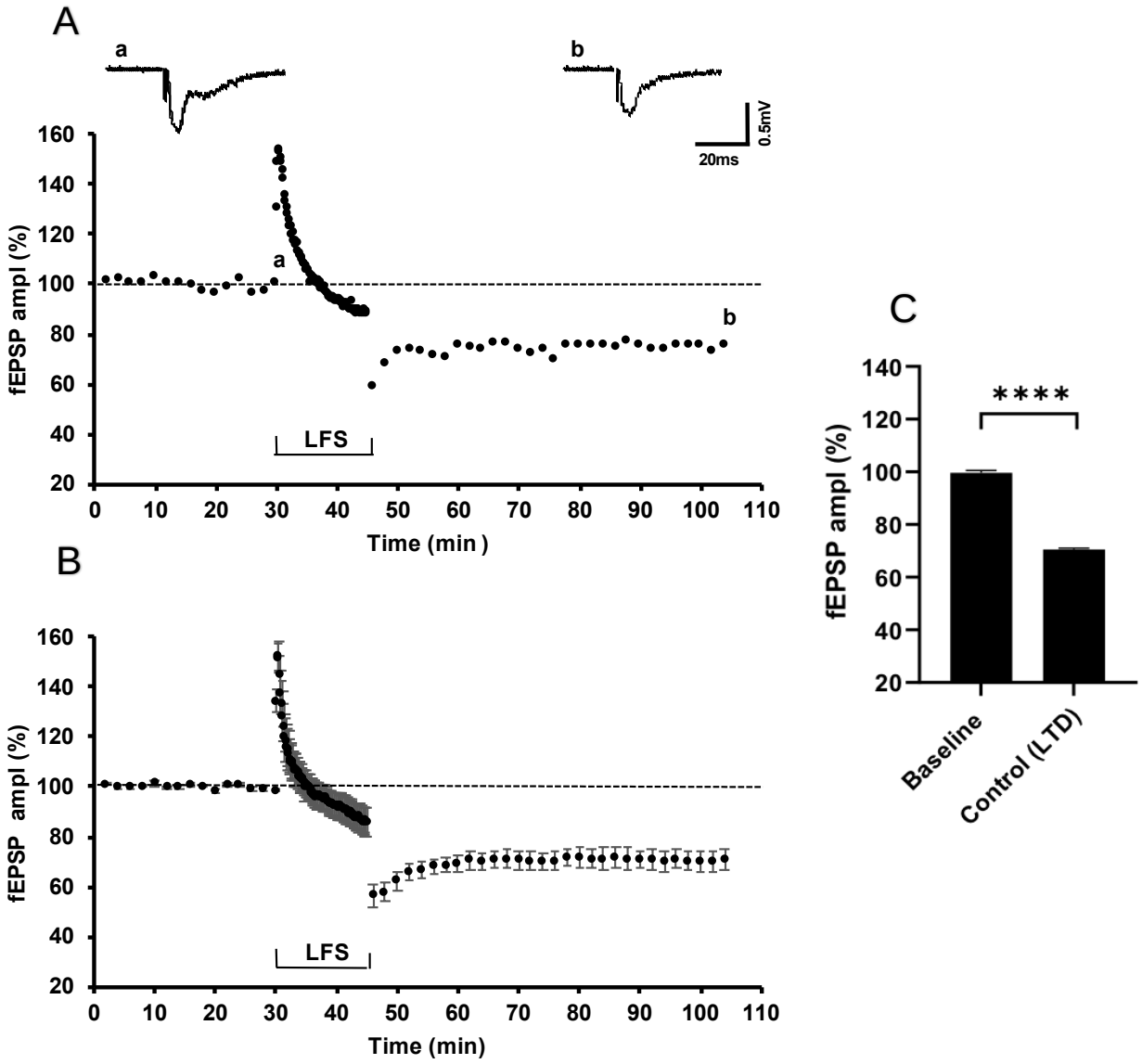


Figure 3.15. Induction of LTD under control conditions*. **(A)** Shows a single experiment in which LTD was induced by low frequency stimulations (LFS) protocol (900 pulses at 1Hz) and followed for 60 minutes. The capped lines display the time period when LTD was induced via LFS. The insets are representative traces extracted at the time point indicated on the plot by letters **(a and b).** Sweeps recorded during the LFS protocol are averaged (every 60 seconds) and plotted**. (B)** Shows the pooled data from all experiments under control condition (mean ± SEM) (n=6). In the bar chart **(C)** demonstrating difference between the last 10 minutes of baseline average fEPSP peak amplitude and the last 10 minutes of average fEPSP peak amplitude following LTD induction via LFS. Level of significance is tested using paired t-test and is revealed as **** = p<0.0001.*

3.6.4 Induction of NMDAR-dependent LTD in the presence of 2-oxo-PCE

The results obtained with the experiments exploring 10μM 2-oxo-PCE (10μM) on LTD induction are shown in figure 3.16. 10μM 2-oxo-PCE significantly reduced the level of LTD induced by the LFS protocol. However, LTD induction was not fully blocked by 10μM 2-oxo-PCE (87.1 ± 2.5% of baseline; last 10 minutes of average fEPSP peak amplitude following LTD induction via LFS in the presence of 10μM 2-oxo-PCE) compared to the effects of 10μM 2-oxo-PCE on LTP induction.($n = 5$, $t_4 = 39.0$, $P < 0.05$, paired t-test vs. baseline, Fig 3.16C). 12.9 ± 0.3% was the level of LTD induced in the presence of 2-oxo-PCE

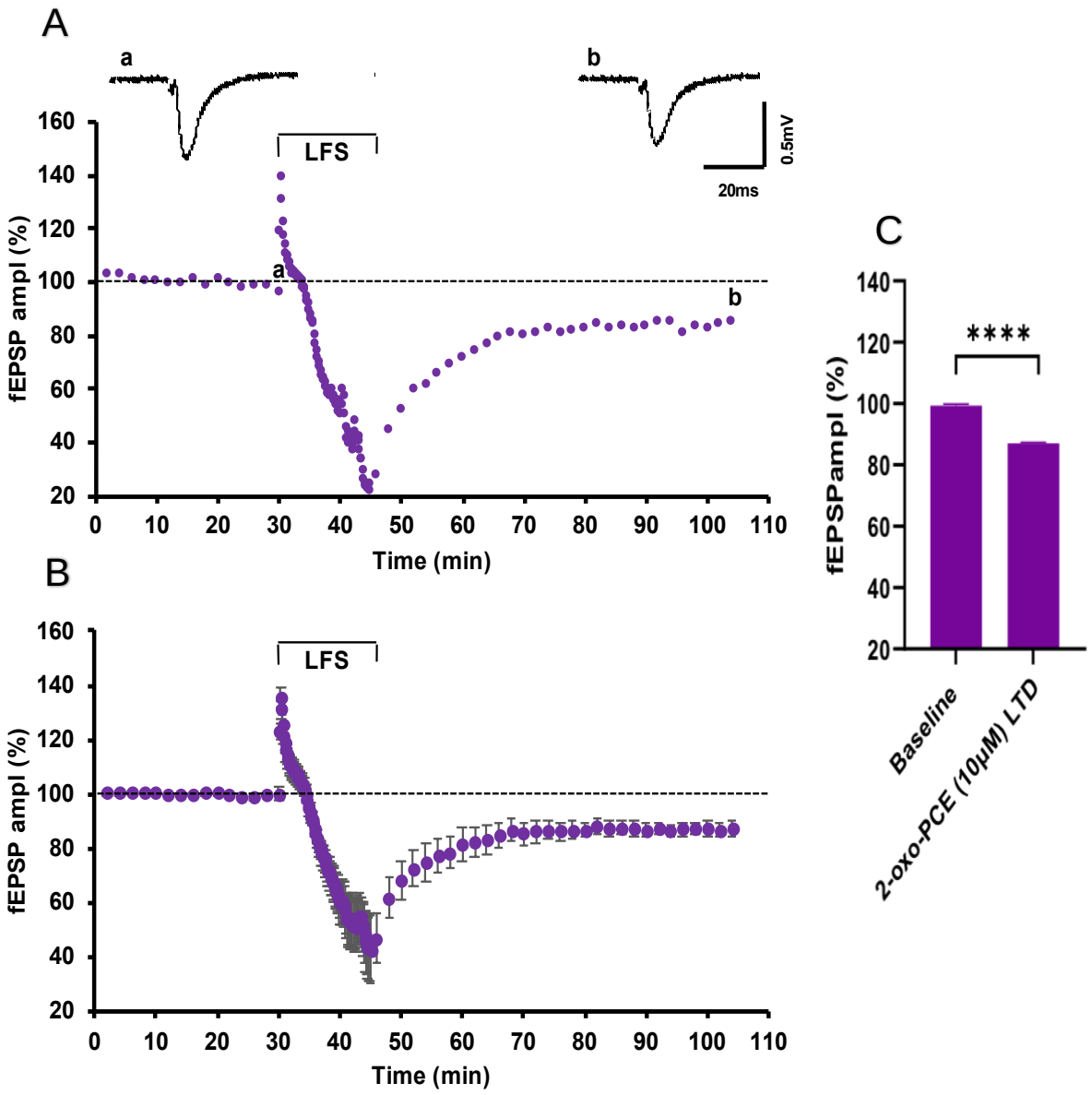


Figure 3.16. Effects of 2-oxo-PCE on NMDAR-dependent LTD. (A) *Represents a single experiment. After 30 minutes stable baseline, LTD was induced by means of LFS protocol (1Hz 900 pulses) and followed for 60 minutes. The capped lines display the time period when LTD was induced via LFS. Sweeps plotted during LFS are averaged (60 pulses). The insets represent averaged traces obtained from the single example at the time point indicated by (**a** before & **b** after LFS) on plot **(A).** **(B)** Illustrates the pooled data comprising of 5 experiments (mean ±SEM) (n = 5). **(C)** A bar chart showing the difference between the last 10 minutes of baseline fEPSP peak amplitude versus the last 10 minutes of average fEPSP peak amplitude following LTD induction via LFS in the presence of 10μM 2-oxo-PCE. Level of significance was tested using paired t-test and is revealed as **** = p<0.0001*

The results about the effects of 10μM 2-oxo-PCE on NMDAR-dependent LTD versus control are summarised on figure 3.17. The amount of the LTD induced in the presence of 2-oxo-PCE is significantly smaller compared to control conditions (t_8 = 60.5, P < 0.05, unpaired t-test vs. baseline, Fig 3.17B). A mean difference of 16.6 ± 0.3%. was calculated from the last 10 minutes of average fEPSP peak amplitude following LTD induction in the presence of vehicle (aCSF) versus the last 10 minutes of average fEPSP peak amplitude following LTD induction in the presence of 2-oxo-PCE. This shows how much 2-oxo-PCE reduced the level of LTD compared to the control experiments.

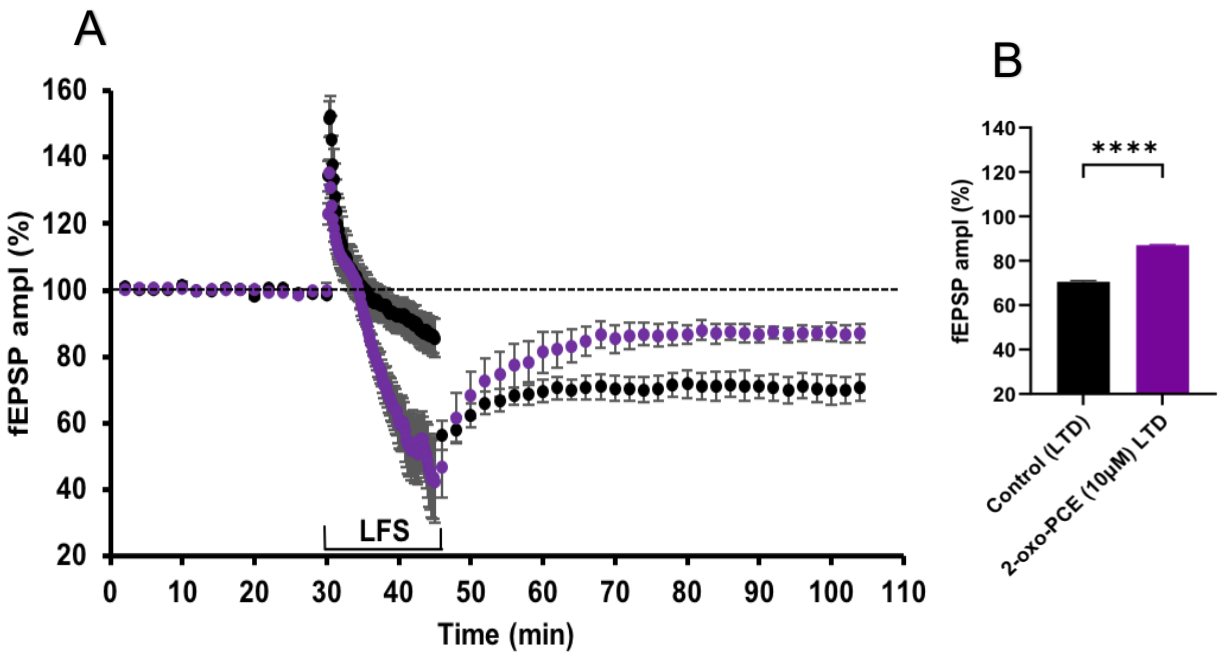


Figure 3.17. Summary of the effects of 2-oxo-PCE on NMDAR-dependent LTD versus control conditions. (A) *Shows the overlapping pooled data plot of the NMDAR-mediated LTD induction under control conditions (n=6) (black) versus in the presence of 10μM 2-oxo-PCE (n=5) (purple). The capped lines display the time period when LTD was induced via LFS.* ***(B)*** *Bar chart assessing the significant difference between the last 10 minutes of average fEPSP peak amplitude following LTD induction in the presence of vehicle (aCSF) versus the last 10 minutes of average fEPSP peak amplitude following LTD induction in the presence of drug. The level of significance was tested using unpaired t-test and **** = p<0.0001.*

4 Discussion

4.1 2-oxo-PCE involvement in NMDAR mediated synaptic transmission

The effects of 2-oxo-PCE on the brain are currently unknown. Therefore, the aim of this project was pivotal to characterise the pharmacology of '2-oxo-PCE' by investigating its functional activity on NMDAR-mediated synaptic transmission in the hippocampus using electrophysiological techniques. 2-oxo-PCE, a newer generation of ketamine derivative, rapidly and potently reduced NMDAR-fEPSPs in a concentration-dependent manner (Fig 3.7). Particularly, a fast-inhibitory onset of kinetics was observed in the fEPSP experiments. Overall, this indicates that 2-oxo-PCE is a fast-acting NMDAR antagonist. This is consistent with the online user anecdotal reports that the effects of 2-oxo-PCE are far more potent than ketamine. (NeuePsychoaktiveSubstanzen.de, 2016; Cheng and Dao, 2020). However, this does not imply that 2-oxo-PCE exhibits less dissociative side effects than established NMDAR antagonists e.g. ketamine. Although the dissociative effects are thought to be largely mediated by the NMDAR blockage (Morris and Wallach, 2014; Lodge and Mercier, 2015) in vitro electrophysiology studies alone, cannot determine whether 2-oxo-PCE exhibits less dissociative side effects than ketamine. Behavioural experiments will be required to verify this.

Furthermore, the results revealed that 2-oxo-PCE did not induce 100% blockade of the NMDAR-fEPSP in CA1 region of the hippocampus. This could be due to various factors, for example possible interactions with other non-glutamatergic synaptic transmission, particularly interacting with the monoamine system (Wallach et al., 2016; Kang et al., 2017) e.g. dopamine receptors. Ketamine, the parent compound of 2-oxo-PCE have shown interactions with dopamine receptors in literature papers (Kapur and Seeman, 2002; Seeman et al., 2005).

3μM, 10μM and 30μM 2-oxo-PCE, both reached plateau level of depression after 3 hours drug perfusion. Interestingly, 0.1μM and 1μM with a slower kinetics profile, failed to reach a plateau after 3 hours drug perfusion respectively (Morris and Wallach, 2014). Consequently, the level of depression calculated cannot be speculated as absolute compared to the 3μM, 10μM and 30μM, which ultimately reached plateau within the time frame of the experimental protocol. The real level of depression will most likely be observed if extended the time course protocol from 3 hours to 6 hours drug perfusion. Thus, further experiments are necessary with an increased duration of perfusion to reach plateau, in order to better determine the level of depression and potency for 0.1μM and 1μM 2-oxo-PCE concentrations respectively. 0.5mg/kg ketamine is the most common clinical therapeutic dose administered intravenously to depressed patients in randomised clinical trials (Berman et al., 2000; Zarate et al., 2006).Also, it has been found that ranges from 0.1mg/kg – 0.5mg/kg revealed antidepressant effects in depressed patients in crossover clinical trials (Lai et al., 2014; Loo et al., 2016). 0.3 - 0.5μM (80 - 150ng/ml) are the subanaesthetic blood levels range that are linked to antidepressant and dissociative effects (Zhao et al., 2012) and ultimately reflects the brain concentration 1 - 10 μM (Cohen et al.,1973; Hartvig et al., 1995; Doyle et al., 2013; Zarate et al., 2006).Consequently, 0.1μM, 1μM, 3μM, 10μM and 30μM 2-oxo-PCE concentrations reflect this and hence explored in this project.

Earlier studies have concluded that non-competitive NMDAR antagonists e.g. ketamine, exhibits various dissociative effects including hallucinations and delusion (Oye et al., 1992; Herrling, 1994; Steinberg et al., 1994). In this thesis, 2-oxo-PCE, a newer generation of ketamine derivative has been shown to potently reduce NMDAR- fEPSPs in the CA1 region of the hippocampus. 2-oxo-PCE, under comparable conditions, appears to be a more rapidly acting and more potent NMDAR antagonist than ketamine, demonstrating a faster onset of NMDAR-fEPSP antagonism and a greater level of depression at 10μM. Interestingly 3μM 2-oxo-PCE resembled a similar level of depression to 10μM ketamine, than 10μM 2-oxo-PCE. Consequently, this could become a concern for abuse liability in humans. Particularly, 2-oxo-PCE is more potent than ketamine. Although ephenidine and 2-oxo-PCE also share similar level of depression at 10μM. 2-

oxo-PCE also exhibits a faster onset of NMDAR-fEPSP antagonism than ephenidine. Ephenidine, a non-competitive NMDAR antagonist with ketamine like properties, requires between 100-500mg to produce a dissociative effect in humans (Kang et al., 2017). Thus, 2-oxo-PCE is likely to require similar dose or possibly lower to induce the dissociative effects seen in humans, based on faster onset of NMDAR-fEPSP antagonism profile and potency. This could further increase the risk of other unwanted potential side effects including thought disorder, attention deficit, and impairments in working memory as well as increasing the risk for overdose (Malhotra et al., 1996; Adler et al., 1998). Another reason why it is critical to pharmacologically characterise 2-oxo-PCE to prevent any fatalities relating to the potency of 2-oxo-PCE.

In this thesis, an earlier preliminary washout experiment of 3μM 2-oxo-PCE was conducted. This was to see whether the recordings would recover back to the NMDAR-fEPSP baseline peak amplitude. After a 90 minute washout period of 3μM 2-oxo-PCE, a notable slow increase of NMDAR-fEPSP peak amplitude was observed. However, with observation and extrapolation suggests that NMDAR-fEPSP peak amplitude could recover back to baseline levels within 4 hours in total. This suggests that 2-oxo-PCE could potentially be a slow reversible drug. Crucially, this finding suggests that 2-oxo-PCE is not only fast acting, but also presents long lasting effects. 2-oxo-PCE could potentially be more favourable than conventional antidepressants which exhibit a delayed onset of action with short lasting effects. Although there are many classes of conventional antidepressants, they all work by targeting the monoaminergic system. For instance, selective serotonin reuptake inhibitors (SSRIs) block the reuptake of monoamines e.g. serotonin. Monoamine oxidase inhibitors (MAOIs), another antidepressant class, work by preventing the breakdown of monoamines e.g. serotonin, dopamine, and noradrenaline (Andrade et al, 2010). However, ketamine, which exhibits fast acting antidepressant effects, is likely to function by mediating the glutamatergic system, specifically blocking NMDARs. Major depressive disorder (MDD) is the number one cause of disability compared to other psychiatric disorders (Collins et al., 2011). Conventional antidepressants have not shown long term remission in the majority of depressed patients (Rush et al., 2006). It is critical to develop more effective antidepressants with a faster

onset and more durable effects. These new drugs could be especially useful for patients who present with treatment resistant depression (TRD), that do not respond effectively to conventional antidepressant drugs. However, more washout experiments are necessary to better understand the duration of NMDAR blockade, in order to validate this interesting finding.

The effects of 10μM ketamine on NMDAR-fEPSP, were consistent with data from previous papers under comparable conditions (Kang et al., 2017). 2-oxo-PCE displays a faster reduction of the NMDAR-fEPSP and faster onset of kinetics compared to ketamine (Fig 3.9). Its faster kinetic properties propose that 2-oxo-PCE is a faster acting NMDAR antagonist than ketamine. However, further in vitro binding affinity studies will provide a better understanding on how potent the 2-oxo-PCE really is, in comparison to ketamine.

Interestingly, the profile of NMDAR-fEPSP inhibition induced by 2-oxo-PCE resembles that induced by fluorolintane more than ketamine based on the potency. Wallach et al. have demonstrated that 10μM fluorolintane reduced the NMDAR-fEPSP peak amplitude by 85.9 ± 1.0% (n=4) (Wallach et al., 2019), compared to 10μM ketamine (63.7 ± 3.2%) (n=3). Both 2-oxo-PCE and fluorolintane demonstrate a faster onset of NMDAR-fEPSP antagonism compared to ketamine. However, 10μM fluorolintane depressed the NMDAR-fEPSP peak amplitude more than 10μM 2-oxo-PCE, under comparable conditions (85.9 ± 1 % vs. 78.8 ± 5.0%). 10μM Fluorolintane depressed the NMDAR-fEPSP 7.1% more than 2-oxo-PCE on the stratum radiatum of the area CA1 in hippocampus slices after Schaffer collateral stimulations. However, 2-oxo-PCE inhibitory kinetics profile is deemed slower than D-AP5, a competitive NMDAR antagonist with fast kinetics. This could be related to the location of drug binding sites. For instance, 2-oxo-PCE needs the channel to open via binding of glutamate and glycine/D-serine as a co-agonist alongside the removal of voltage dependent Mg^{2+} block in the pore channel during AMPAR mediated membrane depolarization in the postsynaptic neuron for 2-oxo-PCE to bind to the channel pore. Whereas, D-AP5 binds to the same site as glutamate at the GluN2 subunit ligand binding site (see Fig 1.3), and competitively antagonises the receptor. Therefore, 2-oxo-PCE and ketamine exhibit similar inhibitory kinetics, and both share the same binding

site, which explains the online user anecdotal reports that 2-oxo-PCE displays comparable effects to ketamine (NeuePsychoaktiveSubstanzen.de, 2016; Cheng and Dao, 2020). Also, this may explain why reports found 2-oxo-PCE in urine samples of a number of patients with a history of ketamine abuse in Hong Kong (Chong et al., 2017; Tang et al., 2018).

Based on the chemical structure of ketamine and 2-oxo-PCE, the major difference is the ethyl group attached on the nitrogen atom of the amino group for 2-oxo-PCE and methyl group on the nitrogen and chlorine attached on the phenyl ring for ketamine (Cheng and Dao, 2020). Previous literature has speculated that 2-Cl-substitution on the phenyl ring could be related to the decreased NMDAR potency (Ho and Dargan, 2016; Corazza et al., 2013). This also relates to the finding that 10μM 2-oxo-PCE, more potently reduces NMDAR-fEPSP than 10μM ketamine. However, binding affinity experiments are necessary to verify the potency of 2-oxo-PCE.

IC_{50} relates to the potency of the drug. The IC_{50} of 2-oxo-PCE is $5.25e^{-7}$ M. This is equivalent to 0.525μM. Thereby, 0.525μM 2-oxo-PCE is required to produce 50% level of depression on NMDAR-fEPSP in the Schaffer collateral CA1 pathway in the hippocampus after 3 hours in drug perfusion. The low IC_{50} reaffirms that 2-oxo-PCE is a very potent non-competitive NMDAR antagonist. 2-oxo-PCE seems to have a more potent effect at the NMDAR than ketamine. Lumsden et al., 2019 study showed that IC_{50} for ketamine was 4.5 μM in mouse hippocampal slices. The IC_{50} value of 2-oxo-PCE is extremely smaller (i.e. 8.57% more) in comparison to ketamine. Therefore, a smaller concentration is required for 2-oxo-PCE compared to ketamine to produce 50% level of depression of NMDARs in the CA1 region of the hippocampus after 3 hours in aCSF perfusion.

In addition, 30μM 2-oxo-PCE blocked the NMDAR-fEPSP by 85.4 ± 0.27% (n=4). Interestingly, extrapolation from the sigmoidal curve indicates that concentrations higher than 30μM will still produce the same level of depression as 30μM. At concentrations of 30μM and higher, it is likely that all NMDAR have been occupied, and so the drug will

most likely not induce a level of depression greater than 85.4%. However, 2-oxo-PCE did not show 100% NMDAR-fEPSPs inhibition in CA1 hippocampal slices and including in the presence of D-AP5. As previously mentioned, possible interactions with other non-glutamatergic synaptic transmission could be a reason for the residual NMDAR response. Another reason could be due to the external noises from the electrophysiological set up used in the lab. For instance, electrical noises can stem from various sources such as: the equipments used such as amplifier, recording electrodes and the computer monitor. Despite, implementation of a faraday cage to help reduce external electrical interferences, electrical noises still existed. Furthermore, another reason for the residual NMDAR response could be due to the saturation of the NMDAR binding sites. This is because at 30μM, a plateau effect was observed. This suggests that approximately nearly all NMDARs that are available have been bound by 2-oxo-PCE at this concentration and that possibly very few unoccupied NMDARs sites are left for the drug to bind to, which affects the ability to induce further inhibition. Similarly, binding specificity can be a reason for the residual NMDAR response. 2-oxo-PCE have shown plateau at various concentrations. It is possible that 2-oxo-PCE may demonstrate different affinities based on different NMDAR subunit composition. Therefore, further research is necessary to determine if 2-oxo-PCE is binding to, and selective for, a certain subunit of the NMDA receptor.

4.2 Implications of 2-oxo-PCE on NMDAR dependant synaptic plasticity.

4.2.1 2-oxo-PCE potently blocked NMDAR-dependant LTP

10μM 2-oxo-PCE completely blocked NMDAR dependent LTP in CA1 hippocampal slices, following a TBS (see Fig 3.13). This is consistent with, and supported by, findings in previous papers that dissociative NMDAR antagonists are able to block LTP induction (Stringer and Guyenet, 1983; Kang et al., 2017; Wallach et al., 2016, 2019). Synaptic plasticity is an important process for learning and memory. In particular, LTP and LTD are thought to be responsible for changes in synaptic weight that underlie memory formation

(Bliss and Collingridge, 1993; Bliss et al., 2014; Volianskis et al., 2015). More specifically, synaptic plasticity is known to influence spatial memory (Morris et al., 1986**)**. The NMDAR's profound involvement in modulating synaptic plasticity, notably LTP and LTD in the CA1 region of the hippocampus, has been widely recognised in previous literature papers (Collingridge et al., 1983; Stringer and Guyenet, 1983) Certain forms of LTP and LTD induction in the CA1 region of the hippocampus are dependent on NMDAR activation and these are some of the most extensively studied forms of synaptic plasticity (Bliss and Collingridge, 1993; Malenka and Bear, 2004). Therefore, I hypothesize that blockade of LTP induction with 10μM 2-oxo-PCE, could produce cognitive deficits in the brain and amnesia (Bliss and Collingridge, 1993). NMDARs are found to be widely expressed in the brain and many different forms of LTP are NMDAR dependent. Therefore, excessive use of 2-oxo-PCE could possibly lead to serious side effects particularly, long lasting episodic memory deficit (Morgan et al., 2004). In conclusion, as the data presented in this thesis show that as little as 10μM 2-oxo-PCE is sufficient to fully block NMDAR dependent LTP in CA1 hippocampal slices and that higher concentrations will have the same effect as 10μM e.g. 30μM. Moreover, this data is consistent with previous findings relating to deficits in LTP induction following the perfusion of ketamine to hippocampal slices. Interestingly, it can be speculated that lower concentrations like 3μM 2-oxo-PCE will also block LTP induction based on the observation that 10μM ketamine resembles similar level of depression to 3μM 2-oxo-PCE than 10μM in fEPSP experiments (Fig 3.4 & Fig 3.9). Literature papers have shown 10μM ketamine to block LTP induction in CA1 hippocampal slices. Hypothetically 1μM would mostly affect and partially block LTP induction given that it blocked NMDAR-fEPSP by 55.5 ± 1.8% respectively (Fig 3.3). However, given that 0.1 2-oxo-PCE blocked NMDAR-fEPSP by 8.8 ± 4.8%, no significant reduction in fEPSP amplitude during the final 10-minute period was found (Fig 3.2C). Thus, most likely will not block LTP induction.

4.2.2 Implications of 2-oxo-PCE on NMDAR-dependant LTD

NMDAR dependent LTD is more difficult to induce in hippocampal slices from older animals. However, previous literature has shown that NMDAR-LTD is more readily induced in Wistar rats than other strains like hooded rats (Manahan-Vaughan, 2000; Manahan-Vaughan et al., 2000). 2-oxo-PCE partially blocked LTD induction in the CA1 region of the hippocampus by 16.6 ± 0.3% in comparison to the control group (Fig 3.17). These data are consistent with preliminary studies that also showed that ketamine did not block NMDAR-LTD in the CA1 hippocampal slices (not shown). Contradictory results have been reported in other research articles (Huang et al., 2016). However, direct comparisons with these papers are not entirely feasible due to differences in the experimental techniques used. For instance, in Huang et al., (2016) ventral hippocampal slices were used, however, in this thesis, parasagittal dorsal hippocampal slices were used. Similarly, in the Huang et al. (2016) paper, the authors induced LTD with NMDA whereas in these experiments I induced LTD via LFS.

It is possible that 2-oxo-PCE exhibits a different sensitivity to LTP and LTD. Thus, it would be worth investigating the 2-oxo-PCE effect on LTD with higher concentrations (e.g. 30μM). This will conclude whether 2-oxo-PCE could exhibit a greater LTD block at higher concentrations. Also, it is important to consider NMDAR subunit specificity. Indeed, ketamine demonstrates greater inhibitory effects at GluN2C/D-containing NMDARs in comparison to GluN2A/B-containing NMDARs (Kotermanski and Johnson, 2009). Interestingly, extra-synaptic NMDARs are often GluN2B-containing receptors and are postulated to play an important role in LTD induction (Cull-Candy et al., 2001; Vyklicky et al., 2014; Traynelis et al., 2010). 2-oxo-PCE is perhaps less effective at antagonising these specific receptor subtypes and this could explain the partial LTD blockade by 10μM 2-oxo-PCE. It could be assumed that the remaining LTD is largely mediated by different NMDAR subtypes that 2-oxo-PCE has no effect on. Nonetheless, further experiments are required to investigate this, and to determine 2-oxo-PCE NMDAR subtype selectivity. This theory is supported by observations in fEPSPs experiments, that showed NMDAR-fEPSP was not fully blocked by 30μM 2-oxo-PCE. Therefore, not blocking all the NMDAR

subtypes. However, when 100μM D-AP5 was added to the perfusion system, subsequently, D-AP5 further blocked NMDAR-fEPSP, exhibiting a near maximal effect. This reaffirms that 2-oxo-PCE could have a selective binding to a particular subunit of NMDAR.

4.3 Implications of 2-oxo-PCE selectivity.

NMDAR dependent synaptic plasticity experiments concluded that 2-oxo-PCE elicits no change to the AMPAR. The peak amplitude of AMPAR-fEPSPs was comparable to baseline values following a 2-hour application of 10μM 2-oxo-PCE (Fig 3.11). This indicates that 2-oxo-PCE binds specifically to the NMDAR and not the AMPA receptor. It is important to establish the specificity of binding of 2-oxo-PCE to NMDAR, as off-target blockade of AMPA receptors could lead to numerous unwanted side effects. For instance, motor dysfunction, nausea, and ataxia (Ko et al., 2015).

Although, in this thesis I have shown that 2-oxo-PCE is selective to the NMDA glutamate receptor, I have not determined whether 2-oxo-PCE is selective for certain NMDAR subunits. Therefore, further experiments are necessary to determine this, and which subunits it might be selective for. Also, being selective to NMDAR does not necessarily mean it does not have affinity for other receptors in the CNS. For example, ketamine a selective NMDAR antagonist, but in binding affinity assays, shown affinity for various CNS receptors e.g. dopamine receptors (Kapur and Seeman, 2002; Seeman et al., 2005).

4.4 Possible mechanism of the effect of 2-oxo-PCE on fEPSPs

The likely mechanism of the effect of 2-oxo-PCE on fEPSP will be based on the NMDARs. For instance, the effects of 2-oxo-PCE on LTP and LTD are both NMDAR dependent and shown to partially and completely block these phenomena respectively. NMDAR activation is extremely important in these processes as this can influence synaptic strength between neurons. 2-oxo-PCE blockade effect on these processes indicates its profound involvement in modulating NMDAR function. Furthermore, sensitivity to NMDAR

subunit compositions can be a possible mechanism of the effect of 2-oxo-PCE on fEPSPs. 2-oxo-PCE may possibly have specific binding affinities based on the NMDAR subunit compositions. This is because 2-oxo-PCE have partially blocked LTD but completely blocked LTP, suggesting a possible sensitivity to NMDAR subunit compositions in its action. Furthermore, open channel blockade is likely mechanism of the effects of 2-oxo-PCE on fEPSP. This is because 2-oxo-PCE closely resembles ketamine, which is an open channel blocker of NMDARs.

Open channel blockers like ketamine bind to the near the asparagine residue of the pore lining M2 loops located inside the channel pore during the open state (Burnashev et al.,1992; Kashiwagi et al., 2002; Kotermanski and Johnson, 2009; Retchless et al., 2012), consequently blocking the NMDAR noncompetitively (Khors and Durieux, 1998). This amplifies its efficacy when NMDARs is excessively activated, leading to more open NMDARs channels for blockade.

Furthermore, 2-oxo-PCE plays an extremely important role in desensitisation modulation. This is because 2-oxo-PCE could possibly modulate NMDARs during the desensitisation state. The desensitisation state is critical as this can influence the effectiveness of the NMDARs. 2-oxo-PCE's modulating effect on NMDARs during the desensitisation state, could enable faster recovery from desensitisation which ultimately could play a role to its impact on the fEPSPs.

Binding affinity of 2-oxo-PCE is important when discussing the likely mechanism of the effect of 2-oxo-PCE on fEPSPs. IC_{50} is indicative of the drug potency and used as a way to measure binding affinity of the drug. IC_{50} calculated for 2-oxo-PCE was 0.525 µM. Low IC_{50} indicates higher binding affinity to the receptor. 0.525 µM value is extremely low and suggests that 2-oxo-PCE binds strongly to the NMDARs and exhibits high binding affinity for NMDARs. 2-oxo-PCE high binding affinity means that at low concentrations can enhance NMDARs modulation. This is because 2-oxo-PCE can strongly and effectively bind to the NMDARs when activated (i.e. during LTP or LTD), and therefore leading to

effective modulation of LTP and LTD function respectively. Thus, 2-oxo-PCE binding affinity for NMDARs can influence its likely mechanism of action on fEPSPs.

2-oxo-PCE closely resembles ketamine, an establish open channel blocker of NMDARs, which involves blocking the channel pore of the NMDARs when it is in the open state. Subsequently, this inhibits the calcium ions which affects synaptic plasticity. Ketamine is as dissociative anaesthetic and recently sparked a worldwide clinical interest for its faster-acting antidepressant effects compared to conventional antidepressants (Berman et al.,2000). Ketamine has shown to reduce excitatory signalling between synapses due to ketamine's NMDAR blockade effect. Antagonism of the NMDARs contributes to the antidepressant affects and the dissociative anaesthetic effects (Morris and Wallach, 2014; Lodge and Mercier, 2015). Fast- acting antidepressant effects are due to NMDARs and additional mechanisms. Ketamine acts as a weak agonist at the D2 receptor (Ingram et al., 2018). These additional interactions with other receptors besides NMDARs contribute to ketamine's fast-acting antidepressant effects.

2-oxo-PCE likely shares similarities in its effect on fEPSPs due to 2-oxo-PCE structurally resembling ketamine. 2-oxo-PCE functions like ketamine by selectively blocking NMDARs which reduces the calcium ion flow in the channel pore and ultimately impacting synaptic plasticity. However, 2-oxo-PCE's likely mechanism of effect on fEPSPs may possibly vary from ketamine to a certain degree. Thus, further research is needed to investigate whether 2-oxo-PCE has additional actions on other receptors similar to ketamine. These additional mechanisms could potentially contribute its effect on fEPSPs

4.5 2-oxo-PCE therapeutic potential

NMDAR dysfunction has been implicated in many neurological disorders e.g. Alzheimer's disease, stroke and epilepsy are thought to be related to overstimulation of NMDAR (Cull-Candy et al., 2001). Therefore, the NMDAR is a crucial therapeutic target in drug developments to treat various neurological disorders. Memantine is a known NMDAR

antagonist, which is often used for alleviating the cognitive impairments associated with Alzheimer's disease (Tariot, 2006; Reisberg et al., 2017).

The involvement of NMDARs in the pathophysiology of depression was first highlighted by research studies involving ketamine (Berman et al., 2000; Zarate et al., 2006). The non-competitive NMDAR antagonist ketamine has been shown to be more favorable than traditional antidepressants for its fast-acting antidepressant effects in major depressive disorder (MDD) patients (Berman et al., 2000; Zarate et al., 2006). Also, it has been shown to be effective in treatment resistant depression, producing rapid antidepressant actions (Caddy et al., 2014). Despite promising therapeutic potential, ketamine can also induce powerful adverse dissociative effects, including hallucination. Consequently, new high efficacy fast acting antidepressant drugs are needed on the market to better treat depression. The new ketamine derivative, 2-oxo-PCE, resembles the pharmacological properties of ketamine. The results from study show that 2-oxo-PCE exhibits a more potent NMDAR-fEPSP antagonism profile than ketamine. Thus, it is possible that 2-oxo-PCE could possibly exert fast-acting ketamine antidepressant effects, which could be tested in behavioural studies involving forced swim tests. If this is the case, then rapid acting antidepressants like 2-oxo-PCE could be more effective for treating high risk suicidal patients that require an immediate antidepressant effect to challenge the suicidal ideation (Lodge and Mercier, 2015; Muir, 2006). Forced swim tests are a widely used assay for depression and are very quick to perform. Autry et al., (2011) showed that ketamine exhibited rapid onset and substained antidepressant effects in mice using the forced swim test (FST) compared to a control group. However, the forced swim test may not be a good approach to measure of depression. For instance, there have been concerns about immobility being a learned behaviour and that rodents may learn that remaining immobile is the best course of action to be rescued from the water. This is referred to as learned immobility (Yankelevitch-Yahav et al., 2015), and this behaviour does not persist after the test is finished. Therefore, immobility behaviour in rodents may not be a true indicator and reflect the symptoms of depression in humans. Moreover, the tail suspension test is also used to measure immobility in rodents. This is also a direct response to the experiment and immobile behaviour is not observed in rodents after the

experiments (Castagné et al., 2009). Therefore, these assays are not suitable as models of depression alone. A better model of depression would be the learned helplessness test. In this test, rodents are given constant foot shock, thus creating an aversive situation for rodents. This creates long lasting neuronal changes in the rodent, comparable to depression. Consequently, the rodents are less able to demonstrate an escape response, even though the test provides an escape route (Castagné et al., 2009; Telner and Singhal, 1984; Vollmayr and Henn, 2001).

Furthermore, I have also shown that 2-oxo-PCE completely blocks LTP induction and partially blocks LTD induction in the CA1 region of rat hippocampal slices. Therefore, 2-oxo-PCE could potentially be effective in the treatment of depression and possibly post-traumatic stress disorder (PTSD) where negative memories are formed after a traumatic and stressful experience. The potential therapeutic uses of 2-oxo-PCE in the treatment of PTSD are supported by a randomized clinical trial that found a single infusion of ketamine reduced the symptoms in patients suffering from chronic PTSD for more than 24 hours (Feder et al., 2014). However, it is still necessary to understand the safety of these drugs beyond a single infusion, and whether chronic PTSD patients will start to experience profound dissociative symptoms. Overall, it remains possible that 2-oxo-PCE would exert the same therapeutic effects observed with ketamine when treating chronic PTSD patients due to their similar pharmacodynamic properties. Furthermore, research by Kang et al., highlighted that under immense stress and when depressed, negative thoughts and memories of unpleasant experiences are possibly created through LTP and LTD (Kang et al., 2017). Therefore, this could mean that the reason why depressed patients continuously experience unpleasant memories is due to abnormal/excessive synaptic plasticity. Given that depression is comorbid with PTSD, this could possibly be an explanation for PTSD patients as well. Previous literature concluded that NMDAR antagonists that inhibit the induction of LTP/LTD can affect the cycle of the pathological plasticity and thereby elevate mood (Collingridge et al., 2010; Kang et al., 2017). However, the findings thus far do not imply that 2-oxo-PCE exhibits less dissociative side effects than established NMDAR antagonists e.g. ketamine. Further behavioural experiments will enable the determination of this.

Based on the 2-oxo-PCE pharmacological profile resembling ketamine, 2-oxo-PCE could possibly have therapeutic implications in the treatment for PTSD, MDD and TRD. However, it is important to take in account that not all NMDAR antagonists (e.g. MK-801) exert ketamine like antidepressant effects (Maeng et al., 2008). It could be speculated that this is due to the fact that MK-801 demonstrates a slower pharmacodynamic profile than ketamine (Wallach et al., 2016). Therefore, further experiments are necessary to advance the understanding of the effects of 2-oxo-PCE in the brain. Particularly, experiments to determine the pharmacokinetics, pharmacodynamics, NMDAR subunit selectivity, and behavioural experiments to better conclude whether 2-oxo-PCE shares similar pharmacodynamic and pharmacokinetic profile to ketamine and whether this is sufficient for the treatment of depression.

4.6 Future experiments

This study thus far provided a framework of 2-oxo-PCE characterization in the brain. However, additional studies are recommended to advance the understanding of the effects of 2-oxo-PCE in the brain and the potential relevance for the treatment of depression. The critical experiments needed to fully characterize 2-oxo-PCE in the brain are listed below:

1. Receptor binding assay experiments are necessary to determine the binding affinity at the NMDAR receptor in comparison to established NMDAR antagonists e.g. ketamine. 2-oxo-PCE did not show 100% NMDAR-fEPSP inhibition in CA1 hippocampal slices. Also, it is assumed that dissociative and therapeutic effects of dissociative drugs are primarily mediated by NMDAR blockade whereas other receptors may also have an effect (Lodge and Mercier, 2015). Ketamine has been shown to have affinity to other receptors in the CNS. Thus, it is worth investigating 2-oxo-PCE binding affinities for additional CNS receptors using the national institute of mental health psychoactive drug screening program assay protocol (NIMH PDSP) (Roth, 2018) to assess off-target inhibitory effects of other non-NMDARs.

2. Ketamine and other NMDAR antagonists have been shown to have a voltage dependent block of NMDARs e.g. ephenidine. MK-801, and memantine (Macdonald et al., 1987; Davies et al., 1988; Kang et al., 2017; Wong et al., 1986; Chen et al., 1992; Frankiewicz et al., 1996). This paper revealed that voltage dependency inhibition of NMDARs has been linked to therapeutic relevance (Frankiewicz et al., 1996). In order to better characterise 2-oxo-PCE and know whether it resembles the profile of ketamine, it is critical to investigate this. In vitro whole cell patch clamp experiment recordings in CA1 pyramidal cell could be conducted to conclude whether 2-oxo-PCE is a voltage-dependant NMDAR antagonist. The effects of 10μM and 30μM 2-oxo-PCE will be measured on NMDAR mediated excitatory postsynaptic currents (NMDAR-EPSCs) in CA1 Pyramidal cells by clamping the voltage at 10mV steps between -60mV to +60mV and stimulate the Schaffer collateral pathway at 0.1Hz. NMDAR-EPSCs will be isolated by adding 10μM NBQX, 50mM picrotoxin and 20mM bicuculine to the aCSF. Mg^{2+} ions will not be present in the aCSF when 10μM 2-oxo-PCE will be added. The same protocol will be replicated in the control experiment but in the absence of 2-oxo-PCE.

3. The experiments thus far do not provide us information on which NMDAR subtype 2-oxo-PCE is binding/selective to. We can confirm this using cell lines to express specific NMDAR subunits using human embryonic cells (HEK-293) cells. From this perform whole cell patch clamp recordings to measure 2-oxo-PCE on NMDAR-EPSCs. Enhanced Green Fluorescent Protein (eGFP) will be used and is a visual marker for the transfection. Alternatively use a specific NMDAR subtype knockout (KO) in mice to also assess 2-oxo-PCE NMDAR subtype selectivity.

4. In vivo behavioural experiments to measure 2-oxo-PCE antidepressant effects and determine the efficacy via forced swim, tail suspension as simple behavioural screening tests but most importantly, learned helplessness test, a better model of depression. Also perform In vivo pre-pulse inhibition (PPI) test in rodents, to test for dissociative side effects. PPI measure sensorimotor gating and previous dissociative

NMDAR antagonists ketamine and PCP have shown to negatively affect the sensorimotor gating (Geyer et al., 2001; Halberstadt et al., 2016). A previous paper has suggested that NMDAR antagonist dissociative effects are associated with decrease in subcortical gating and subsequently causing a sensory overload (Vollenweider and Geyer, 2001). Furthermore, decreases in PPI have been shown to negatively affect the sensorimotor gating. Thus, the dissociative effects of these known NMDAR antagonists could potentially be caused by the same information processing deficit causing the PPI disruption (Wallach et al., 2019). Therefore, it is crucial to assess 2-oxo-PCE effects on sensorimotor gating via PPI and compare the effects to ketamine.

4.7 Limitations

The time frame of the experimental protocol relating to the fEPSP experiments was a limitation for this study. It was not possible for 0.1µM, 1µM and 3µM 2-oxo-PCE to reach plateau in that time window based on its slow kinetic profile. Thus, the 0.1µM, 1µM and 3µM measured level of depression cannot be certain and becomes more difficult in predicting the potency. It might be favourable to extend the time course of the protocol from 3 hours to 6 hours in the aCSF perfusion for better judgement on potency relating to these specific concentrations and improve our understanding of the pharmacology of 2-oxo-PCE.

5 Conclusion

Although 2-oxo-PCE exhibits a similar chemical structure to ketamine, the effects of this novel drug on the brain are currently unknown. This study was set out to pharmacologically characterise 2-oxo-PCE by investigating the effects on NMDAR-mediated synaptic transmission and plasticity. Using in vitro electrophysiological field recordings in the CA1 region of rat hippocampal slices, I have shown that 2-oxo-PCE more rapidly and more potently reduces NMDAR-mediated synaptic transmission than ketamine in a dose-dependent manner. Furthermore, 2-oxo-PCE affected the induction of LTP and LTD in CA1 hippocampal slices, without affecting AMPAR-mediated synaptic transmission. Thus, 2-oxo-PCE is a faster and more potently acting antagonist than the selective NMDAR antagonist ketamine. Based on the small level of variation in all the data obtained (i.e. small SEM), these data are valid and credible.

This study may be of assistance to future experiments to ultimately investigate therapeutic potential in the treatment of depression. Particularly, binding affinity experiments alongside behavioural experiments could fully elucidate our understanding of the effects of 2-oxo-PCE in the brain better.

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