FACULTY OF MEDICINE AND HEALTH SCIENCES

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in the Biomedical Sciences

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Voorwoord

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SAMENVATTING

ACHTERGROND

Temporale kwab epilepsie(TKE) wordt gekarakteriseerd door een onevenwicht in excitatoire en inhibitoire neuronale activiteit met buitensporige elektrische ontladingen als gevolg. Ongeveer 1/3 van de patiënten is niet gevoelig voor klassieke anti-epileptica, dus is er nood aan nieuwe behandelingsmethoden. In dit onderzoek werd gebruik gemaakt van Designer Receptor Exclusively Activated by Designer Drugs(DREADDs) om aanvallen te onderdrukken. De hM4Di-receptor wordt geëxpresseerd in CaMKIIα-excitatoire neuronen van de hippocampus. Activatie van deze receptor, door middel van clozapine en clozapine-N-oxide(CNO), kan mogelijks via inhibitie van excitatoire neuronen aanvallen voorkomen. Modulatie van deze neuronen kan echter interfereren met hippocampale functies. Naast het anti-epileptisch effect van DREADD-activatie, wordt er getracht om mogelijkse effecten van clozapine en clozapine-N-oxide(CNO) op gedrag na te gaan.

METHODEN

Intrahippocampaal kainaat-injectie(IHKA) is een geverifieerd model voor TKE bij muizen. Het anti-epileptisch effect van hM4Di-activatie is onderzocht in muizen met meerdere epileptische ontladingen per uur. Muizen met minder epileptische ontladingen zijn benut geweest om gedragseffecten te evalueren. Spatieel geheugen is bestudeerd aan de hand van de novel object location test(NOL). Angst en locomotie zijn geëvalueerd in de open field test (OFT).

RESULTATEN

Zowel clozapine als CNO kunnen via hM4Di-activatie een daling in totale aanvalsduur veroorzaken. Bovendien werden geen specifieke neveneffecten (a.g.v. hippocampale inhibitie), noch aspecifieke effecten (off-target binding) van clozapine en CNO met betrekking tot gedrag geobserveerd bij IHKA-muizen.

CONCLUSIE

Een daling in totale aanvalsduur is mogelijk via inhibitie van excitatoire neuronen in de hippocampus. Bovendien hebben clozapine en CNO geen invloed op het resultaat van gedragstesten.

SUMMARY

BACKGROUND

Temporal Lobe Epilepsy(TLE) is characterized by a disbalance in excitatory and inhibitory neuronal activity, resulting in excessive electrical discharges. Approximately one out of three patients is insensitive to classic anti-epileptic drugs(AED), thus there is a pressing need for novel therapies to target TLE. In this research, Designer Receptor Exclusively Activated by Designer Drugs (DREADD)-technology is employed to suppress seizures. Activation of the hM4Di-receptor, expressed in CaMKIIα-excitatory neurons of the hippocampus, might be able to silence excitatory neurons and subsequently suppress seizures. However, modulation of hippocampal neurons might interfere with hippocampal functioning. Besides evaluating the anti-epileptic effect of DREADD-activation, it is also aimed to evaluate potential behavioural effects of clozapine and clozapine-N-oxide(CNO), ligands used for activation of the hM4Di -receptor.

METHODS

Intrahippocampal kainic acid(IHKA) is a verified model for TLE in mice. The anti-epileptic effect of hM4Di-activation was assessed in mice with high-frequent epileptic activity. Mice with low-frequent epileptic activity were used to evaluate behavioural effects. The novel object location test (NOL) was executed to examine spatial memory, while anxiety and locomotion was tested in the open field test (OFT).

RESULTS

Both clozapine and CNO are able to diminish the total seizure-duration by inhibiting excitatory hippocampal neurons. Additionally, no specific effects (hippocampal inhibition) or non-specific effects (off-target binding) of clozapine and CNO concerning behaviour are observed in IHKA-mice.

CONCLUSION

DREADD-mediated inhibition of excitatory neurons in the hippocampus is capable of reducing the total seizure-duration. Furthermore, no behavioural effects of clozapine and CNO are observed in epileptic-mice.

1 INTRODUCTION

1.1 DEFINITION OF EPILEPSY

Approximately 1% of the population worldwide suffers from epilepsy [1]. Epilepsy is defined as a neurologic disorder characterized by recurrent unprovoked epileptic insults. According to ILAE patients can be diagnosed with epilepsy if the patient suffered from two or more spontaneous seizures more than 24h apart or if *an* unprovoked seizure *has taken place and there is a probability of 60% or higher that in the following 10 years a seizure will recur*. Seizures can elicit impaired consciousness, strange sensitizations, automatisms and have an impact on the cognitive, psychological and social behaviour of the patient [2].

Epilepsy is characterized by abnormal and excessive discharges, which might be due to an increase in excitatory or a decrease in inhibitory neuronal activity. This disbalance results in synchronous and excessive signalling of neuronal clusters. If the epileptic onset is restricted to a discrete area within a hemisphere, the seizure is classified as focal (60%). In generalized seizures (40%) abnormal electrical activity is widespread throughout the brain. Known examples of the latter are tonic-clonic seizures, absence epilepsy, and myoclonic seizures [3,4].

1.2 EPILEPTOGENESIS

Epileptogenesis is an insidious and gradual process characterized by molecular and cellular alterations in the brain after a stressful event has taken place. This phenomenon makes the brain more susceptible to aberrant neuronal functioning and contributes to the transformation from a healthy into an epileptiform network.

Initially, the occurrence of a stressful event, for example trauma or infection, induces inflammatory reactions and immediate neuronal cell loss. The acute phase is followed by a latent period which slowly facilitates the formation of a hyperexcitable network. The latent period is characterized by structural and functional alterations such as neurodegeneration, mossy fiber sprouting, neuro-inflammation, gliosis, neurogenesis, and alterations in the permeability of the blood brain barrier (BBB). In some patients chronic epilepsy is established, characterized by frequent seizure activity. Epileptogenesis is not limited to the latent period. Neuronal changes continue to happen even after the appearance of recurrent spontaneous seizures and is part of the disease progression. Hence epileptogenesis is a dynamic yet subtle process [4–6].

1.2.1 Temporal lobe epilepsy

For approximately 20% of all patients who suffer from epilepsy, the temporal lobe is the zone of onset. The hippocampus is a deep structure within the temporal lobe that consists of a consecutive trisynaptic excitatory organization that is highly regulated by various negative feedback loops to prevent hyperexcitation[4].

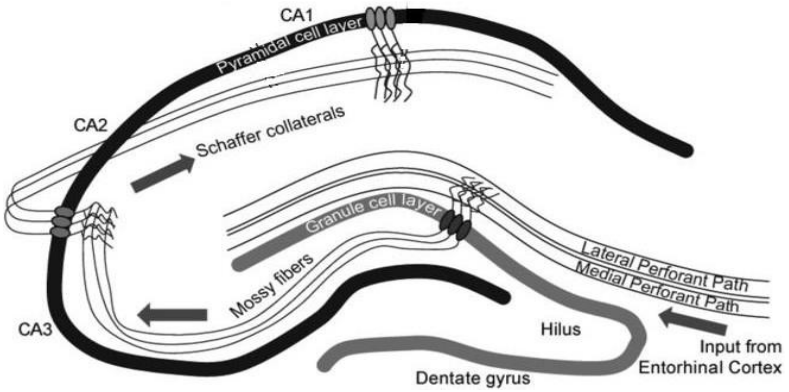

***Figure I* Organization of the hippocampus in mice.** Entorhinal cortex connects to the granule cells of the dentate gyrus. The axons of granule cells, respectively the mossy fibers, project to pyramidal neurons of the CA3 region which subsequently connects with the pyramidal cell layer of CA1 via Schaffer collaterals. Figure adopted from [7].

The anatomical structure and corresponding connections of the hippocampus were thoroughly investigated. The granule cells of the dentate gyrus receive input from the entorhinal cortex. Axons of the granule cells project to the CA3 region which subsequently connects to pyramidal neurons of the CA1 region in a glutamatergic manner (figure I). Axons of the CA1 pass the signal on to other extrahippocampal structures. This consecutive glutamatergic pathway is referred to as the trisynaptic excitatory formation.

Besides glutamatergic excitatory connections, negative feedback loops formed by GABA-ergic interneurons are also present, as depicted in figure II. Furthermore, granule cells have an additional pathway to prevent hyperexcitation. Granule cells connect in a bidirectional glutamatergic manner with mossy cells. These neurons make excitatory synapses with basket cells that eventually project back to the dentate gyrus in a GABA-ergic manner. Thus basket cells are also able to block excitatory activity of granule cells [8,9]. A schematic overview of hippocampal synapses is provided in figure II below.

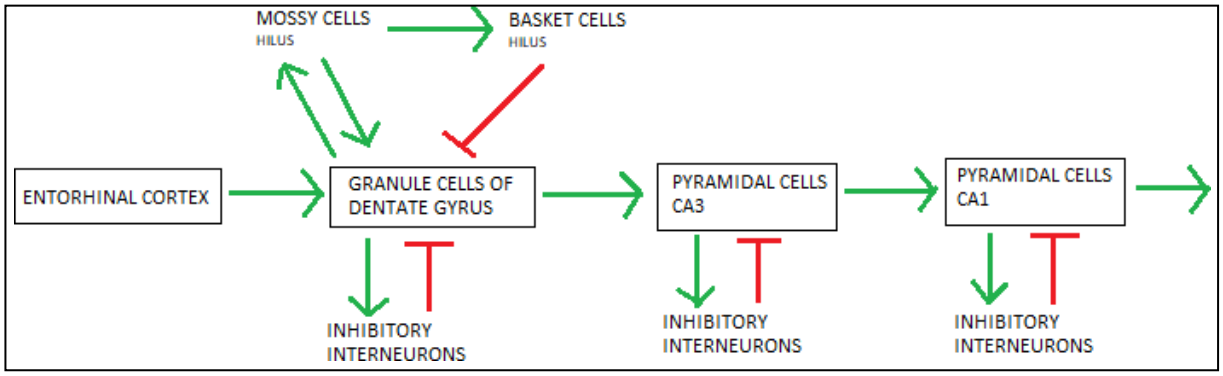

Figure II: **Schematic overview of hippocampal synapses in a healthy subject.** The entorhinal cortex provides excitatory input for the granule cells which subsequently project to neurons of CA3 and CA1. Granule cells and pyramidal cells of CA3 and CA1 are under control of a bidirectional negative feedback loop. The additional feedback loop of the granule cells is also illustrated. Excitatory synapses (Glutamate) are indicated with a green arrow, inhibitory synapses (GABA) are indicated in red. Figure based upon [8,9]**.**

A hallmark of TLE is hippocampal sclerosis and neuronal death in the hilar region, which consists of both excitatory mossy cells and inhibitory neurons. Mossy cells are extremely susceptible to excitotoxicity as they can receive excitatory input of 30 granule cells. This might explain why mainly loss of this cell population is observed. Three theories are proposed for the explanation of hyperexcitability in patients with TLE (figure III).

The first hypothesis suggests that a decline of mossy cells results in mossy fiber sprouting. Under normal circumstances mossy fibers or axons of the granule cells project to the mossy cells of the hilar region. Due to loss of mossy cells, the mossy fibers sprout and connect with surrounding neurons, even with other granule cells resulting in a self-stimulating positive feedback loop. However, this hypothesis is based upon indirect evidence. Not only is it uncertain whether mossy cell loss causes mossy fiber sprouting, loss of hilar interneurons might be involved as well. Furthermore, there are also indications that hyperexcitability can occur without sprouting.

The second theory is referred to as the dormant basket cell hypothesis. As a result of absent innervations of the basket cell, due to mossy cell loss, inhibitory basket cells become dormant. A part of the inhibitory feedback loop fails to control excitatory synapses, which makes the hippocampal circuit more prone to hyperexcitation.

Lastly, the irritable mossy cell hypothesis considers the relation between surviving mossy cells and hyperexcitability. After a decrease in mossy cells, the surviving mossy cells conceivably adjust their activity and become more responsive to stimuli [8].

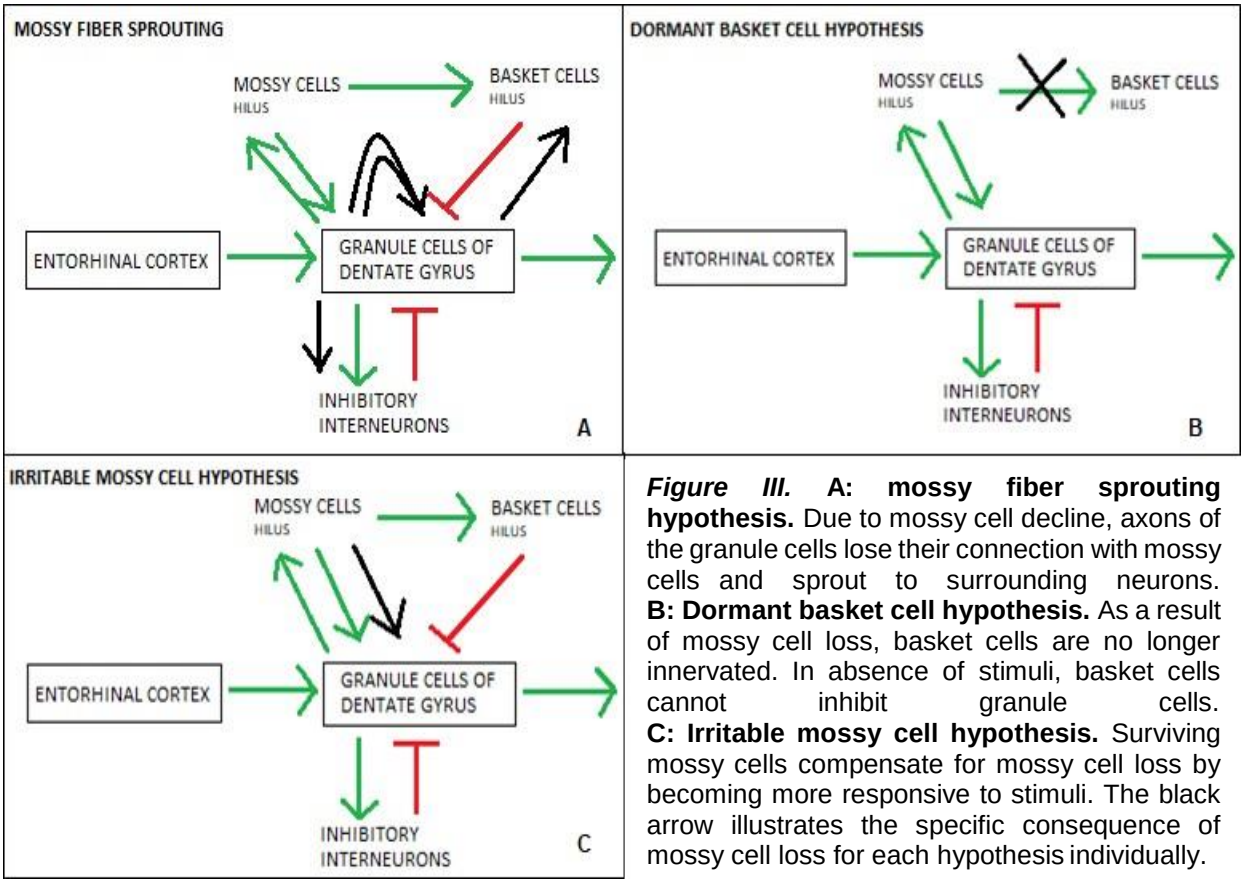

Figure III. **A: mossy fiber sprouting hypothesis.** Due to mossy cell decline, axons of the granule cells lose their connection with mossy cells and sprout to surrounding neurons. **B: Dormant basket cell hypothesis.** As a result of mossy cell loss, basket cells are no longer innervated. In absence of stimuli, basket cells cannot inhibit granule cells. **C: Irritable mossy cell hypothesis.** Surviving mossy cells compensate for mossy cell loss by becoming more responsive to stimuli. The black arrow illustrates the specific consequence of mossy cell loss for each hypothesis individually.

1.3 ICTOGENESIS

It is important to distinguish the terms epileptogenesis and ictogenesis. Epileptogenesis, as described above, is a gradual process characterized by neuro-inflammation, gliosis, neurogenesis, etc. Epileptogenesis favours the occurrence of ictogenesis or seizure-generation by inducing subsequent alterations in ion voltage-gated channels (Na^+, K^+,Ca^{2+}), neurotransmitter-release, AMPA and NMDA- receptor activity etc. In some cases these changes are followed by a higher probability of sodium-voltage gated channels opening and subsequent opening of calcium-channels. Modulation of sodium- and calcium-influx might contribute to excessive electrical discharges [10,11].

Epileptic neurons, modified by the process of epileptogenesis, are more prone to generate a paroxysmal depolarizing shift (PDS). PDS is defined as a prolonged depolarization generating multiple action potentials at a high rate. In terms of a neuronal network, PDS in one neuron can generate synchronized PDS in adjacent neurons which can result in seizures. A subsequent hyperpolarization via Ca^{2+}-dependent K channel activation ends burst firing. PDS is the intracellular correlate of interictal spike, which can be measured with electroencephalopgraphy (EEG). Interictal spike activity is a trait of focal epilepsy as it is closely related to the onset of spontaneous seizures[12–14].

Epileptogenesis and ictogenesis are strongly associated. As illustrated above, epileptogenesis might facilitate the occurrence of electrical seizure activity. Vice versa, interictal spike activity stimulates the process of epileptogenesis. Combined burst firing of neurons in the dentate gyrus might interfere with the secretion of molecules that guide axons of the granule cells during mossy fiber sprouting. Besides, interictal spikes are able to potentiate synaptic communication, which facilitates burst-firing even more [13,15]. Hence, an appropriate treatment might be able to hamper progression of the disease.

1.4 TREATMENT

Prescription of anti-epileptic drugs (AED) is the traditional approach to suppress seizure. AED interferes with neurotransmitter release and ion-channel properties, albeit the exact mechanisms of action are often not fully understood. The class of drugs used depends on the type of seizures. Combination of multiple AED is regularly prescribed to patients for whom one type of AED is not sufficient. Approximately 66% of the patients are seizure-free as a result of pharmacological therapy. However, this implies that one third of the patients with epilepsy are refractory and do not benefit from classical therapies. Furthermore, AED is associated with negative side effects such as nausea, fatigue, cognitive impairments. Thus there is a pressing need to develop specific treatments. The list of AED that is discussed in this thesis is limited to commonly used AED for partial seizures. Other techniques to control seizures such as surgery and neuromodulation are also briefly discussed [11,16].

1.4.1 Drug treatment for partial seizures

The type of AED described in this section is commonly used as anti-convulsant in patients who suffer from partial seizures. Most AED aim to either increase synaptic inhibition, modulate voltage gated channels or decrease synaptic excitation. Both carbamazepine (Tegretol) and phenytoine (Dilantin) enhance the inactive steady-state of voltage-gated sodium channels, which complicates the influx of Na^+-ions, and thus prevents depolarization. Another example of AED for the treatment of partial seizures is valproic acid (Depakote) which is metabolized to valproate. The latter increases synaptic inhibition, presumably by facilitating GABA-receptor activity. Besides usage of first line drugs, alternative agents are prescribed to patients as well. This is however beyond the scope of this thesis [17].

1.4.2 Epilepsy surgery

It is estimated that 50-60% of the patients who suffer from TLE are suitable for epilepsy surgery. The type of epileptic surgery recommended is discussed for each individual patient. In resective surgery, the area responsible for the initiation of seizures is removed, whereas for disconnective surgery the nerve fibers are disrupted in order to prevent spread of epileptic activity. Prior to epilepsy surgery, the area involved in seizure-generation has to be determined. Anamnesis, video-EEG monitoring and neuro-visualization techniques (MRI, PET and SPECT) are part of the presurgical evaluation and contribute to identification of the zone to which seizures are confined. This region should be well delineated and must not include functional areas, as resection might lead to cognitive or physical impairment [18].

Approximately 66-70% of patients who underwent mesial temporal resective surgery in combination with AED remains seizure-free at short term follow-up (<5 years). The immediate post-operative success-rate is linked to the duration of the epilepsy. Patients who suffered from epilepsy for a longer period of time from are less likely to have a beneficial post-operative outcome, possibly due to secondary epileptogenesis. On the other hand, early remission after surgery is the only indication for long-term freedom of seizures [19,20] .

Disconnective surgery is mainly performed in patients with an epileptogenic zone localized in a functional area. Multiple subpial transsections include the disconnection of horizontal orientated fibers through which excessive neuronal activity spreads. Disconnection of the horizontal fibers hampers transcortical spreading of seizures with minimal impairment of neurological functioning. The purpose of disconnective surgery is reducing the frequency and severity, instead of rendering freedom of seizures in patients. Corpus callosotomy is another type of disconnective surgery, typically performed in patients who suffer from generalized seizures. Epilepsy surgery offers a valuable alternative for the treatment of refractory patients [19,20].

1.4.3 Neurostimulation

This technique can be used in non-responders to AED or patients that suffer from severe side effects. Neurostimulation is based upon application of electrical currents in order to prevent excessive neuronal signalling. Vagus Nerve Stimulation (VNS) and Deep Brain Stimulation (DBS) are types of neurostimulation that are frequently used to treat epilepsy. In VNS a stimulator is implanted under the clavicle of the patient. The stimulator is connected to a helical electrode that intermittently stimulates afferent fibers of the vagus nerve. The exact anti-epileptogenic effect is not fully understood, although it is suggested that anti-inflammatory effects might play a key role. Approximately one out of three patients experience a >50% reduction in seizure-frequency. Furthermore in 5-10% of patients long-term seizure freedom is

reported. Besides its application in refractory epilepsy, VNS is also used to treat depression, dementia and pain.

Another successful neurostimulation technique is DBS, which is even more invasive than VNS. Electrodes are implanted in deep structures of the brain. For example, the seizure-focus or the subthalamic nucleus, a structure involved in pacemaker activity of neuronal signalling. Electrical stimulation of these structures are capable of controlling epileptic seizures, whereas in a pilot study 70% of the patients had a >50 % reduction in seizure-frequency [21].

1.5 MOUSE MODEL FOR TLE

1.5.1 Intrahippocampal kainic acid (IHKA) model

Intrahippocampal injection of kainic acid (KA) is a verified model for epilepsy, more precisely, chronic TLE [22]. Immediately after KA injection, status epilepticus (SE) occurs. KA is an analogue of L-glutamate, which binds to ionotropic KA-receptors. KA-receptors are located at the presynaptic and postsynaptic site of the neuron and are highly expressed in the hippocampus. KA1R and KA2R, subunits with high affinity for KA, are mainly expressed in pyramidal cells of CA1 and CA3, therefore this region is more sensitive to KA-induced excitotoxicity [23]. Besides pyramidal cells of CA1 and CA3, neurodegeneration is also apparent in mossy cells of the hilus, cells within the dentate gyrus, and interneurons of CA1 and CA3. In the subsequent weeks, during the so-called ‘latent period’, a series of neuronal alteration takes place. For instance: Granule cell dispersion and hypertrophy, mossy fiber sprouting, reactive gliosis, reorganization and upregulation of GABA-A and AMPA receptors of granule cells of the dentate gyrus [4]. In the third stage spontaneous abnormal paroxysmal discharges are visible on EEG and hippocampal sclerosis is developed, corresponding to the pathogenesis of TLE-patients. Another important feature of the seizures in the KA-model is its nonreactivity towards AED such as carbamazepine, phenytoine, and valproic acid[24]. IHKA-injection is a suitable model for the preliminary evaluation of novel therapies targeting refractory TLE [24].

1.5.2 Pilocarpine model

Pilocarpine, a cholinergic agonist of the muscarinic receptors, is able to induce SE. The cellular and molecular changes induced by pilocarpine are similar to the KA-model: loss of neurons in the hippocampal region, particularly in the CA1 and CA3 region. Cell death has also spread to amygdala, olfactory cortex, thalamus, and layers 2, 3, and 5 of the neocortex. The spread is more extensive in comparison to KA-injection. In some experiments lithiumchloride is injected prior to pilocarpine injection for the development of more homogeneous seizures. This model differs from the IHKA-model as pilocarpine induces acute epileptic brain activity and EEG-

signal returns back to baseline 24h after pilocarpine-injection. The pilocarpine model is typically used to examine mechanisms of seizure-generation and propagation[4].

1.5.3 Kindling model

Usage of the kindling model allows to control the induction of seizures. Electrical currents are repeatedly applied to certain regions in the brain like the amygdala or the hippocampus. In response to electrical stimulation, an insult is evoked. The advantage of kindling in comparison to other models is the temporal control of seizure-induction. Kindling of limbic regions evokes complex partial seizures or generalized seizures, analogous to TLE in humans. Continuous application of electrical currents might result in neurodegeneration of the limbic area and a decline in seizure-treshold. Thus in a minority of the cases, spontaneous seizures might also emerge eventually. In conclusion, kindling represents an acute model for epilepsy [25].

1.6 CHEMOGENETICS

Chemogenetics is the genetic modification of proteins in such a way that these proteins are now able to interact with small chemical ligands in a dose-dependent manner. G-protein coupled receptors (GPCRs) are frequently the subject of chemogenetic engineering. There are many different chemogenetic techniques such as Receptor Activated Solely by Synthetic Ligands (RASSLs), allele specific activation of genetic encoded receptors etc. However, these techniques resulted in high basal activity and have a variable success ratio. In this thesis the use of Designer Receptor Exclusively Activated by Designer Drugs (DREADD) is discussed. A number of studies have extensively described DREADD-technology and its application in neuromodulation [26].

1.6.1 DREADDs

DREADDs are modified GPCRs that are not reactive to the native ligand, acetylcholine, but are solely activated by exogenous ligands, as summed up below. In the active configuration DREADDs are associated with heterotrimeric G-protein (Gα,β and γ subunit) or with β-arrestin. Both proteins have an impact on the downstream signalling-cascade. DREADDs are selected for their insensitivity to native ligands, low basal activity and high specificity for nanomolar concentrations of chemical molecules [26–28].

1.6.1.1 Excitatory DREADDs

Random mutagenesis of the human muscarinic receptor (hM3) led to the discovery of hM3Dq, the first DREADD. In presence of a potent ligand, hM3Dq is activated which initially results in stimulation of phospholipase C, an enzyme that promotes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in inositol trisphosphate (IP_3) and diacylglycerol. IP_3 diffuses through the cytosol and binds to IP_3- receptors of the smooth endoplasmic reticulum, with a subsequently release of Ca^{2+}-ions. This increase in intracellular Ca^{2+} facilitates depolarization of the cell and thus the net effect of hM3Dq- activity is enhancement of neuronal firing. Other types of Dq with a similar action are hM1Dq and hM5Dq [26,28,29].
The Gs- DREADD is an example of an excitatory DREADD with a slightly different mechanism of action. Gs-DREADD is a combination of hM3 and turkey erythrocyte β-adrenergic receptor. The signalling molecule Gα-olf induces cAMP-activation and thus enhances depolarization [28].

1.6.1.2 Inhibitory DREADDs

Besides enhancement of neuronal signalling, silencing is also possible. In this thesis, the hM4Di-receptor is employed. Activation of the hM4Di-receptor is followed by release of the Gβγ-subunit. This signalling complex promotes opening of G-protein inwardly rectifying potassium (GIRK) channels. An efflux of positively charged potassium ions causes hyperpolarization and thus synaptic inhibition. Apart from hyperpolarization, limitation of glutamate-release of presynaptic L2/3 neurons contributes to the synaptic silencing properties of hM4Di as well. Although hM4Di is mostly adopted, the use of KORD might quickly emerge since co-expression of hM3Dq and KORD can be achieved, thus both activation and inhibition of neuronal firing in a sole neuron might be obtained [28].

1.6.2 Ligands

KORD is activated by Salvorin B, whereas CNO is the prototype ligand used for activation of modified muscarinic receptors. Up until recently, CNO was considered inert, as no interaction with native receptors was observed. However, in humans and guinea pigs, CNO is back-metabolized into clozapine and N-desmethyl-clozapine (NDMC), which might interfere with native receptors in the central nervous system. The uptake of CNO is complicated by the P-glycoprotein-efflux pump, present in endothelial cells of BBB. Currently the penetration of the BBB has not been confirmed in vivo for CNO. The possibility that metabolites of CNO, rather than CNO itself might be involved in DREADD-activation has to be considered [30–32]. Gomez et al. have confirmed that clozapine manages to pass the (BBB) more easily and shows a higher affinity for DREADDs in comparison to CNO. Saturation binding experiments and

experiments carried out in rodents have also pointed out that clozapine binds preferentially to DREADD-receptors rather than CNO. Presumably, CNO is rapidly converted to clozapine and subsequently clozapine penetrates the BBB and binds to DREADD-receptors. Clozapine is 100 times more potent than CNO and might also bind endogenously expressed receptors in a dose-dependent way, therefore low concentrations are needed [30]. Small doses of clozapine (<0.1mg/kg) might be able to sufficiently activate hM4Di, although the minimal effective dose should be determined to avoid possible off-target binding issues. Clozapine is already approved for usage in humans as an antipsychotic, which facilitates the translational potential as its safety is already demonstrated. Other ligands that are able to activate DREADDs are compound 21 and perlapine. Similar to clozapine, Perlapine, a sedative used in Japan to treat insomnia, is also approved for human application. If the dose-response range is wide, this might be a possible novel prototype ligand for DREADD-activation as well [27,28,30].

1.6.3 DREADD-expression and visualisation

Hippocampal cells are transfected through injection of adeno-associated viral vector (serotype AAV 2/7) that carries a plasmid with the CaMKIIα-hM4Di-mCherry construct (figure IV). Transfection of neuronal cells can be obtained by using for example an adeno-associated virus (AAV), lentivirus, or herpes simplex virus. Toxicity, cloning capacity, stability, and specificity of transfection are just a few properties that have to be taken into account for the choice of viral transduction method. AAV is able to transfect both dividing and non-dividing cells. In the latter, the modified single stranded DNA long-term viral expression is achieved up to 2 years in rodents and 6 years in primates.

Since the viral DNA exists in an episomal form, the probability of insertional mutagenesis is significantly reduced. In this experiment AAV 2/7 is used. Gene-expression and viral replication is regulated by replication genes derived from AAV 2, whereas genes of AAV 7 codes for structural proteins of the viral capsid [33]. Gene-expression is largely driven by the type of promotor. For example: Human synapsin (hSyn) is a universal promotor which allows gene-expression in different types of neurons. By contrast, CaMKII is expressed in excitatory neurons of the brain. Triggering of excitable neurons induces an increase of intracellular Ca2+ and subsequent activation of calcium binding protein and calmodulin. In excitatory neurons, the latter activates Ca2+/CaM-activated protein kinases II (CaMKII). The α -subunit CaMKIIα is even more specific as it is only expressed in excitatory neurons of the forebrain. More precisely, if AAV, containing a CaMKIIα-promotor, is injected in the hippocampal region, the granule cells of the dentate gyrus and the pyramidal cells of the CA3 and CA1 region are mainly targeted. The ability to target specific cell-populations might be of great use in the search for therapeutic agents [34,35].

The fluorescent marker mCherry is fused to the hM4Di-receptor and thus allows visualization of DREADD-expression. This is an improvement in comparison with other proteins such as the HA-tag that only displays cells that are transfected, which does not guarantee with absolute certainty that DREADDs are expressed [36].

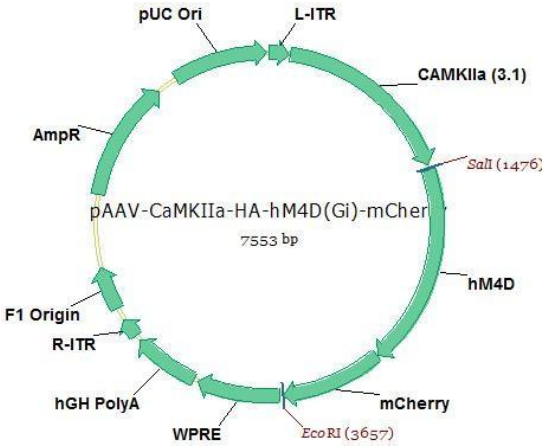

Figure IV: **AAV-CaMKII-hM4Di-mCherry plasmid.** AmpR is a reverse primer coding for the ampicilin resistence gene. The plasmid contains an origin of replication-site (Puc ORI). Multiple genes within ORI are mutated in order to prevent ligation and subsequent replication of the plasmid. The promotor and gene of which transcription is required lays within two inverted terminal repeats (ITR). The CaMKII-promotor controls expression of the hM4Di-receptor,which is only expressed in excitatory neurons of the forebrain. The gene mCherry is fused to the the hM4Di-gene thus is co-expressed with the DREADD. Between Sal1 (5' cloning site) and EcoR1 (3' cloning site) lays the Open Reading Frame (ORF). Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) is able to enhance expression of the transcribed gene. Human growth hormone polyadenylation signal sequence preludes the transcriptional termination. Finally F1 origin indicates the direction of (+) strand synthesis. Figure derived from [28] Bryan Roth ; Addgene plasmid#45548

1.7 FUNCTIONS OF THE HIPPOCAMPUS

1.7.1 Spatial memory

In light of memory formation there are three stages: encoding, consolidation and retrieval. The encoding process, which can be impaired due to lack of attention, consists of acquiring information. Afterwards, information is conserved and stored during consolidation and at last, memories can be actively recalled in the retrieval-process [37]. Short term memory or working memory is able to recollect information for minutes, during which protein-synthesis does not take place. Long term memory is capable of recalling information after a longer period of time, requiring de novo transcription and translation [38]. Several studies suggest the involvement of the hippocampus in spatial memory formation [37,39]. In rodents, hippocampal functioning, especially the dorsal CA1 region, can be tested by executing the novel object location (NOL), which implicates all three phases of memory-formation [37,38,40].

1.7.3 Locomotor behaviour

Locomotor behaviour is modulated on several levels: the motor cortex, basal ganglia, cerebellum, etc. The focus of this thesis is the engagement of the hippocampus in locomotor behaviour. Several studies state that the hippocampus is associated with running speed. This hypothesis is based upon the occurrence of theta-rhythm (5-12 Hz) in CA1 and CA3 neurons during locomotion. Theta oscillations of pyramidal cells presumably play a role in speed regulation. However the impact of excitatory hippocampal on the state of mobility is not fully established [41].

1.7.2 Anxiety

Anxiety is a reaction induced by the occurrence of a potential danger, which is evolutionary seen as an important function as the animal is alerted and inclined to avoid dangerous situations. Anxiety is characterized by an incertitude and an ambiguous feeling, for example the conflict between staying closely to the walls in a safe environment (avoidance) or giving in to the urge for exploration (approach). It is important to distinguish anxiety from fear, which is the reaction to a more threatening condition. For example in fear conditioning where a foot-shock is preceded by a tone, fear and subsequently freezing-behaviour is induced in presence of a tone [42]. There is gaining evidence that besides dorsal hippocampal involvement in spatial memory formation, the ventral regions of the hippocampus are involved in anxiety-related behaviour. Ventral regions of the hippocampus are closely linked to the prefrontal cortex, amygdala, bed nucleus, and other structures involved in the hypothalamic-pituitary-adrenocortical axis (HPA-axis), which plays an important role in the generation of a reaction in response to a stimulus. Some studies are suggesting that hippocampal lesions might result in reduction of anxiety-related behaviour. However, it remains rather difficult to assess the contribution of the ventral hippocampus in anxiety due to compensation mechanisms of other structures associated with the HPA-axis [43].

1.8 SOFTWARE ANALYSIS OF BEHAVIOURAL TESTS

Another important section of this thesis is devoted to the validation of Optimouse for the analysis of behavioural tests, in specific the open field test (OFT) and novel object location test (NOL). OptiMouse is a free available program written in Matlab R2017b. This software allows automatized nose-body-tail detection of rodents with an option for manual adjustment if needed. In order to validate automatic detection, video-files were manually corrected frame by frame and compared to the results of the automatic detection in Optimouse and to the automatic detection of EthoVision XT, a commercially available video-tracking technology that is frequently used to evaluate a wide range of behavioural tests [44].

2 RESEARCH QUESTIONS

2.1 ACUTE EFFECT OF HM4DI-ACTIVATION ON TOTAL SEIZURE DURATION IN FUNCTION OF TIME

The objective of this thesis is to investigate whether CNO and clozapine are capable of efficiently reducing the total seizure duration in IHKA mice by activation of the hM4Di-receptor, expressed in excitatory neurons of the hippocampus. The total seizure duration for baseline EEG-signals of epileptic mice are compared to the total duration of seizures 1-8h after treatment, 9-16h after treatment, and 17-24h after treatment. Besides evaluating the effect of silencing excitatory neurons on total seizure duration, it was also to identify the most potent treatment.

2.2 SPECIFIC SIDE EFFECTS OF CLOZAPINE/CNO IN IHKA MOUSE MODEL FOR TLE

The hippocampus is mainly known for its role in spatial memory but it is also involved in anxiety and locomotor behaviour. DREADD-mediated inhibition of hippocampal neurons might hamper functions of the hippocampus. Behavioural effects of vehicle, clozapine and CNO-injections on spatial memory functioning, anxiety and locomotor behaviour are compared within DREADD-expressing mice. The open field test (OFT) is used to assess anxiety and locomotion behaviour, while spatial memory functioning is evaluated in the NOL.

2.3 ASPECIFIC SIDE EFFECTS OF TREATMENT IN IHKA MOUSE MODEL FOR TLE

Besides evaluating specific effects of hippocampal neuromodulation, potential off-target effects of drugs should be assessed as well. As previously mentioned, clozapine is a drug with dose-dependent effects. Besides its antipsychotic activity, it might also induce drowsiness, hypotension, etc. depending on the dosage. Due to back-metabolization of CNO to clozapine, it is unclear if CNO causes analogous side effects [28,45]. In this thesis possible behavioural impairments of CNO and clozapine application, in doses strong enough to activate DREADDs, are evaluated in sham-mice.

2.4 SOFTWARE COMPARISON FOR ANALYSIS OF BEHAVIOURAL TESTS

For the evaluation of automatic detection of body- and nose-points in Optimouse and Ethovision, the results are compared to manual frame by frame corrections of the same files in Optimouse both for OFT (n=13) and NOL(n=8).

3 MATERIALS AND METHODS

3.1 ANIMALS

Male C57BL/6jax mice (n=30; Envigo, UK) are used. Mice are allowed to recover from stress due to transportation and to adapt to the novel environment 1 week prior to KA-surgery. They are single housed at the time of the experiments and are kept under controlled conditions (temperature: 20-24°C; humidity: 45-65%; 12 hours light-dark cycle with lights on at 6.00 A.M; food and water provided ad libitum).

3.2 EXPERIMENTAL DESIGN

A schematic overview of the experimental design is provided in figure V. Furthermore each topic is chronologically discussed in the sections below.

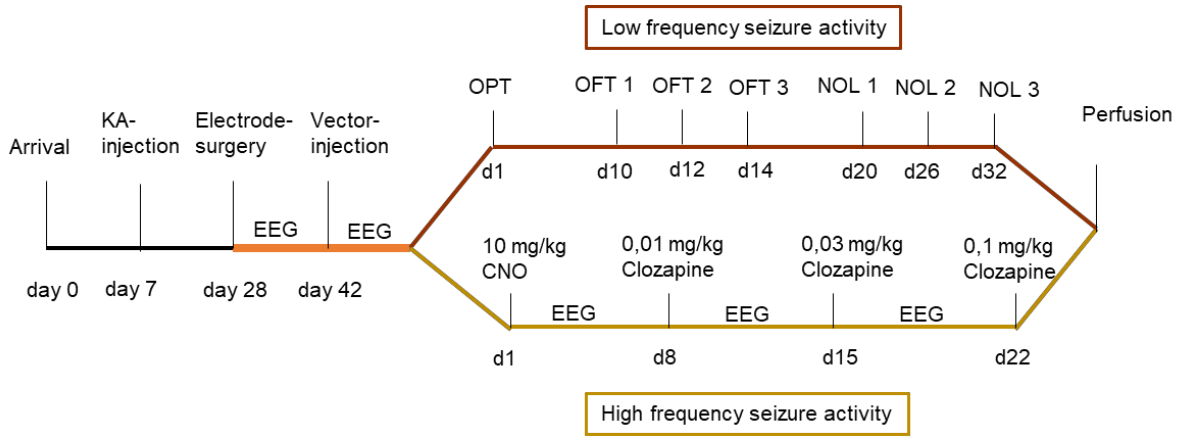

Figure V: **Timeline of the experimental design.** 7 Days after arrival in the laboratory KA-injection is executed. In the following 3 weeks, some mice developed recurrent spontaneous seizures. Electrodes and a cannula are implanted to measure brain activity (start EEG-recordings: orange). After they have recuperated from electrode- implantation surgery mice are injected through the cannula with either a DREADD- or sham-vector. Based upon EEG-signals the mice are divided in groups according to seizure-activity frequency: low frequency (brown) or high frequency (yellow). The latter are needed to evaluate the effect of different treatments on seizure-activity. Animals with low frequency of seizure-activity are used to perform behavioural tests under influence of drugs. The following behavioural tests are carried out: Object preference test (OPT), open field test (OFT) and novel object location (NOL). At the end of the experiment, mice are transcardially perfused.

3.2 INTRAHIPPOCAMPAL KAINIC ACID SURGERY

In order to create a mouse model for TLE, kainic acid (Tocris Bioscience, USA) is injected in the dorsolateral right hippocampus. KA resembles L- glutamate and causes hyperexcitation of neurons. After KA injection, status epilepticus is induced and recurrent epileptic seizures occur. In the following weeks epileptogenesis takes place and mice develop epilepsy-like symptoms.

Before each surgery surgical instruments are disinfected. The Hamilton syringe is cleansed five times with Hamilton cleaning solution, five times with distilled water and once with 70% ethanol.

Anaesthesia is induced in mice using 5% Isoflurane (IsoFlu®, Abbott, Belgium) and 8l/min oxygen until the animal is sedated. The mouse is placed in the stereotactic frame and anaesthetics is lowered to ±1.5% Isoflurane and 1l/min oxygen.

The head is shaved with a razor blade and Isobetadine (MedaPharma, Switzerland) is subsequently employed to disinfect the skull. In order to protect the eyes against beams shining directly in the eyes, eye ointment (Duratears®, Alcon-Couvreur, Belgium) is applied and a bandage is used to cover the eyes. In addition, a heat lamp is needed to prevent hyperthermia. Next, an incision is made from cranial to caudal at the dorsal side of the head, the skull is cleaned with cotton wipes and aceton is used to improve the visibility of the sutures. The coordinates of bregma, the intersection of the coronal and sagittal suture, are read out and allows to determine the coordinates of the dorsolateral right hippocampus (-0.2 cm AP; -0.15 cm ML and -0.18 cm DV relative to bregma [46]).

A hole is drilled through the skull at the intersect of -0.2 cm AP;-0.15 cm ML. Prior to injecting KA in the hippocampus, the Hamilton 7000 1 µl syringe is filled with KA and tested to make sure it releases fluid when needed. Next, the needle is placed against the dura and slowly lowered 1.8 mm until it reaches the hippocampus. Each animal is injected with 50 nl of a 4 mg/ml KA solution in the hippocampus at a rate of 100 nl/min. Five minutes after injection the syringe is slowly removed to prevent spillage of KA. The incision is carefully sewed up and a disinfecticia (Neobacitracine®, Erfa, Belgium) and analgetica (Xylocaine®, AstraZeneca, Belgium) are applied. Mice are regularly checked after surgery.

3.3 ELECTRODE AND CANNULA IMPLANTATION SURGERY

In some cases the occurrence of spontaneous recurrent seizures is established. Electrodes, which are used to visualize brain activity, are handmade (Fig. I in addendum). For fabrication of bipolar depth electrodes stainless steel wire with a polyimide coating (California Fine Wire Company, USA) was used. Scalp electrodes were made with isolated, copper wire (Violet 28 Gauge Solid, Kynar Electronic; Marvac Electronics) and attached to the screw (head Ø 2.5 mm; shaft Ø 1.57 mm; shaft length 2.4 mm, Plastics One, USA).

Mice are sedated with Isoflurane and placed in the stereotactic frame, similar to the previous surgery. Each mouse receives a subcutaneous injection of 0.25ml Temgesic (0.003 mg/ml Temgesic ®, Reckitt Benckiser, UK), a supplementary analgesic. After shaving, Isobetadine is applied to the skull and the eyes are protected with Duratears. A cut is made from cranial to caudal and acetone is used to improve the visibility of bregma.

Figure VI provides an overview of the location of electrodes and screws. The cannula (ID 0.32 mm; OD 0.64mm; Ø pedestal 3,5 mm; Plastics One USA) and bipolar depth-electrode are attached to each other during the fabrication and simultaneously implanted. A supplementary figure of the cannula and electrode-implantation in the coronal-plane is attached in the addendum (figure 2). After implantation of electrodes and screws, the depth electrode, reference scalp, and the ground scalp are attached to the connector, which is fixated with UV-cement and dental cement to the skull. A volume of 0.1 ml of a 0.2 mg/ml Metacam solution (Metacam®, Boehringer, Ingelheim), a non-steroidal anti-inflammation drug (NSAID), is i.p. injected if the surgery is terminated.

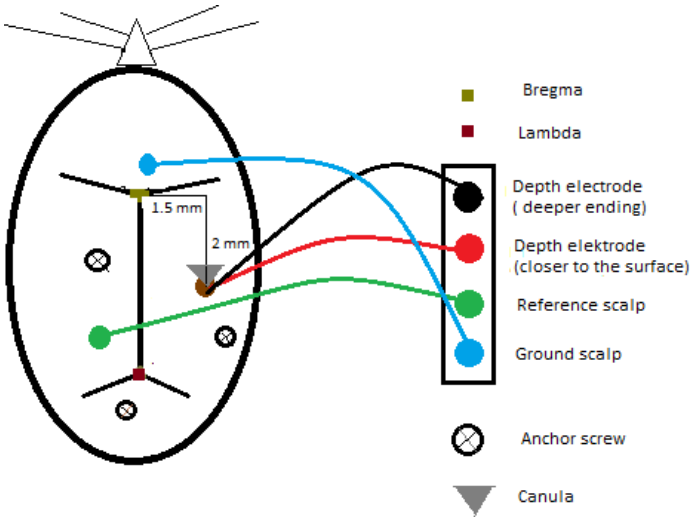

Figure VI: **Overview of electrode- and screw placement.** The ground scalp, which is placed anterior, reduces noise by cancelling out artefacts such as chewing. The reference scalp measures baseline activity in the contralateral hemisphere. The depth-electrode and cannula are located respectively at (-0.2 cm AP, -0.15 cm ML, -0.18 cm DV) and (-0.2 cm AP, -0.15 cm ML, -0.03cm DV) in reference to bregma. The cannula is connected to an anchor screw using UV-cement, for reinforcement of the cannula. Other anchor screws make sure that the head-cap is properly attached to the skull.

3.4 VECTOR INJECTION

Animals are either injected with a viral DREADD-vector (4.66*10^13 viral genome copies/ml) containing a plasmid with a CaMKIIα-hM4Di-mCherry construct or injected with a sham vector (2.08*10^13 viral genome copies/ml) that codes for CaMKIIα-spacer-mCherry. All preparations as described in the KA-surgery are carried out. A small alteration is made to the cleansing of the Hamilton syringe. Prior to rinsing the syringe with the cleaning solution, it has to be rinsed 5 times with RBS (Sigma-Aldrich, Belgium) for the removal of biological residues such as viruses. The settings of the QSI are also slightly different: a volume of 500 nl is selected at a rate of 100 nl/min. The animal is sedated as described above and placed in the stereotactic frame. The needle is placed on the surface of the cannula and is slowly navigated towards the centre of the cannula. The vector has to be injected at 0.18 cm below the dura, which can be precisely executed as the length of the cannula (1.47 cm) and the place of implantation (-0.03

cm) is known (figure VII). The syringe is lowered 1.62 cm and subsequently 500 nl is injected. After five minutes the syringe is carefully pulled out the cannula and the dummy is placed on.

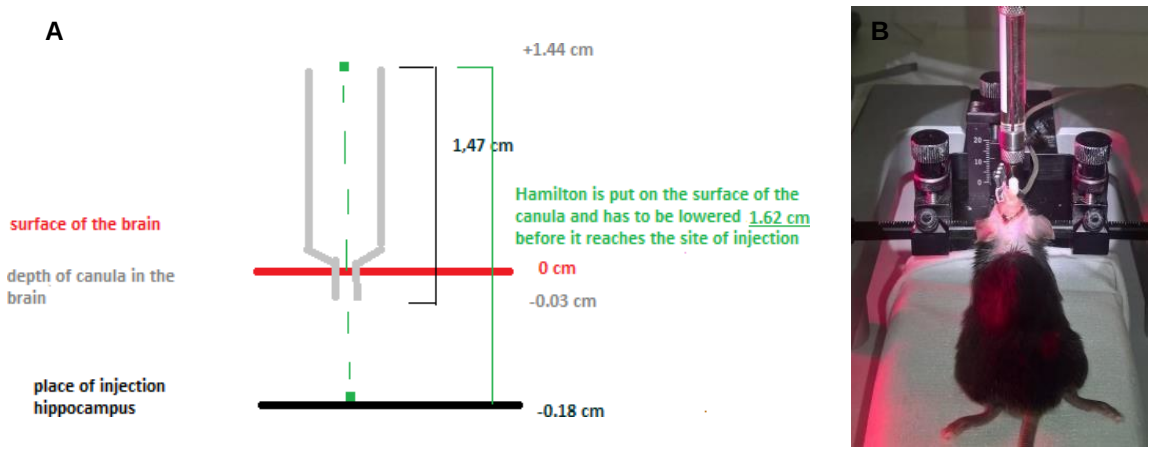

Figure VII: **Vector injection. A:Schematic visualization of the location of the cannula.** The ending of the cannula is located 0.03 cm below the dura. Because the length of the cannula is known to be 1.47 cm, it is possible to calculate the position of the cannula-top above the surface, respectively 1.44 cm (1.47 cm-0.03 cm). The desired injection place is situated 0.18 cm underneath the dura, which means that when the Hamilton syringe is placed at the top of the cannula it has to lower 1.62 cm(1.44 cm+ 0.18cm) for injection in the hippocampus. **B: Picture taken during vector injection.** The needle of the Hamilton syringe is lowered into the cannula. A volume of 500 nl vector is injected in the hippocampus at a rate of 100 nl/min.

3.5 BEHAVIOURAL TESTING

3.5.1 Object preference test (OPT)

The goal of this test is to evaluate the curiosity of mice towards different objects. In order to prevent confounding of the NOL-outcome by a certain preference or dislike for a specific object, items that are significant more are less appealing to mice in comparison to other objects are excluded from the NOL. Each item is evaluated at least 4 times in different mice (Table I in addendum). Predilection for a certain object was calculated by the formula below.

$$\text{Mean percentage exploration time} = \frac{\text{time spent exploring object 1}}{\text{total time spent exploring (object 1+ object 2)}} * 100$$

The object preference test is carried out in IHKA- mice (n=13) at day time. The testing cage (30 x 23 x 30 cm) is made of plexiglass and objects are placed approximately 3 cm away from the border. Prior to testing, green surgery paper is placed at the bottom of the cage in order to enhance contrast between the animal and the environment. Between each trial, the transportation cage, test cage, and objects are cleaned with a 70% ethanol solution and air dried for 10 minutes. All actions are carried out with gloves. An infrared camera has recorded all trials, which are manually analysed afterwards.

First, mice are habituated to the test cage for 10 minutes per day for three consecutive days without any objects present in the cage. After habituation, mice are placed in the test cage with

2 different objects for 5 minutes. During the first OPT it became clear that the objects were too small, as excessive climbing and sitting on top of objects was observed, which could confound results. Thus in the second OPT, the height of certain items were adjusted to +/- 10 cm. Interaction is defined as the time that the nose of the mouse is within 1 cm of the object. The exact setup and results for each object can be found in the addendum.

3.5.2 Open field test (OFT)

OFT is able to estimate behavioural alterations, such as locomotion and anxiety. The test is conducted in IHKA- mice (n=13) between 7.00 PM and 1.00 AM. OFT is performed in a PVC-cage (60 x 60 x 35 cm), which is fictively subdivided in 25 squares of which the inner 9 are defined as centre of the arena. Similar to the OPT, green surgery paper is placed at the bottom of the cage, every action is conducted with gloves and each cage is disinfected between trials. Mice are habituated to the test cage for 10 minutes per day for three consecutive days. One hour prior to execution of the OFT, mice are intraperitoneally (i.p.) injected with 10 mg/kg CNO, 0.1 mg/kg clozapine or vehicle in a randomized design (Table I; Table II in addendum). The OFT is conducted 3 times in total. To evaluate the effect of treatment on anxiety, only the first 5 minutes of the OFT is analysed, whereas locomotion is assessed throughout the entire OFT. Recordings are made with the infrared camera and are afterwards analysed with Ethovision XT software.

Table I: **Dose and concentration of treatment.** Overview of volumes and concentration used for i.p. injection of treatment in behavioural tests.

Treatment	Dose	Volume injected	Concentration
CNO	10 mg/kg	0.3 ml	1 mg CNO/ ml (2.5% DMSO; 97.5% saline)
Clozapine	0.1 mg/kg	0.3 ml	0.01 mg clozapine/ ml (2.5%DMSO; 97.5% saline)
Vehicle		0.3 ml	(2.5%DMSO; 97.5% saline)

3.5.3 Novel object location (NOL)

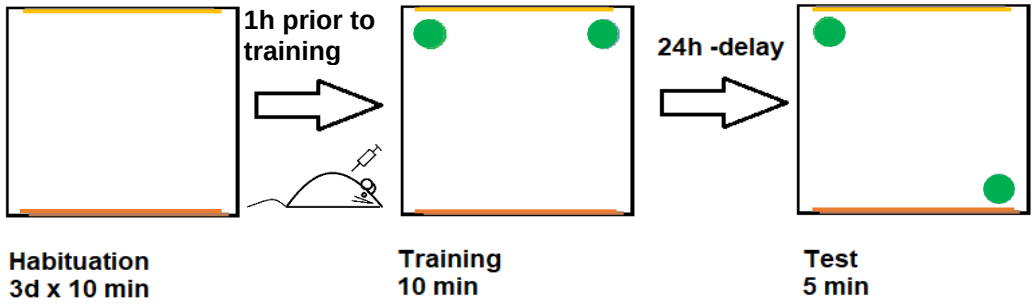

Figure VIII: **Schematic representation of NOL.** Mice are habituated three consecutive days in presence of visual stimuli (yellow and brown line). 1h prior to training, Mice received an i.p. injection. Subsequently mice are exposed for 10 minutes to 2 identical objects. The NOL test (5min) is conducted with a 24h delay, whereas 1 object is relocated to a different area in the cage.

The NOL is a validated test to evaluate spatial memory functioning of rodents. The test is conducted in IHKA-mice (n=13) between 7.00 PM and 1.30 AM. NOL is executed in a testing cage (30 x 23 x 30 cm) that consist of plexiglass. Similar to the OPT, green surgery paper was placed on the floor, cages and objects were disinfected and actions were carried out with gloves. The discrimination index (D.I.) calculates the ability of mice to distinguish the relocated object from the fixed object and is used as an indication for spatial-memory functioning.

$$D.I\ (\%) = \frac{\text{(time spent exploring relocated object- time spent exploring fixed object)}}{\text{(total time spent exploring objects)}} * 100$$

The setup of the experiment is visualized in Figure VIII. NOL consist of a habituation, training, and testing phase. One hour before training, mice are i.p. injected with clozapine (0.1 mg/kg), CNO (10 mg/kg) or vehicle, in a randomized design (Table II in addendum). Each mouse was subjected to the NOL three times with various objects, so different treatments could be compared within the same mouse, the exact set-up can be found in the addendum (Fig. III in addendum). Interaction, as defined in the section OPT, is assessed with Ethovision XT - software; automatic detection.

3.6 DATA-ANALYSIS

3.6.1 EEG

Prior to EEG-registration, the EEG-setup is calibrated. As described in the protocol of electrode surgery, the connector is fixed with UV and dental cement to the cranium. The connector is linked to a pre-amplifier, which lowers the impedance of the signal. The pre-amplifier is connected to the swivel with a cable, which allows animals to move freely whilst recording brain-activity. After a second amplification of the signal 510x, data is converted in a binary-code, digitalized with a data-acquisitation card (NI DAQ) and saved on a hard drive. Analysis of EEG-signals is possible in Neuron, a script of the MATLAB 2007b software. A doctoral student helped with the quantification of epileptic seizures.

3.6.2 Behavioural tests

There are different programs available for the analysis of behavioural tests. This thesis is limited to the comparison between ‘Optimouse’, freely accessible, and the commercially available Ethovision XT 11.5 (Noldus).

3.6.2.1. Optimouse

The objective of this program is accurate, automatic detection of nose-, tail- and body-positions in mice. Besides mice, detection of rats is also feasible, given that the test conditions, such as contrast, are optimized. Figure IX is an illustration of the Optimouse interface.

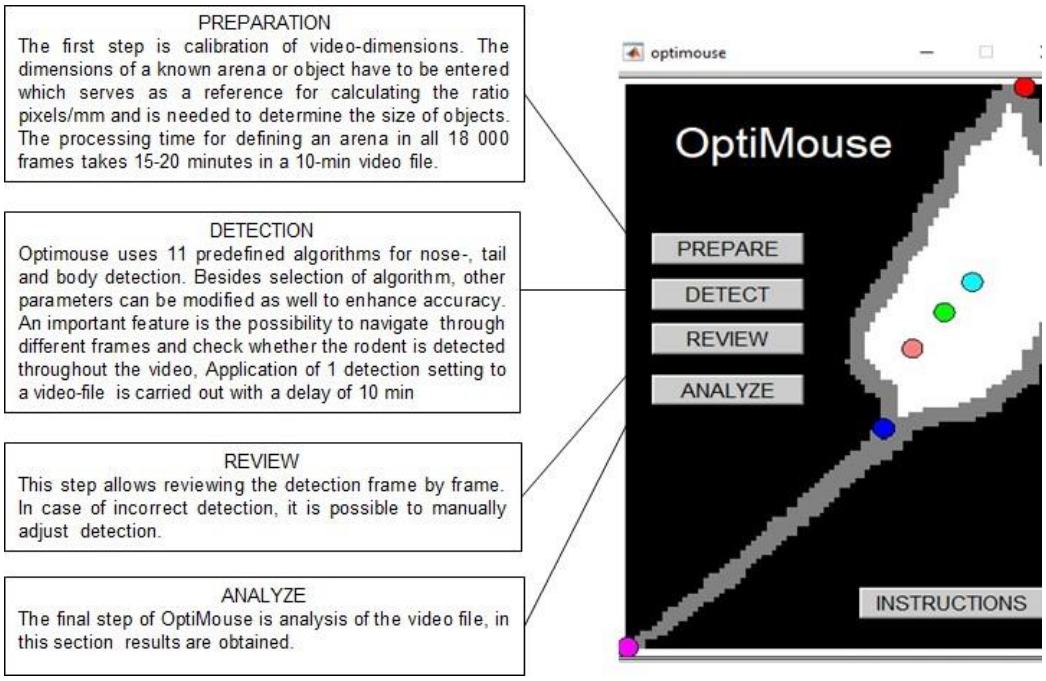

Figure IX: **Optimouse interface.** Overview of different stages needed to analyse video files.

DETECTION ALGORITHMS

The method of identifying the nose varies between different algorithms, although the body-point is calculated in the same way. The number of peeling cycles and binary images are used as input for the detection algorithms. When changing the algorithm, other parameters should be revisited as well. All algorithms are based upon the assumption that the rodent is the bulkiest mass in the arena.

TRESHOLD

The threshold is able to separate the rodent from the background. Large threshold values results in failure of detection, too small values however results in aberrant detection of rodents and confounding with objects. Each pixel is converted to a binary code (1: intensity > threshold; 0: intensity < threshold). Binary data is used as to apply detection settings to the video file.

PEELING

Nose-detection is far more difficult than body- and tail-detection. The intention of peeling is to erode pixels at the borders of the mouse until the tail, which is considered the thinnest part of the mouse, is eventually excluded. The difference between images with and without tail are

needed for correct identification of the tail. This is a very important step, as nose-detection is based on tail-detection. Excessive peeling results in confusion between nose and tail or complete detection failure (figure X)[44,47].

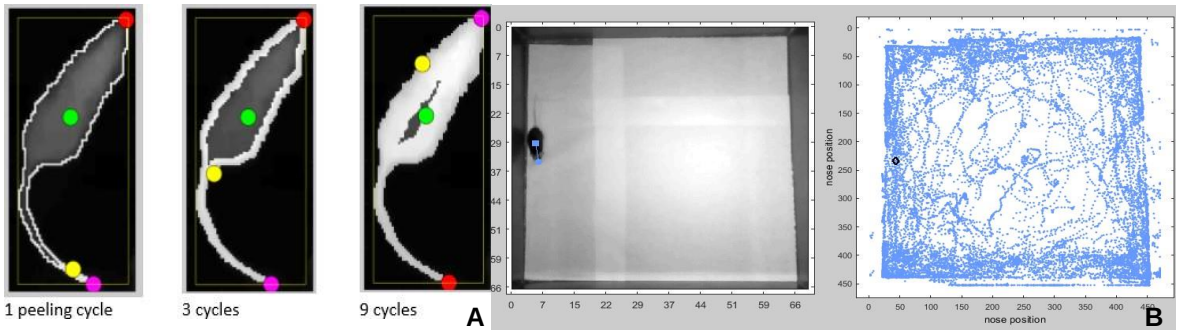

***Figure X*: Detection in Optimouse. A: Illustration of the effect of peeling cycles on nose-detection.** The difference between a mouse with and without tail forms the essence of nose-detection. The red circle indicates the nose-point as presumed by Optimouse. Nose-detection failed when peeling consists of 9 cycles. Albeit nose-detection is correct in both 1 and 3 peeling cycles, the preferred number of cycles is three. A peeling of 3 cycles shows a tail that is completely peeled away(indicated in white). Figure adapted from Optimouse user manual[44,47]. **B: Illustration of nose- and body-detection during the OFT.** Left: Correct identification of both nose- and body position (algorithm 7; threshold 1.1; peeling cycle 3). Right: overview of the followed path in OFT.

ANALYSIS

The final step is data-analysis. A quick overview of the options that can be analysed are provided below :

- Distance in function of time
- Tracks for body and nose-position
- Heatmap (body and nose positions)
- Body angle
- Speed
- Zone based analysis in function of time (body and nose positions)
- Automatic zone preference index (body and nose positions)

It is important to note that one specific detection setting does not yield flawless detection in all frames[44,47]. In this thesis 8 video-files of mice treated with vehicle performing the NOL are analysed in MATLAB with automatic detection (algorithm 7, threshold 1.1, and peeling cycle 3), afterwards with manual nose- correction in Optimouse and with Ethovision XT 11.5 (NOLDUS). For the OFT, the same detection settings were employed in all 13 files. Files were analysed automatically in Optimouse, analysed with Ethovision and also manually corrected for body-positions.

3.6.2.2 Ethovision

OVERVIEW OF DETECTION SETTINGS

A clear-cut contrast between the subject and the background is key for good automatic detection. In this case, use of the automated setup is appropriate, which involves delineating the animal with exclusion of its tail. It is recommended to check whether automatic detection is accurate throughout the whole arena. If an automated setup is not satisfactory, modulation of the advanced settings might help. Fig XI provides an overview of setting that can be modulated in order to improve detection.

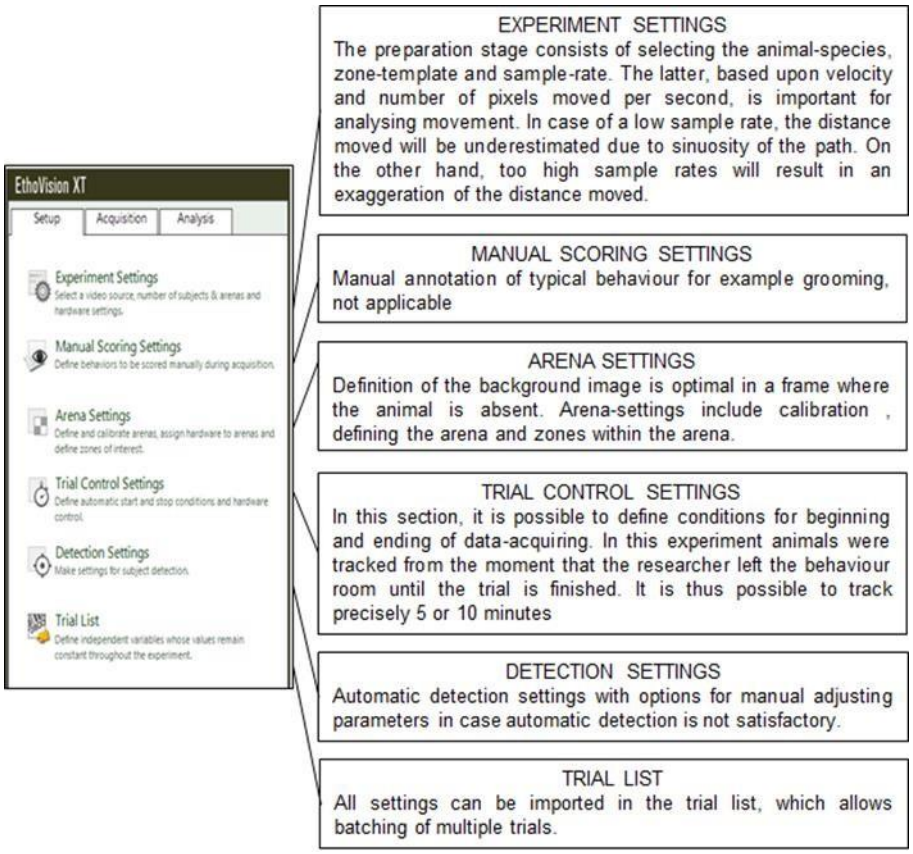

Figure XI: **Interface of Ethovision XT.** Overview of different stages needed to analyse video files.

BACKGROUND

Similar to Optimouse, alteration of background can enhance detection. Gray scaling is only advised if the contrast is good and lighting is stable. This method uses a range of grayscale values. White is assigned to the highest value (225) and black to the lowest (0), corresponding to the colour of the subject which allows detection of this subject. Static subtraction is used when there is an overlap between the grayscale values of the subject and the arena. In case of background changes, dynamic subtraction is advised.

DETECTION

The Shape based model XT4, is the oldest detection-algorithm that is supported and is inferior to the other detection models. If the subject is always fully visible, the use of Model based XT5-algorithm is encouraged. The Advanced based model is set as default and allows nose-detection even if the subject is partially visible (Fig XII)

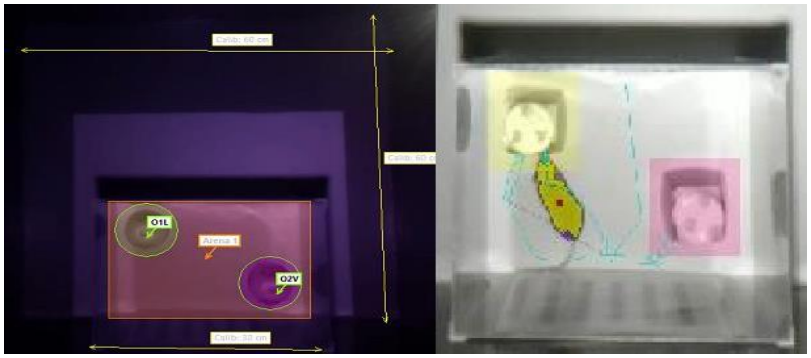

Figure XII: **Example of settings in Ethovision XT during NOL. Left: Illustration of arena settings.** Yellow arrows represent calibration lines of the open field cage (60 x 60 cm) and a side of the NOL-cage (30 cm), preferably a horizontal and a vertical line. The orange area is the NOL-arena. When the nose-point of the mouse enters the green or puple zone, this is defined as interaction. **Right: Illustration of detection settings applied in a video file.** The nose is indicated in blue, body-point is indicated in red and tail-base is indicated in purple. The dashed line is a representation of the temporarily followed path.

ACQUISITION AND ANALYSIS

Unfortunately, detection in the acquisition phase is slightly different than during the detection phase of analysis. This incoherence between detection and acquisition is very time-consuming. After acquiring data, it is possible to analyse different functions, as described below:

- Distance moved
- Velocity
- Movement (the duration for which the body point was changing location)
- Acceleration
- Time spent in zone + distance to zone (body and nose-positions)
- Angular velocity
- Mobility (the percentage of mobility, even when the body-point remains the same)
- Heatmaps

In this thesis, all 116 video-files are analysed with Ethovision XT 11.5 software. For the NOL, each arena setting was personalized as the zones differed in function of the object. For detection each file was annotated personally using an automated setup and changing advanced settings until detection was adequate during the preparation and was evaluated again during acquisition. Because only the body-point is needed for the OFT, one detection setting was applied for all videos. In some videos this setting failed, thus for these videos

settings were adjusted and personalized in order to achieve a good nose-detection after all. An automated setup was used in OFT and generated the model dynamic subtraction and model-based XT5 method. For data-analysis in OFT, the following dependent variables were selected: distance moved, velocity, time spent in centre, and time spent at the border. For NOL the same functions were evaluated except that time spent in centre or at the border is replaced by time spent in zone 1 and 2.

3.6.3 Statistical analysis

The Friedman test, a non-parametric variant of Repeated Measures One-way Analysis of Variance (ANOVA), with correction for multiple comparison (post-hoc Dunn test) is carried out in the majority of experiments. Use of other statistical tests is explicitly mentioned in the section Results.

For the evaluation of hM4Di-activation on total seizure duration in function of time the Friedman-test is used. Within each treatment, different time-periods are compared to each other, e.g. within CNO, baseline, 1-8h, 9-16h and 17-24 h after injection are compared to each other. Furthermore, the effect of different treatments is also evaluated within a time period. For example the effect of CNO and clozapine is compared within 1-8h after injection. Behavioural tests are carried out with Friedman test as well and subsequent post-hoc Dunn test, whereas the effect of clozapine, vehicle and CNO are assessed within the DREADD- or SHAM group. The results of the OPT are analysed with the non-parametric Kruskal Wallis, post-hoc Dunn tests, as data is not paired (Fig V and Table V in the addendum). In addition, the addendum (Table VI and fig VII in the addendum) contains also the comparison of vehicle vs. DREADD-activation (mean value of clozapine and CNO) for behavioural tests. The latter is analysed with the Wilcoxon matched-paired signed rank 2-sided non-parametric t-test.

To evaluate different softwares, the results of 13 saline-treated animals (SHAM+DREADD) were used. Anxiety is assessed with the Friedman-test; post-hoc Dunn, whereas data of distance travelled, velocity and D.I. followed a Gaussian-distribution and are analysed with Repeated Measures One-way ANOVA; post-hoc Bonferroni.

Data was analysed in the program Graphpad Prism. If applicable, data is shown as mean ± standard error of the mean (S.E.M.). Results are considered significant if $p<0.05$ and are indicated with '*'.

4 RESULTS

4.1. SEIZURES INDUCED BY IHKA-INJECTION

Mice (n = 30) received IHKA-injection at the age of 8 weeks. None of the animals died during surgery, however three animals were found dead a few days after surgery. The unnatural positions in which these mice were found allow to suspect that they died as a result of SE. Three weeks after IHKA-injection, electrodes are implanted to measure EEG-signals. In 48% (13/27) of the mice, rhythmic high voltage sharp waves and paroxysmal electrical discharges were frequently observed. Eventually only 8 mice remained in this group, since 3 mice broke their head cap and 2 mice died after SE. The group with high frequent epileptic activity was used to evaluate the anti-epileptic effect of hM4Di-activation, whereas the group with low frequency of seizures was used to assess potential side effects. One animal of the behavioural group was perfused after the head cap broke prior to testing and another animal died during a training session of the NOL after injection. To note, for the group with high frequent epileptic activity, only the results of DREADD-expressing mice are discussed (n = 4).

4.2 ACUTE EFFECT OF HM4DI-ACTIVATION ON TOTAL SEIZURE DURATION IN FUNCTION OF TIME

A decrease in total seizure duration was observed after i.p. injection of CNO and clozapine (figure XIII). Firstly, the effect between different treatments was evaluated within 1-8h after injection, 9-16h after injection, 17-24h after injection and for baseline (mean of 24h, 16h, 8h and 1h prior to injection), which served as a control. A significant difference between 0.1 mg/kg and 0.01 mg/kg clozapine during the first 8h after injection ($p = 0.0243$) was revealed (figure XIV). Individual values are depicted for each treatment in Fig. IV in the addendum. Furthermore, the time-dependent effect was also evaluated within each treatment (Table II). A table of mean values ±S.E.M for different treatments in function time together with a table of the p-values with the comparison between treatments within a time-period is attached in Table III and IV in the addendum.

Table II : **p-values for evaluation of the time-dependent effect on total seizure duration within treatments**. The effect of different treatments on total seizure duration compared between different time periods. Significant differences are marked (* $p < 0.05$).

	CNO 10 mg/kg	Clozapine 0.1 mg/kg	Clozapine 0.03 mg/kg	Clozapine 0.01 mg/kg
Baseline vs 1-8h	p = 0.0156 (*)	p = 0.0825	p = 0.171	p = 0.6025
Baseline vs 9-16h	p = 0.3317	p > 0.999	p > 0.999	p > 0.999
Baseline vs 17-24h	p > 0.999	p > 0.999	p > 0.999	p > 0.999
1-8h vs 9-16h	p > 0.999	p > 0.999	p > 0.999	p > 0.999
1-8h vs 17-24h	p = 0.0825	p = 0.0156 (*)	p = 0.0156 (*)	p = 0.3317
9-16h vs 17-24h	p > 0.999	p = 0.3317	p = 0.6025	p > 0.999

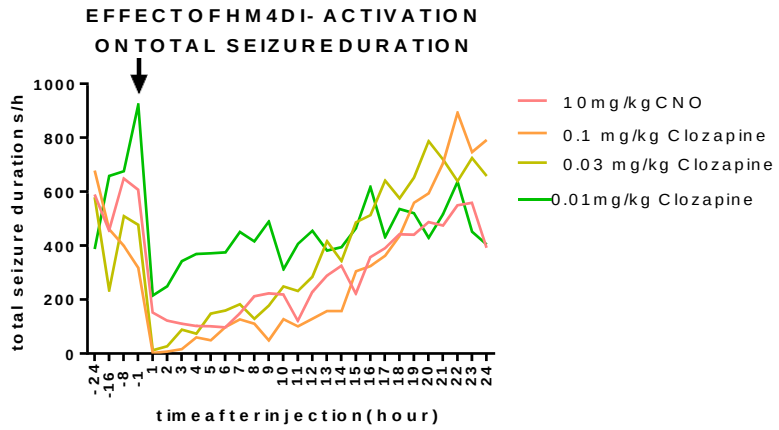

Figure XIII: **Acute effect of hM4Di-activation on total seizure duration in function of time.** DREADD-animals (n=4) were treated with CNO (10mg/kg) and Clozapine (0.1 mg/kg; 0.03 mg/kg ; 0.01 mg/kg). The moment of injection is indicated with the black arrow. The total seizure duration of DREADD-animals are shown in function of time.

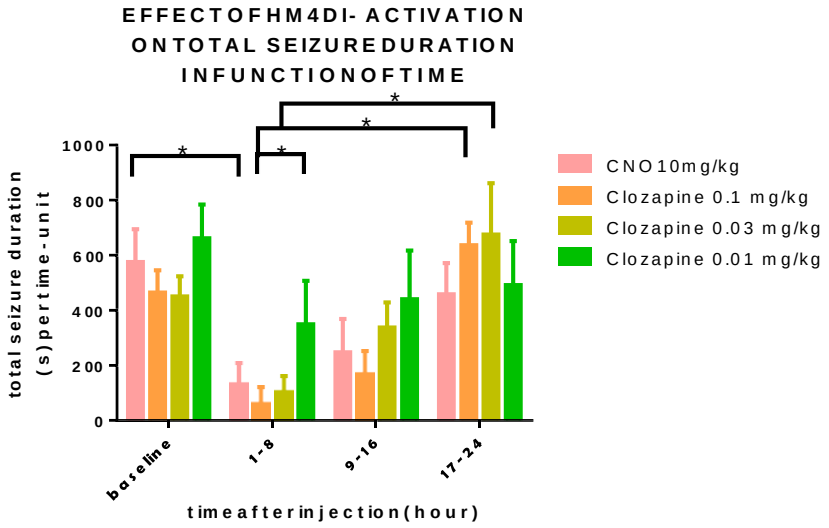

Figure XIV: **Analysis of the acute effect of hM4Di-activation on total seizure duration in function of time.** The histogram provides a visual representation of total seizure duration for each treatment in function of time. Error bars indicate the S.E.M. Significant reductions are marked ($p<0.05$*).

4.3 BEHAVIOURAL EFFECTS OF TREATMENT

4.3.1 Effect on locomotion

Locomotion was evaluated throughout the entire OFT (10 min). Data of DREADD-animals (n=6) and sham-animals (n=7) was collected for evaluation of distance and velocity. No significant difference in the DREADD-group was found between treatments for distance and velocity, nor in the sham-group. Individual results are obtained by detection in Ethovision and are shown in figure XV (distance) and figure XVI (velocity). Similar to the observations of time spent in the centre, a relatively large variation in distance travelled and velocity is observed. A more detailed list of p-values are shown below in table III. Descriptive are shown in table VII and VIII of the addendum. Furthermore the mean effect of DREADD-activation was compared to vehicle-treatment (Fig. VII and Table VI in the addendum).

***Table III*: p-values for analysis of the effect on locomotion between different treatments.**
A:Provides an overview of the comparison between treatments within DREADD-animals and sham-animals on distance travelled. No significant differences ($p<0.05$) are observed between treatments
B: Provides an overview of the comparison between treatments within DREADD-animals and sham-animals on average velocity. No significant differences ($p<0.05$) are observed between treatments.

A) DISTANCE

	Vehicle – Clozapine	Vehicle - CNO	Clozapine - CNO
DREADD (p = 0.9563)	p > 0.999	p > 0.999	p > 0.999
SHAM(p = 0.9640)	p > 0.999	p > 0.999	p > 0.999

B) VELOCITY

	Vehicle – clozapine	Vehicle - CNO	Clozapine - CNO
DREADD (p >0.999)	p > 0.999	p > 0.999	p > 0.999
SHAM (p = 0.9640)	p > 0.999	p > 0.999	p > 0.999

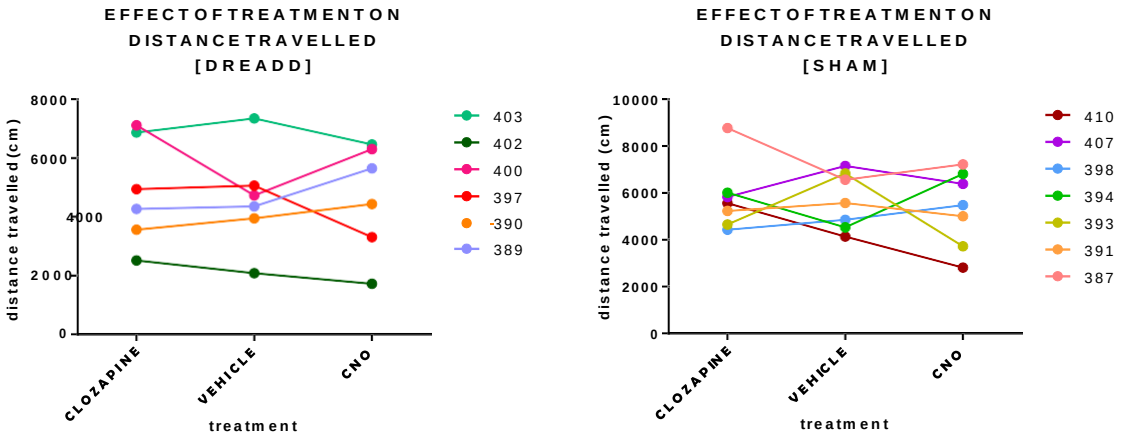

***Figure XV:* Effect of treatment on distance travelled.** Mice received i.p. injection 1h before executing the OFT. Individual values of distance travelled (cm) are shown for 0.1mg/kg clozapine, vehicle and 10 mg/kg Clozapine in both DREADD-mice (n = 6) and sham-mice (n =7).

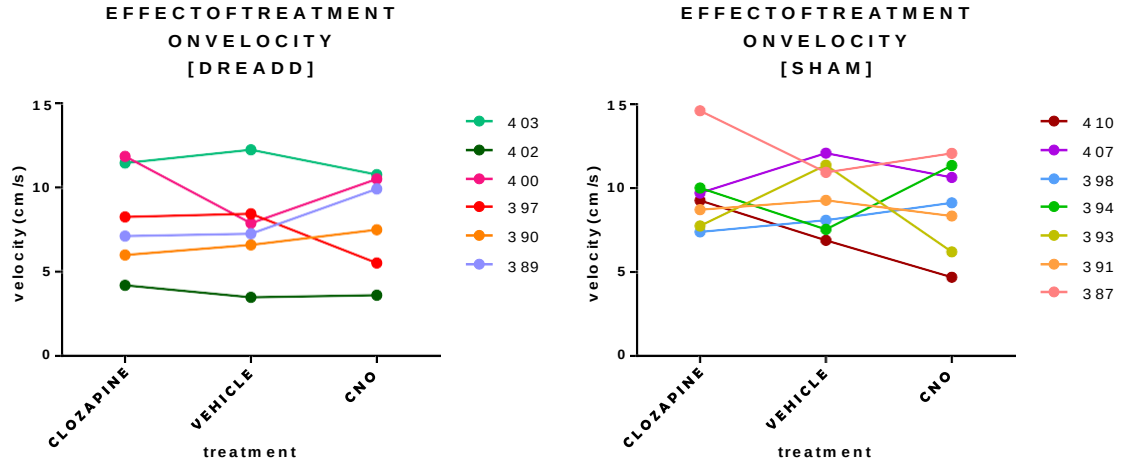

Figure XVI: **Effect of treatment on velocity.** Mice received i.p. injection 1h before executing the OFT. Individual values of the mean velocity (cm/s) are shown for 0.1 mg/kg clozapine, vehicle and 10 mg/kg Clozapine in both DREADD-mice (n=6) and sham-mice (n=7).

4.3.2 Effect on anxiety

The effect of treatment on anxiety was evaluated both in a DREADD-group (n=6) and sham-group (n=7). Anxiety is inversely related to the time an animal spent in the centre of the OFT-arena. Behavioural tests were analysed using Ethovision software. The results illustrated below were obtained during the first five minutes of OFT. In both DREADD- and SHAM- group, a few outliers and a relatively large spread in data-distribution were identified. No effect on anxiety-related behaviour was observed between different treatments in DREADD-mice and SHAM-mice as depicted in figure XVII. A list of p-values is shown in table IV. A table of descriptive results is attached in addendum Table IX. Furthermore the mean effect of DREADD-activation was compared to vehicle-treatment (Fig. VII and Table VI in the addendum).

Table IV: **p-values for analysing the effect on time spent in the centre between different treatments.** Within the DREADD and sham-animals there is no observable difference between clozapine, CNO or vehicle treatment. ($p < 0.05$ is considered significant)

	Vehicle – Clozapine	Vehicle - CNO	Clozapine - CNO
DREADD (p=0.7402)	p > 0.999	p > 0.999	p > 0.999
SHAM (p=0.4861)	p = 0.8553	p = 0.5445	p > 0.999

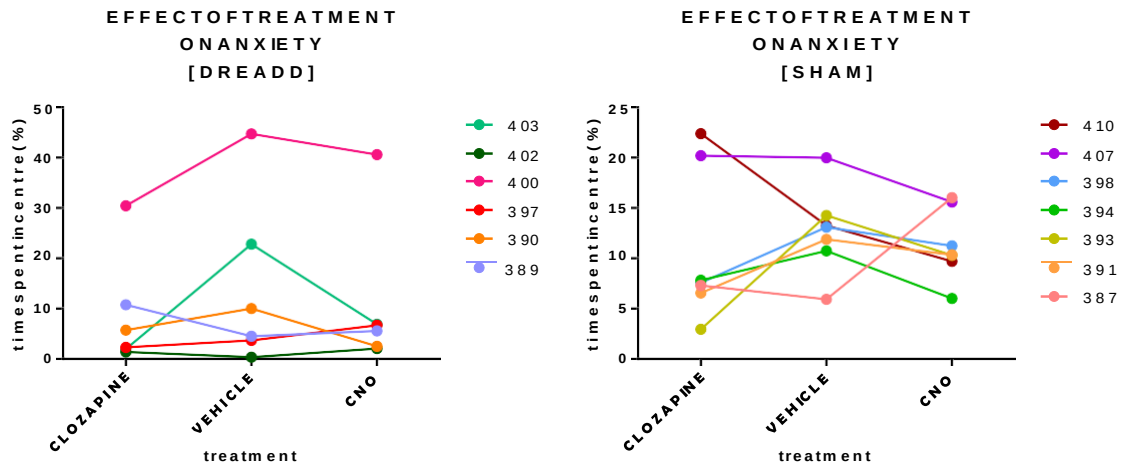

Figure XVII: **Effect of treatment on percentage of time spent in centre.** Mice received i.p. injection 1h before executing the OFT. Individual values are shown for 0.1 mg/kg clozapine, vehicle and 10 mg/kg Clozapine in both DREADD-mice (n=6) and sham-mice (n=7).

4.3.3 Effect on spatial memory

Results of the NOL-test for DREADD-group (n=6) and sham-group (n=6) shown in figure XVIII, are obtained by detection in Ethovision. Originally the sham-group consisted of 7 animals but 1 animal died during training-session, results from previous injections of this animal were excluded. One of the DREADD-animals (397) had a clear behavioural seizure while performing the NOL-test (the day after clozapine injection) thus data was collected until 30s before visual signs of the epileptic insult. In some cases, behavioural seizures were observed during the transportation from home-cage to the test cage. If such behaviour is noticed, mice were tested after a delay of 10 minutes. The latter was reported during the transportation of animal 400 prior to testing in the vehicle-group, animal 410 prior testing in the clozapine group and animal 403 prior to training and testing in the vehicle condition. However, the occurrence of clinical seizures during transportation had no obvious effect on D.I., presumably because the brain-activity returned to normal after a 10 minute delay. The effect of treatment is not significant in the DREADD-group, nor in the sham-group (Table V). A table with descriptive results is provided in table X of the addendum. Furthermore the mean effect of DREADD-activation was compared to vehicle-treatment (Fig. VII and Table VI in the addendum).

Table V: **p-values for analysis of the effect on spatial memory between different treatments.** Within the DREADD and sham-animals there is no observable difference between clozapine, CNO or vehicle treatment. (p< 0.05 is considered significant)

	Vehicle – Clozapine	Vehicle - CNO	Clozapine - CNO
DREADD (p =0.9563)	p > 0.999	p > 0.999	p > 0.999
SHAM (p =0.1840)	p = 0.250	p >0.999	p =0.250

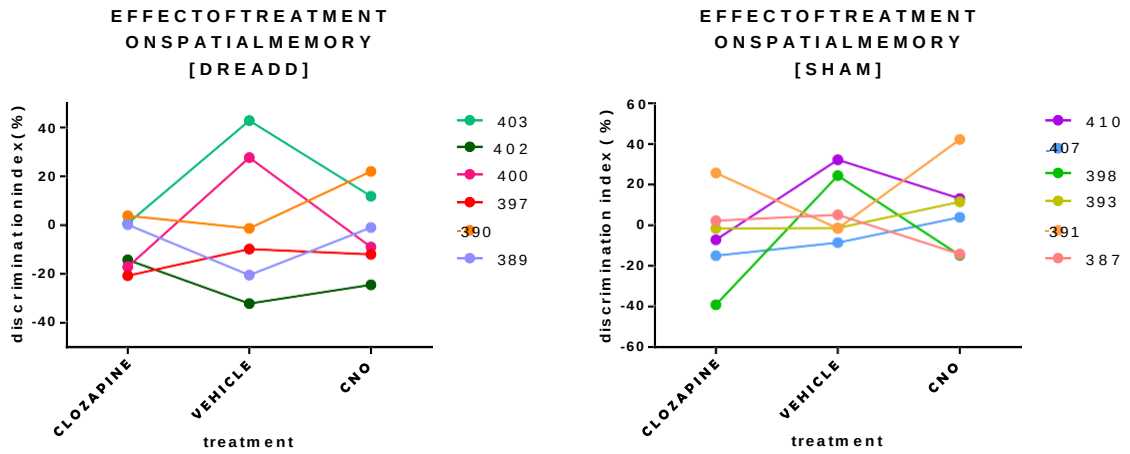

Figure XVIII: **Effect of treatment on discrimination index(%).** Individual values for the D.I. during the testing-phase of NOL are shown.

4.4 COMPARISON BETWEEN SOFTWARE PACKAGES

4.4.1 Software validation for OFT

4.4.1.1 Locomotion

For the evaluation of locomotion, only correction for the body-position was carried out in Optimouse Corrected. Locomotion was assessed during the whole 10 minutes of OFT. Both distance and velocity values followed a normal distribution, therefore Repeated Measures one-way ANOVA was used with Bonferroni post-hoc test both for distance ($p<0.0001$) and velocity ($p<0.0001$). Statistical analyses revealed that Optimouse and Optimouse Corrected differed significantly from Ethovision. The results of each software detection for distance and velocity is depicted in figure XIX. A list of detailed p-values is provided in table VI. A list of descriptive results can be found in table XI of the addendum.

Table VI: **p-values for analysis of differences in distance and velocity between software.** Significant differences between software are indicated with p< 0.05 * ; p<0.01; ** ; p<0.001 *** ; p<0.0001 ****

	Optimouse–Optimouse Corrected	Optimouse - Ethovision	Optimouse Corrected - Ethovision
Distance	p = 0.4243	p = 0.0002 (***)	p = 0.0003 (***)
Velocity	p = 0.4450	p < 0.0001 (****)	p = 0.0001 (****)

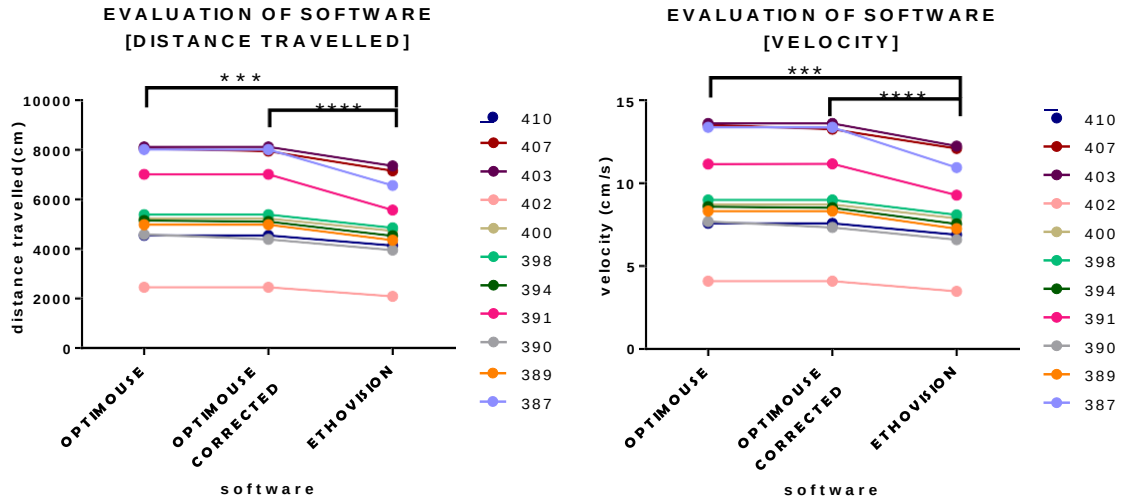

Figure XIX: **Comparison between software for evaluation of locomotion. Left:** Effect on distance travelled; **Right:** Effect on average velocity. Video recordings of vehicle-treated mice (n=13) were analysed with Optimouse, Optimouse corrected and Ethovision. Significant differences were indicated (p< 0.05 * ; p<0.01; ** ; p<0.001 ***; p<0.0001 ****)

4.4.1.2 Time spent in centre

Recordings of vehicle-treated mice were analysed with three different detection-methods. The results shown in figure XX is the percentage of time spent in the centre during 10 min OFT. For Optimouse-correction of the OFT, only body-position was manually annotated instead of nose-correction because the time spent in centre was calculated by the position of the body-centre. A significant difference between softwares (p= 0.0001) is observed. To be precise, differences were significant between Optimouse vs. Ethovision and Optimouse corrected vs. Ethovision (table VII). A list of descriptive results can be found in table XI of the addendum.

Table VII: **p-values for the analyses of differences in time spent in the centre between software.** Significant differences between software is indicated with p< 0.05 * ; p<0.01; ** ; p<0.001.

Optimouse – Optimouse corrected	Optimouse - Ethovision	Optimouse corrected- Ethovision
p >0.9999	p = 0.0029 (**)	p = 0.0006 (***)

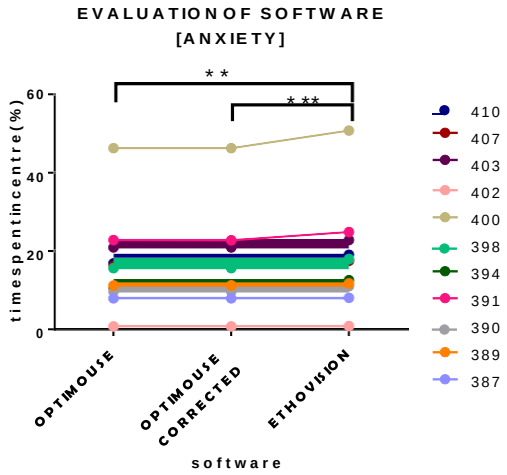

Figure XX: **Comparison between software to evaluate time spent in centre.** Video recordings of vehicle-treated mice (n=13) were analysed with Optimouse, Optimouse corrected and Ethovision. Significant differences were indicated (p< 0.05 * ; p<0.01; ** ; p<0.001 ***)

4.4.2 SOFTWARE VALIDATION FOR NOL

For NOL, the D.I. between different software packages was compared during 10 minutes of training (figure XXI). Detection of vehicle-treated animals performing the NOL-training was analysed by Optimouse, by Optimouse corrected and by Ethovision. In contrast to Optimouse corrected in the OFT, correction in the NOL included nose-point correction as well. The manually corrected nose-position was considered the reference-value as each frame was visually checked and corrected if needed. Data followed a normal distribution and a 1-way Analysis Of Variance (ANOVA) repeated measures was conducted with a Bonferroni-correction. A significant difference was reported between softwares (p = 0.0123), more specifically between Optimouse detection vs. Ethovision (table VIII). A list of descriptive results can be found in table XI of the addendum.

Table VIII: **p-values for analysis of differences in D.I. between software.** Significant differences between software is indicated with p< 0.05 * ; p<0.01; ** ; p<0.001 ***

Optimouse– Optimouse Corrected	Optimouse - Ethovision	Optimouse Corrected - Ethovision
p = 0.6441	p = 0.0439 (*)	p = 0.1605

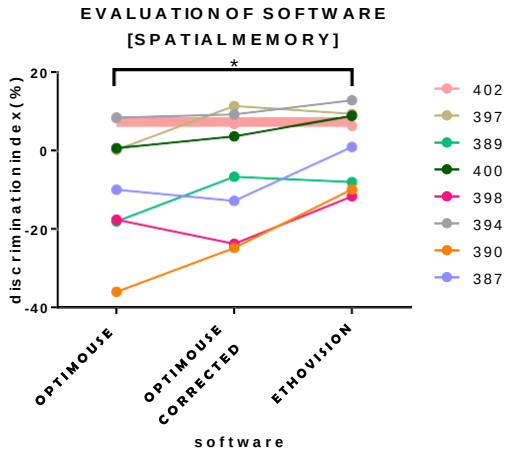

Fig XXI: **Comparison between software for discrimination index** Video recordings of vehicle-treated mice (n=8) were analysed with Optimouse automatic detection, Optimouse manually nose-corrected and Ethovision automatic detection. Significant differences were indicated (p<0.05 * ; p<0.01 ** ; p<0.001 ***)

5 DISCUSSION

5.1 SEIZURES INDUCED BY IHKA MODEL

Approximately 45% of the IHKA-injected animals developed seizures that were clearly visible on EEG, which is lower than the reported success rates of 60% [22]. Nonetheless, the observed success rate within our study is presumably an underestimation, as mice with low-frequent epileptic brain activity suffered from clinical seizures while performing behavioural tests. Furthermore elements such as strain and anaesthetics could possibly interfere with the development of TLE. Literature recommends the use of Isoflurane, as it does not interfere with excitotoxic properties of KA. Furthermore, the probability that seizures can be caused by electrode implantation and concomitant brain damage was reduced to a minimum as control animals implanted with electrodes did not exhibit epileptic brain activity[24]. Seizures were defined by the occurrence of high voltage sharp waves (signal to noise ratio >2:1). Studies in IHKA-treated C57/BL6 mice have reported a rate of approximately 38 seizures/h [48,49], however the seizure rate induced by IHKA -injection in mice is known to vary quite largely, as other studies have reported a seizure-rate of 20-100 sz/h [50–53]. It is noteworthy that the mean seizure duration is shorter in studies that reported a high frequency of seizures per hour. The mean incidence of seizures/h observed in this experiment (28,35 ± 1,631593) was inferior to the reported incidence of 38 seizures/h in C57/BL6 mice. However, the overall observed total seizure duration per hour (591.85s ± 34.45) in this experiment was comparable to reported total seizure duration (865 – 560s/h) in IHKA-treated mice [49]. Furthermore, the mortality rate of IHKA was about 12%, which was in line with our findings(10%) [23].

5.2 BEHAVIOURAL EFFECTS OF IHKA-INJECTION

5.2.1 Effect of IHKA on locomotion

Literature studies have reported inconsistent results in regard to the effect of IHKA on locomotion. A number of studies using i.p. or intranasal injection of KA in mice and rats have observed a slight increase in hyperexcitability [54–57]. However, a few studies in IHKA mice and i.p. rats have reported no observable effect on distance travelled and velocity [22,58]. Differences in literature studies might be due to the manner of inducing SE and subsequential morphological differences in hippocampal damage.

5.2.2 Effect of IHKA on anxiety

There are contradictory studies concerning the effect of KA on anxiety. The percentage of time spent in the centre is reversely related to anxiety, meaning that animals that spent more time in the centre of the arena were less anxious. The majority of literature did not report a significant

difference in time spent in the centre of the OFT between IHKA and vehicle-treated animals [22,54,56]. Several of these studies have executed additional tests to evaluate anxiety, such as elevated plus mazes and light-dark boxes. The results of these additional tests were in line with the results of the OFT. By way of contrast, a study executed in KA-treated rats has reported a decrease in anxiety-like behaviour in comparison to control animals. Rats received intraperitoneal KA-injection until SE was induced. Behavioural tests such as OFT were carried out 8 weeks after SE, which indicated a significant increase in time spent in the centre for KA-rats [58]. Differential results might be explained by the method of KA-administration, as the ventral hippocampus and amygdala are more affected by i.p. KA injection than by intrahippocampal injection [58].

5.2.3 Effect of IHKA on spatial memory

TLE is associated with co-morbidities like depression, anxiety, and memory-impairment. Studies have suggested that chemoconvulsants used to mimic TLE in rodents might alter behavioural manifestations, corresponding to the co-morbidities in patients.

According to Hubens et al. [56] KA-injections and subsequent hippocampal damage did not affect the outcome of the NOL-test, implying that spatial memory in rodents remains intact during the chronic phase of TLE. However, this study has calculated the difference in exploration time of the relocated object between training and testing for the evaluation of spatial memory. An overall increase in exploration of both objects might also have occurred simply as a result of a decrease in anxiety and does not intrinsically prove intact spatial memory. Few studies have examined behavioural alterations in mice as a result of KA- injections. Gröticke et al. [22] have reported similar findings, stating that IHKA injection in mice did not impair memory functioning. Nonetheless, this observation cannot be generalized to spatial memory as memory was assessed in the novel object recognition (NOR) test. Usage of the latter is somewhat controversial for the assessment of spatial memory functioning as other structures such as the perirhinal cortex, insular cortex and ventromedial prefrontal cortex are involved as well and possibly compensate for a detrimental functioning of the hippocampus. Studies in rats have investigated the effect of KA-treatment on memory during the acute phase of TLE both in the NOR and NOL. KA-treated rats showed a short (i.e 5 min delay) and long-term (i.e 60 min delay) spatial memory deficit in NOL, whereas for NOR the short term memory, but not long term memory, was considered intact [54].

Besides the NOL, other tests such as the Morris Water Maze (MWM) and Radial Arm Maze can be used to evaluate spatial memory. IHKA mice did also perform the MWM in the study of Gröticke, which showed impaired spatial memory. Nonetheless the results of MWM might be confounded due to stress, anxiety and aversity of mice against H_20 [56].

5.3 THE ACUTE EFFECT OF HM4DI RECEPTOR ACTIVATION CONCERNING SUPPRESSION OF SEIZURES

As previously mentioned in section 1.2.1 all TLE-hypotheses assume morphological and cellular changes as a result of mossy cell death, followed by an excessive activation of granule cells in the dentate gyrus. Consequently, hyperexcitation of CA1 and CA3 pyramidal neurons is also acquired through the trisynaptic glutamatergic organization of the hippocampus. In this experiment it was shown that activation of the hM4Di-receptor, which counteracts excessive neuronal firing of excitatory neurons by a GIRK-mediated hyperpolarization and limitation of axonal glutamate-release, was capable of suppressing seizures [28].

The total seizure duration per hour was calculated by multiplying the mean seizure duration with the incidence of seizures. A decrease in total seizure duration of >75% was obtained in the 1-8h time period by injection of 10 mg/kg CNO and 0.1mg/kg and 0.03 mg/kg clozapine (Table IV addendum). Nonetheless, only the dose of 10 mg/kg CNO was able to cause a significant decrease when comparing baseline to 1-8h after injection. It is plausible that a significant decrease in total seizure duration for an 8h time-period could also be observed for the doses 0.1 mg/kg and 0.03 mg/kg clozapine if more animals with high seizure frequency were recruited.

In addition, a significant difference was observed between 1-8h after injection and 17-24h after injection for both 0.1mg/kg and 0.03 mg/kg clozapine. After 17-24h, the effect of clozapine had faded and even a small increase in mean seizure duration was observed in comparison to baseline-recording. This increase was a temporary observation and did not establish a long-term augmentation in total seizure duration.

Furthermore, in the 1-8h after injection, a significant difference was present between 0.1 mg/kg and 0.01 mg/kg clozapine (Table III addendum). The 0.1 mg/kg clozapine elicited a 100% response rate in four animals and a total suppression of seizures in 3 out of 4 animals animal for at least eight hours, whereas the dose of 0.01 mg/kg clozapine caused a more moderate decrease in total seizure duration and 1 animal did not respond to treatment. The effect of treatments in individual mice is shown in Fig IV of the addendum.

The first successful application of chemogenetics in modulation of seizure activity was performed in rat models for focal neocortical seizures. Activation of the hM4Di-receptor, expressed in excitatory neurons of the forelimb area of the primary motor cortex, caused attenuation of seizures in acute (pilocarpine, picrotoxin) and chronic models (tetanus toxin). In this experiment i.p injection of 1 mg/kg CNO was sufficient to significantly reduce seizures for over 90 min without causing behavioural impairment [59]. Similar results have been achieved in animal models for TLE-models through inhibition of excitatory neurons in thalamus and hippocampal slices [60,61]. Alternatively, it has been reported that activation of hippocampal

GABA-interneurons also had favourable effects on the spread of seizure activity throughout the brain, but the role of inhibitory neurons concerning induction of seizures remains more controversial [62,63].

5.3.1 CAVEATS OF DREADD-THERAPY

It is noteworthy that DREADD-expression was not limited to the dorsal ipsilateral hippocampus. Spreading of the vector was also observed in the surrounding cortex, the ventral area of the hippocampus, the contralateral hippocampus, and projecting regions of the hippocampus such as the thalamus. Silencing of excitatory neurons in the thalamus might have been partly responsible for the observed effects in this experiment concerning suppression of seizures. Nonetheless the thalamus is more involved in propagation of seizures rather than initiation[60]. In contrast to optogenetics, which allows neuronal modulation in a range of milliseconds, the temporal control of chemogenetics depends on the clearance of the ligand [26,62]. As explained in section 1.6.2 clozapine is probably the true actuator of DREADDs instead of CNO. Given that clozapine was already FDA-approved and safety tested, the clinical potential of this treatment is simplified. Clozapine is known to interact with endogenously expressed muscarinic receptors, histamine receptors, α-adrenergic receptors, and serotonin receptors. Yet is more likely to bind to muscarinic-DREADD receptors [62]. Nevertheless it is strongly recommended to employ low doses of clozapine in order to prevent endogenous receptor binding [64]. Obviously, the influence of low clozapine concentrations concerning behaviour should be evaluated to exclude off-target effects. Therefore, in this experiment sham-animals (non-expressing DREADD) were used. Interpretation of these results must be made carefully as not all behavioural effects can be observed within sham-animals. For example, possible cross-talk between DREADD-receptor and an endogenous receptor might occur, which cannot be evaluated in non-expressing DREADD-animals [65].

Furthermore, there is a concern regarding constitutive activity of engineered receptors. In absence of ligand binding, dimerization of GPCR might occur with the activation of G-protein signalling cascade as a result. Although the level of basal activity in DREADDs is limited in comparison to other chemogenetic techniques, precautions must be taken. Usage of a lower viral titer, a weaker promotor, or deletion of the WPRE might decrease the probability of basal activity [28].

Another phenomenon that should be considered is the desensitization of DREADD-receptors. Both arrestin and kinase phosphorylation binding places are preserved in the modified muscarinic receptor. Thus, similar to endogenously expressed receptors, they might be subjected to phosphorylation, desensitization, and internalization [60,62]. Phosphorylation of muscarinic receptors by GPCR-receptor kinases (GRK) might occur after chronic ligand occupation of the receptor. Subsequently, β-arrestin binds to the third intracellular loop of the

phosphorylated receptor, which blocks interaction with G-proteins. Desensitization provides a possible explanation for the diminished effect in DREADD-studies when treating chronically [28,60]. The latter might be anticipated by increasing expression of the receptor. In case of high receptor density, a maximal response is elicited without full occupation of receptors, which is referred to as receptor reserve and lower concentrations of ligands are needed. High receptor density might be able to achieve an adequate long-term effect when treating chronically, even if receptors are partly desensitized or internalized. However, high expression of receptors increases the probability of constitutive activity [28,62].

5.4 BEHAVIOURAL EFFECTS OF DREADD-ACTIVATION

5.4.1 Locomotion related effects of clozapine and CNO administration in DREADD- and SHAM-mice.

Bender et al. have clarified the influence of hippocampal discharges on locomotor behaviour. The hippocampus receives input from the medial septum (MS), needed for the establishment of theta oscillations in the hippocampus. Theta-rhythmic stimulation of GABA-ergic MS axons projecting to inhibitory interneurons of the CA1 region is capable of eliciting theta oscillations (5-12 Hz) in pyramidal neurons of the hippocampus. Regularity of both frequency and amplitude of hippocampal theta-oscillations plays a key role in the modulation of running speed but not in induction of locomotion. A slower and more constant running speed was observed during regular optogenetic stimulation at frequencies of 7 and 9 Hz, in comparison to spontaneous theta at a corresponding frequency rate. The latter is more variable as spontaneous theta oscillations are regulated by speed-correlated afferents of the hippocampus. Pyramidal neurons connect with neurons of the lateral septum (LS), which subsequently project to the lateral hypothalamus and other subcortical regions involved in locomotion.

Inhibition of CA1 pyramidal neurons has illustrated the importance of hippocampal contribution in regard to velocity, whereas regulation of theta stimulation in MS could not exert a decrease in running speed when pyramidal neurons are silenced. Elongated inhibition of CA1 neurons with chemogenetics is even associated with an increase in speed, thus intact functioning of the hippocampal-LS pathway is necessary for controlling running speed [41]. The observation that regular hippocampal theta-oscillation causes a decrease in velocity might provide an explanation for the hyperactivity reported in some studies among animals with hippocampal damage. Furthermore it would have been interesting to examine differences in speed distribution or acceleration between hM4Di-receptor activation and vehicle-injected DREADD-animals in this thesis.

The results observed in this experiment are in contradiction to the findings described above, as there was no observable difference on distance travelled and velocity when activating the hM4Di-receptor. Furthermore, no off-target effects of clozapine or CNO were discerned in sham animals. To note, vector expression and the execution of consecutive trials had no influence on locomotion (fig VIII-IX and table XII-XIII addendum).

Literature studies have reported inconsistencies in regard to aspecific clozapine and CNO side effects on locomotor behaviour, however these side effects were largely dose-dependent. Dosage of clozapine (0.1 mg/kg) did not induce change, whereas a dose of 1 mg/kg had a negative influence on the distance travelled[30]. A dose of 5 mg/kg CNO, the presumed inert ligand, was reported to diminish amphetamine-induced hyperlocomotion. Presumably CNO interferes with amphetamine-receptor binding and prevents hyperlocomotion, which complicates the inert properties of CNO[66]. However no off-target locomotor effects of 1 mg/kg and 10 mg/kg CNO were reported in studies lacking amphetamine induced hyperlocomotion [30,67].

5.4.2 Anxiety related effects of clozapine and CNO administration in DREADD and SHAM-mice

Activation of the hM4Di-receptor had no impact on anxiety-related behaviour, nor did clozapine or CNO affect the percentage of time spent in the centre of the sham-group. It is noteworthy that a relatively high frequency of outliers was observed both in DREADD- and sham-group. Furthermore, the impact of multiple testing on the total time spent in the centre was also evaluated. Between each trial, 1 habituation day was enabled. The total time spent in the centre remained relatively stable throughout different trials (Fig IX and table XIII addendum) To assess the effect of receptor expression, vehicle conditions between DREADD- and sham animals were compared. As expected DREADD-expression had no impact on anxiety (Fig VIII and table XII addendum)

There is increasing evidence that hippocampal functioning is region-dependent. Spatial memory and possibly exploratory behaviour is regulated by the dorsal hippocampus, whereas the ventral hippocampus is known for its role in anxiety-related behaviour such as avoidance [42,43,68]. Although the viral vector was injected in the dorsal-site of the hippocampus, histology has confirmed that limited expression of the hM4Di-receptor in the ventral hippocampus was also present. Thus potential alterations in anxiety-related behaviour should be taken into consideration, as modulation of excitatory neurons in the ventral hippocampus also occurs. However, in this experiment, the percentage of time spent in the centre was not significantly influenced by hM4Di-activation, perhaps because the vector spread to the ventral hippocampus was very limited or due to compensation mechanisms of other structures associated with the HPA-axis. The results of this thesis were consistent with a literature study

that showed no altered anxiety-related behaviour when bilaterally inhibiting excitatory neurons of the ventral dentate gyrus [67], although it should also be noted that this study used a 1 mg/kg dosage of CNO to activate the exogenous receptor.

No off-target effects of clozapine and CNO [67] were revealed, which is in line with other literature studies. It should be noted that an acute low dose of clozapine 0.1 mg/kg did not exert an anxiolytic effect in single-housed rodents. However, there were indications that there might be a minor impact on anxiety within a low dose range (0.1 mg/kg - 0.4 mg/kg)[69]. Hence, determination of the exact clozapine dose for obtaining the largest anti-epileptic effect must be carefully considered, in regard to possible off-target effects of clozapine.

5.4.3 Spatial memory-related effects of clozapine and CNO administration in DREADD- and SHAM- mice.

As previously stated, hippocampal neurons are known to play a significant role in encoding, consolidation, and retrieval [70]. The potency of the hippocampus concerning memory-processes has been illustrated by the stimulation of a subgroup of neurons within the dentate gyrus. Stimulation of the latter, that were previously engaged in fear-conditioning test, was able to significantly increase freezing-behaviour in comparison to the non-stimulated condition [71]. Numerous studies have illustrated the involvement of the hippocampus in memory processes, ranging from memory recall to memory suppression by neuromodulation techniques[62,70,71]. Spatial memory might be impaired by inactivation of hippocampal neurons. For example, hippocampal administration of an AMPA/kainate receptor antagonist blocked fast glutamatergic synaptic transmission. Consequently, detrimental effects on spatial memory were observed in the MWM test [70]. A similar outcome was reported in a study that employed the hM4Di-receptor under control of the hSyn-promotor, which was able to transfect both excitatory and inhibitory neurons within the hippocampus [40]. The illustrations above have demonstrated the possible impact of neuromodulation within hippocampal neurons on spatial memory and emphasize the need for caution when executing similar experiments.

To our knowledge, only one article inhibited excitatory neurons of the dorsal hippocampus using viral vector (1µl) transfection for expressing the hM4Di-receptor under control of the CaMKIIα-promotor. Activation of the DREADD-receptor with a 3 mg/kg CNO-dose did hamper long-term memory formation in NOL[40].

In this thesis, mice were injected 1h prior to training, thus potential effects of treatment on encoding and consolidation of spatial memory could be evaluated. Mice with intact spatial memory are presumed to spend significantly more time to the relocated object, resulting in a D.I. that varies between 20-45% [38]. Activation of the hM4Di-receptor did not result in a significant difference in D.I compared to vehicle-treated DREADD-expressing animals. The majority of vehicle-treated animals were not capable of discriminating both objects, making it

more difficult to draw conclusions concerning therapeutic effects on spatial memory. The most logical explanation for failure of NOL in vehicle-treated animals would be impairment of spatial memory due to IHKA-injection. Unfortunately the setup of this experiment did not allow to test this hypothesis. Besides IHKA-injection other determinants might have to be taken into consideration as well.

The objects that were used in different trials might bias interpretation of results. Aversion or predilection for a certain object has an impact on the total exploration time during training, which can subsequently influence the establishment of memories. Prior to the NOL, the OPT was executed in order to exclude confounding of the NOL by using various objects in different trials. No significant differences in the percentage of exploration time between objects were observed, although 2 objects were rejected from the NOL due to excessive climbing and sitting on top of the objects (fig V-VI and table V addendum).

Besides a predilection for objects, place-preference might also influence the NOL. In this experiment no evidence was acquired that indicated a preference for a certain place. However due to the design of the experiment all areas except for one were evaluated during training (fig X and table VI addendum).

A possible explanation for not exceeding the expected D.I. values might also be due to insufficient exploration of objects during the training session. Other studies have employed a training session criterion of 38 s exploration time of each object in lesioned mice[37]. In 34 out of 38 trials, the required 38 s of exploration during training was achieved. However, the animals in the 4 remaining trials had almost attained the desired exploration time. No obvious effect on D.I. during testing was revealed in the 4 animals that did not reach the 38s of exploration during training (table XIV addendum). Next, the effect of treatment on exploration time during training was also evaluated, however there was no evidence that the type of treatment influenced exploratory behaviour (fig XI and table VI addendum).

A small decrease in total exploration time during training of the NOL was observed in trial 3 in comparison to trial 1 (fig IX and table XIII addendum). This finding was somewhat surprising as each trial consisted of three days habituation in absence of objects, 1 day training, 1 day testing, and 1 resting day. In the future, it might be considered to implement 2 resting days between trials. This observation did not affect the interpretation of treatment effects as a cross-over design was used. (Table II addendum). In addition, the total exploration time during testing was not significantly different between trials (fig IX and table XIII addendum).

Lastly, the effect of vector expression was also evaluated by comparing vehicle-treated DREADD-mice with non-expressing DREADD-mice. As expected, hM4Di-expression had no impact on spatial memory (Fig VIII and table XII addendum). Furthermore, one observation that stood out was the CNO-treated animal with ID number 390, where the D.I. was higher in comparison to both clozapine and vehicle. Looking back at the observations, the training phase

included 20 minutes instead of 10 minutes because the first 10 minutes had not been recorded due to technical problems. This might be interesting when working with IHKA-mice in the future as for now the majority of IHKA-mice failed the NOL, which made it difficult to assess the effect of different treatments. Thus, increasing the time of the training phase might improve the D.I. of testing as observed in animal 390. When the majority of animals succeeds in discriminating the relocated object, results concerning the effect of treatment will be more representative. However this hypothesis is based upon 1 observation and is difficulted by individual variation in total exploration time. After evaluating possible factors that might interfere with spatial memory, it is likely that the majority of mice fail NOL as a consequence of the KA-induced phenotype. Another alternative hypothesis to explain negative values of D.I. is neophobia: animals being anxious towards novelty [38]. Yet this hypothesis is less likely as no clear anxiety-related behaviour was observed in the OFT.

To evaluate of off-target effects of treatment on long term spatial memory, SHAM-mice were also injected with vehicle, clozapine, and CNO. Similar to the observations in DREADD-mice, the majority of mice fail to discriminate the relocated objected. In this experiment there was no clear evidence that clozapine or CNO had an effect on spatial memory functioning in comparison to vehicle-treated mice. However, vehicle-treated SHAM-mice did also fail the NOL, which makes it difficult to assess the effect of treatment on spatial memory. Literature studies have reported a lack of aspecific side effects for doses of 2 mg/kg CNO [72] and 1 mg/kg clozapine[73] on memory-functioning.

5.4.4 Bottlenecks of behavioural tests

5.5.4.1 IN GENERAL

Behavioural tests in animals should be executed with care and attention to details as they are subjected to many confounding factors. Considering the fact that mice are nocturnal animals, behavioural tests were carried out at night. Besides correcting for the active period, it was also possible to diminish the effect of stress induced by sounds of other experiments, as the building was empty at night. It was not feasible to correct for noise originating from weather-phenomena as the behavioural room was not properly isolated.

Stress is a major confounding factor in behavioural tests, thus some precautions were taken in order to reduce stress as much as possible. Cages were cleansed with ethanol and air dried for 10 min to avoid stress induced by unfamiliar odours. Another preventative measure to cancel out stress was adaptation of the animal to the novel environment for three consecutive days prior to testing. One of the most substantial determinants of stress in this experiment was i.p. injection. Both scruffing and injection were experienced as unpleasant and induced a lot of stress in mice, which made mice more difficult to handle and more prone to seizure activity

when touched during the final days of testing. There are little alternatives for acute administration of drugs that will lessen stress in animals. One study administered CNO using eye-drops, which retained the speed of CNO-delivery to the brain. Mice were scruffed, which also caused stress, but the pain of injection was avoided. Eye drop administration had one major drawback as the exact dose was difficult to determine as the animal closed its eyes while administering [74]. Another option might have been administration of drugs in the drinking water as this did not induce stress, but again it will not be possible to correct for differences in doses between treatments, which was essential in our experiment as drugs had a dose-dependent effect. Another challenge in behavioural tests was correct nose detection of the animal. Both Ethovision and Optimouse did not succeed in a 100% accurate nose detection. The percentage of accurate detection greatly depended on the contrast between the animal and the environment. Green surgery paper was placed at the bottom of the cage for contrast enhancement and absorption of infrared light. Furthermore, the walls of the NOL-cage were pre-treated with acetone for the purpose of reducing reflection of the animal, which was sometimes detected by the software instead of the animal.

5.4.4.2 SPECIFICATIONS FOR OFT

Anxiety is a possible confounder in the assessment of locomotor activity in the OFT. Data of the first five minutes was used to evaluate the effect of drugs on anxiety-related behaviour, whereas locomotion was assessed during the entire trial.

5.4.4.3 SPECIFICATIONS FOR NOL

For the choice of objects it is important to take into account the texture and colour of the items. Objects should be easy to clean, easy to fixate, non-odourless and distinguishable from the fur of the mouse. The proximity of objects to the wall might also influence the observed interaction time. In this experiment objects might have been located too close to the border as thigmotaxis, the tendancy to stay close to the wall, was observed. The animal stayed between the object and the wall without actually exploring the object, which was mistakenly defined as interaction. The perspective of the infrared lamp and camera is also key to a good detection result. The problem in some experiments was shadow formation around the object, particularly in high objects. The shadow zone of an object is in keeping with the location of the object and its relation to the camera point-of view. It is more difficult to determine a 1 cm border around the object when a shadow is present. Especially if the shadow depends on the location of the object and differs within one trial. In the future, this might be prevented by drawing a 1 cm border around the object prior to testing.

Each trial of the NOL started with picking up the animal by its tail to transfer the mouse from transportation-box to the test cage, which was not the case in OFT. Physical contact between the researcher and the animal can induce stress and thus impair behavioural testing, especially in IHKA mice.

5.5 COMPARISON BETWEEN SOFTWARES FOR BEHAVIOURAL TESTING

An important section of this thesis is devoted to the validation of Optimouse, in regard to automatic detection of nose- and body-points for analysing behavioural tests. For NOL, the D.I. of 8 vehicle-treated animals was assessed with manual correction in Optimouse (referred to as Optimouse Corrected) and automatic detection in both Ethovision and Optimouse. For OFT, the effect of detection software on distance, velocity, and anxiety was assessed in 13 vehicle-treated mice.

It should be noted that manual annotation in NOL implemented both nose-and body- point correction, whereas for OFT only body-point correction was included as locomotor and anxiety behaviour was only analysed in terms of body-position. Because correction of body-points is far less intensive, it was feasible to manually correct detection in all vehicle-treated animals for OFT. The Optimouse Corrected version was considered the most accurate estimation as each frame was individually validated and corrected. Optimouse Corrected was used as a reference to validate softwares in NOL and OFT.

5.5.1 Optimouse automatic detection vs. optimouse manually corrected

Automatic detection of the body-point in mice was very accurate in a series of OFT-trials. Manual correction of body-points was unnecessary, except when the animal lifted the surgery paper at the bottom of the cage as detection failed due to lack of contrast between the environment and the animal. No significant difference in time spent in centre, distance, and velocity was observed in comparison to Optimouse Corrected.

In the NOL no significant difference in D.I was observed between Optimouse and Optimouse Corrected. Within these trials it would not have been necessary to individually annotate each frame as it was a very time-consuming process. However, it is strongly recommended to monitor every trial in detail and to adjust detection settings if detection is completely off-track for a significant period of time. For example, in a few animals (397, 389 and 390) rather large differences in D.I. between the automatic and the corrected Optimouse were present, meaning that automatic detection was more erroneous. Perhaps because of the contrast or the individual difference such as nose or tail thickness. It is highly likely that other detection settings were more suitable for these animals, thus rendering a result closer to the exact D.I.

5.5.2 Ethovision automatic detection vs. Optimouse Manual Corrected

A significant difference between Ethovision and the reference software was reported for time spent in the centre, distance and velocity. The data distribution showed a relative consistent error between the two software packages. The results obtained by Ethovision were systematically smaller for distance, and velocity than the results reported by Optimouse. This is in contrast to the time spent in the centre, which was consistently higher in comparison to the reference software. These observations were likely due to a small difference in calibration and delineation of the test cage between software packages. It was rather difficult to precisely draw calibration lines for determining the size of the cage, which made calibration of the cage more prone to errors than originally assumed. It was recommended by Ethovision to delineate the arena bulkier than the exact arena in order not to lose any data when the animal stands for example on its hindlegs. For Optimouse the walls of the arena were not included in the arena because reflection of the animals resulted in unreliable automatic detection. This discrepancy in arena size had implications for the time spent in the centre. The centre part was defined as the inner nine squares of the whole arena. Since the arena defined in Ethovision was somewhat larger than in Optimouse, the centre arena was more voluminous as well, consequently the time spent in the arena is a fraction longer. Both inaccurate calibration and discrepancy in arena surface might explain the consistent error that resulted in a significant difference between Optimouse and Ethovision for the time spent in centre, distance, and velocity.

Notwithstanding the fact that detection between Optimouse and Ethovision was significant distinct, this does not imply that the difference in detection was relevant. As the error was consistent and the difference in calibration was likely responsible for the observed difference, the body detection of Ethovision might have been quite exact. The acquiring phase of Ethovision allowed to review detection afterwards, which seemed relatively good and confirmed the suspicion that incorrect detection did not account for the observed difference. Although Ethovision is a validated program for assessing behavioural tests, the software sometimes resulted in inaccurate nose-detection. In Ethovision no optimal detection setting, applicable to the majority of trials, was found thus settings were individually adjusted until an acceptable detection was acquired.

A possible explanation might be the lack of the researcher’s experience in Ethovision or the influence of environmental factors on detection such as contrast and lighting. Once the detection was satisfactory, the D.I. could be calculated. In a few animals (387,390, and 398) there was more variance in D.I., presumably not attributed to detection-settings but more likely to be a consequence of the difference in zone area. It was not feasible to copy the zone area

from Optimouse to Ethovision and vice versa thus zones were determined on sight, which might explain the relatively larger differences in D.I. for some animals.

5.5.3 Conclusion for validation of software packages

Neither Optimouse nor Ethovision were able to automatically identify the nose with a 100% success rate. Detection depends on the type of settings and environmental factors. It was not feasible to assess which automatic detection system was better since there are many options to enhance detection. For Optimouse, only one setting was used, while for Ethovision each animal had a different setting. Besides, even when the detection method was chosen, it was not possible to objectively evaluate the fraction of errors between Optimouse and Ethovision as the reviewing detection in Ethovision was accelerated and in Optimouse there was a three-fold delay. Without a doubt Ethovision is more user friendly. The preparation and detection is less time-consuming in comparison to Optimouse. When conducting a series of behavioural tests it is preferable to use Ethovision.

The findings discussed above allow us to conclude that Optimouse Automatic Detection is a validated software package for assessing behavioural tests, although each trial should be visually evaluated and potentially different detection settings should be applied, in agreement with the needs for each individual detection.

In this thesis it was not possible to validate Ethovision with reference to Optimouse since determinants such as arena size were differently defined. As Ethovision is widely used in all kinds of animals for a series of behavioural tests, it is safe to assume that Ethovision was able to correctly identify nose- and body points.

6 FUTURE PERSPECTIVES

CNO is capable of reducing the total seizure duration for a significant period of time in DREADD-animals. In following experiments, more animals should be recruited for the establishment of long-term (> 8h) seizure reduction by clozapine in hM4Di-animals, as a clear trend is already apparent. Furthermore, it is also aimed to tackle bottlenecks concerning DREADD-technology. One of the hypotheses that should be tested is increasing the receptor density by altering the viral titer in order to overcome desensitization. However, increasing DREADD-expression should be executed with caution as this might have implications for basal receptor activity. In addition, potential side effects of clozapine and CNO in control animals that did not receive IHKA-injection should be considered, especially concerning spatial memory. The effect of treatment on spatial memory is difficulted by the observation that animals treated with vehicle also failed the NOL. Assessing spatial memory in control animals that received DREADD- or SHAM-injection might give a more truthful and accurate representation of specific and off-target effects concerning treatment.

7 CONCLUSION

Activation of the hM4Di-receptor, expressed in CaMKIIα-excitatory neurons of the hippocampus, is capable of suppressing seizures in IHKA-mice. A dosage of 10 mg/kg CNO significantly reduces the total seizure duration in DREADD-expressing mice for at least 8h, whereas for clozapine, a clear trend is apparent. Presumably a significant reduction of total seizure-duration is also probable when recruiting more animals. In this thesis a dose of 10 mg/kg CNO and 0.1 mg/kg clozapine did not exert any behavioural impairments. However, specific and off-target effects of treatment concerning spatial memory should be re-evaluated in control-mice.

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FACULTY OF MEDICINE AND HEALTH SCIENCES

Addendum Chemogenetic approaches for unravelling the specific contribution of hippocampal neurons in the epileptic brain

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1 LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AED	Anti-epileptic drugs
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	anterioposterior
ArchT	archaerhodopsin
BBB	Blood brain barrier
Ca	calcium
CaMKII	Ca/calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
ChR2	Channelrhodopsin
CNO	Clozapine-n-oxide
D.I.	Discrimination index
DBS	Deep Brain Stimulation
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
DREADD	Designer Receptor Exclusively Activated by Designer Drugs
DV	dorsoventral
EEG	Electroencephalography
FDA	Food and drug administration
GABA	Gamma Aminobutyric Acid
GIRK	G protein- coupled inwardly-rectifying potassium channels
GPCR	G-protein coupled receptor
HA	Human influenza hemagglutinin
hM1Dq	Human M1 muscarinic DREADD coupled to Gq
hM3Dq	Human M3 muscarinic DREADD coupled to Gq
hM4Di	Human M4 muscarinic DREADD coupled to Gi
hM5Dq	Human M5 muscarinic DREADD coupled to Gq
HPA	Hypothalamic-pituitary-adrenal axis
hSyn	Human synapsin
I.D.	Identification number
i.p.	intraperitoneal
ID	Inner diameter
IHKA	Intrahippocampal Kainic Acid
ILAE	International League Against Epilepsy
IP3	Inositol trisphosphate
ITR	Inverted terminal repeats
K	potassium
KA	Kainic acid
KAR	Kainic acid receptor
KORD	Kappa-opioid-receptor DREADD

LS	Lateral septum
ML	mediolateral
MRI	Magnetic resonance imagiging
MS	Medial septum
MWM	Morris water maze test
Na	sodium
NDMC	N-desmethyl-clozapine
NI DAQ	National instruments data acquisition
NMDA	N-methyl-D-aspartic acid
NOL	Novel Object Location test
NOR	Novel object recognition test
NpHR	Natronomonas halorhodopsin
NSAID	Nonsteroidal anti-inflammatory drug
OD	Outer diameter
OFT	Open field test
OPT	Object preference test
ORF	Open reading frame
ORI	Origin of replication
PDS	Paroxysmal depolarizing shift
PET	Positron Emission Tomography
PIP2	Phosphatidylinositol 4,5-bisphosphate
PVC	polyvinylchloride
QSI	Quintessential stereotaxic injector
RASSL	Receptor activated solely by a synthetic ligand
SE	Status Epilepticus
S.E.M	Standard error of the mean
SPECT	Single photon emission computed tomography
TLE	Temporal Lobe Epilepsy
VNS	Vagus nerve stimulation
WPRE	Woodchuck posttranscriptional regulatory element

2 MATERIALS AND METHODS

2.1 ELECTRODE AND CANNULA IMPLANTATION SURGERY

Fig I: **illustration of a bipolar depth electrode (left) and scalp electrode(right).** The bipolar depth electrode is used for measuring EEG-signals inside the seizure-focus, whilst scalp-electrodes are employed to cancel out artefacts and correct for non-epileptic brain-activity.

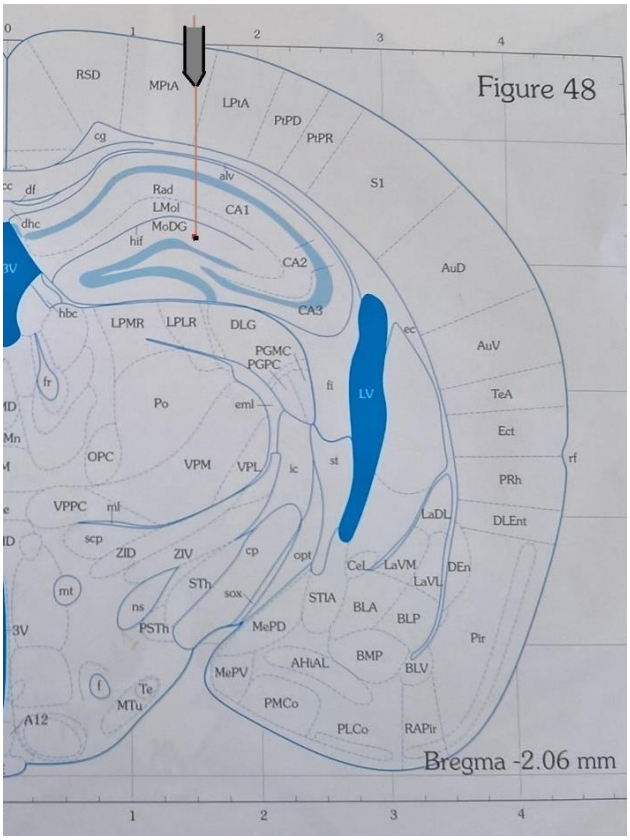


Fig II: **Location of bipolar depth-electrode and cannula in the hippocampus: coronal plane.** The cannula, indicated with the grey tube, is situated at -0.2 cm AP, -0.15 cm ML, -0.03 cm DV relative to bregma. The depth-electrode (indicated in brown) is implanted at -0.2 cm AP, -0.15 cm ML, -0.18 cm DV relative to bregma. The black and red tip are 200 µm apart. Figure adopted from Paxinos and Franklin mouse brain atlas [46].

2.2 BEHAVIOURAL TESTS

2.2.1 Object preference test

Table I: **Overview of items used for each animal during the OPT.** The OPT consisted of 2 sessions (1 session per day) for 5 minutes. Each animal was exposed to 4 objects in total. To note, during the NOL, animals were exposed to different objects than the 4 objects they had explored in the OPT.

ID	Items session 1	Items session 2	ID	Items session 1	Items session 2
387	Pumpkin - blocks	Coffee-sticks	397	Pumpkin - blocks	Cups - tube
389	Lamp - coffee	Penholder – pink water	390	Coffee - cups	Pumpkin - lamp
400	Blocks - tube	Jam - coffee	410	Blocks - tube	Pink water – penholder
407	Tube - sticks	Blocks- jam	393	Pink water - jam	Pumpkin – tube
398	Lamp - cups	Coffee - lego	394	Jam - penholder	Lamp - lego
402	Penholder - lamp	Jam - lego	403	Cups - pumpkin	Coffee- pink H20
391	Lego - pink water	Lamp - coffee			

2.2.2 Overview of the received treatment per trial for OFT and NOL

Table II: **Overview of the received treatment per trial for each mice.** The type of treatment was assigned to each individual animal in a randomized design. One animal died after during trial 3 of NOL after injection. The latter is indicated with ǂ

ID	Vector	Trial 1 OFT	Trial 2 OFT	Trial 3 OFT	Trial 1 NOL	Trial 2 NOL	Trial 3 NOL
387	SHAM	Vehicle	Clozapine	CNO	Vehicle	Clozapine	CNO
389	DREADD	CNO	Vehicle	Clozapine	CNO	Vehicle	Clozapine
400	DREADD	Vehicle	Clozapine	CNO	Vehicle	Clozapine	CNO
407	SHAM	Clozapine	CNO	Vehicle	Clozapine	CNO	Vehicle
398	SHAM	Vehicle	Clozapine	CNO	Vehicle	Clozapine	CNO
402	DREADD	Clozapine	CNO	Vehicle	Clozapine	CNO	Vehicle
391	SHAM	Clozapine	CNO	Vehicle	Clozapine	CNO	Vehicle
397	DREADD	CNO	Vehicle	Clozapine	CNO	Vehicle	Clozapine
390	DREADD	Vehicle	Clozapine	CNO	Vehicle	Clozapine	CNO
410	SHAM	CNO	Vehicle	Clozapine	CNO	Vehicle	Clozapine
393	SHAM	CNO	Vehicle	Clozapine	CNO	Vehicle	Clozapine
403	DREADD	Clozapine	CNO	Vehicle	Clozapine	CNO	Vehicle
394	SHAM	Vehicle	Clozapine	CNO	Vehicle	Clozapine	ǂ

2.2.2.1 Specifications for NOL

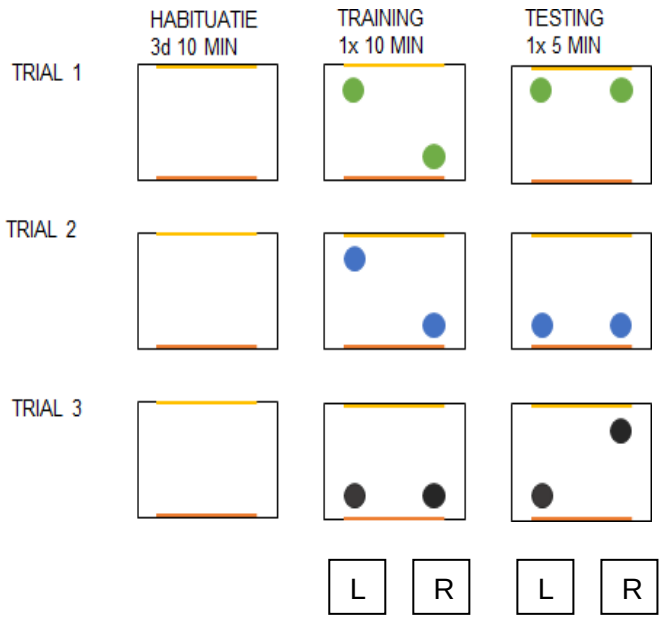


Fig III: **Schematic illustration of the NOL setup per trial.** The yellow and brown lines represent visual cues. The coloured circles represent the location of objects for each trial. L= left; R= right.

3 RESULTS

3.1 ACUTE EFFECT OF H4MDI-ACTIVATION ON TOTAL SEIZURE DURATION

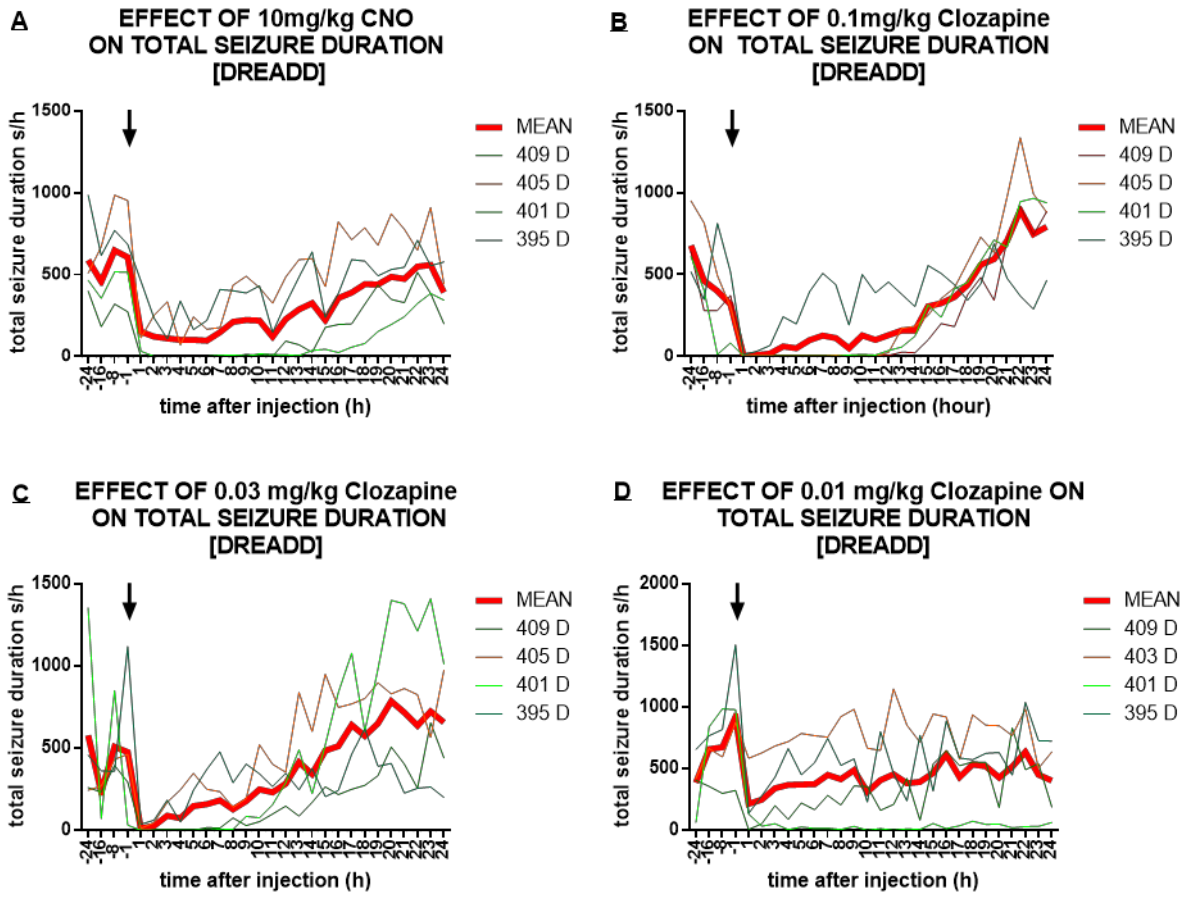


Fig IV: **Effect of treatment on total seizure duration: individual values. A:the effect of 10 mg/kg CNO; B: the effect of 0.1 mg/kg clozapine ; C: the effect of 0.03 mg/kg clozapine; D: the effect of 0.01 mg/kg clozapine.** The moment of injection is indicated with a black arrow. Individual values are shown for each mouse (n=4) . The total seizure duration is shown in red.

Table III: **Overview of detailed p-values for the comparison between treatments within a time period.** The effect of treatment on total seizure duration compared between different treatments within a fixed time period. Significant differences are marked (* $p < 0.05$)

	Baseline	1-8h after injection	9-16h after injection	17-24h after injection
10 mg/kg CNO vs 0.1 mg/kg Cloza	p > 0.999	p > 0.999	p > 0.999	p = 0.6025
10 mg/kg CNO vs 0.03 mg/kg Cloza	p > 0.999	p > 0.999	p > 0.999	p > 0.999
10 mg/kg CNO vs 0.01 mg/kg Cloza	p > 0.999	p = 0.7924	p > 0.999	p > 0.999
0.1 mg/kg Cloza vs 0.03 mg/kg Cloza	p > 0.999	p > 0.999	p > 0.999	p > 0.999
0.1 mg/kg Cloza vs 0.01 mg/kg Cloza	p > 0.999	p = 0.0243 (*)	p > 0.999	p > 0.999
0.03 mg/kg Cloza vs 0.01 mg/kg Cloza	p > 0.999	p = 0.1710	p > 0.999	p > 0.999

3.1.1 Descriptive results of the effect of treatment on total seizure duratio

Table IV: **Descriptive results of the time-dependent effect on total seizure duration within treatments** Total seizure duration (s) per hour ± SEM.
→ reduction of total seizure duration in comparison to baseline (%)

	Baseline	1-8h after injection	9-16h After injection	17-24h After injection
10 mg/kg CNO	575.4 ± 118.8	130.7 ± 75 →77.3 %	248.1 ± 120.6 →56.9 %	457.6 ± 113.7 →20.5%
0.1 mg/kg cloza	464.4 ± 80.76	59.3 ± 59 → 87.2 %	168.1 ± 82.5 →63.8 %	636.4 ± 81.88 → -37.0 %
0.03 mg/kg cloza	450.5 ± 73.68	102.2 ± 55.27 → 77.3 %	337.7 ± 91.29 →25.0%	674.5 ±186.4 → -49.7%
0.01 mg/kg cloza	661.3 ± 122.1	349 ± 157.7 →47.2 %	446 ± 176.5 →32.6 %	491.3 ± 159.8 →25.7 %

3.2 RESULTS OPT

Table V: **Descriptive results of the mean exploration time for each object during the OPT.** Mean exploration time for each object (s) ± S.E.M.

Object	Mean exploration time	Object	Mean exploration time (%) ± SEM
Pumpkin	47.13 ± 3.306	Cups	58.39 ± 1.544
Blocks	45.86 ± 1.398	Penholder	56.55 ± 3.951
Lamp	48.67 ± 2.651	Lego	48.82 ± 3.001
Coffee	48.80 ± 2.278	Pink water	48.73 ± 2.160
Tube	49.36 ± 2.59	Jam	47.05 ± 5.195
Sticks	53.31 ± 1.87		

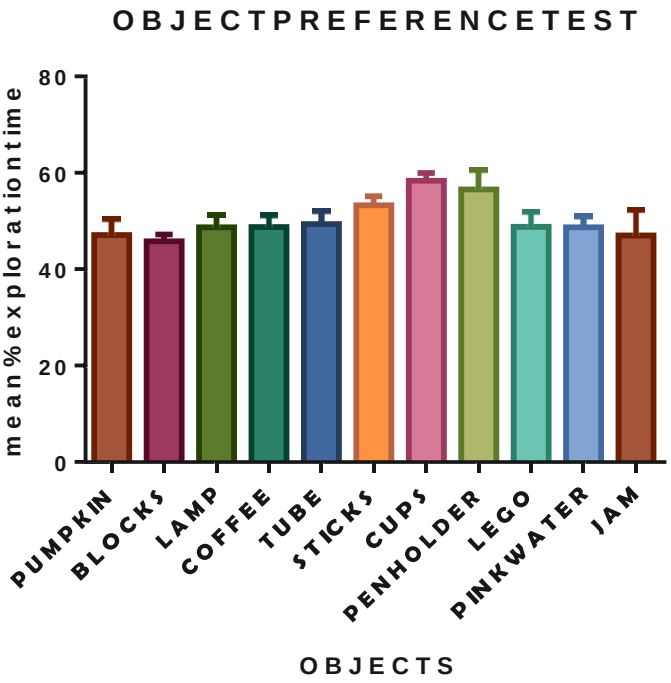


Fig V: **Results of OPT presented in a bar-chart.** Each bar illustrates the mean percentage exploration time per object. Error bars represents the S.E.M. Significant differences are indicated ($p < 0.05$ *) Statistical analysis (Kruskal- Wallis; post-hoc Dunn test) showed no significant differences between objects ($p = 0.1867$)

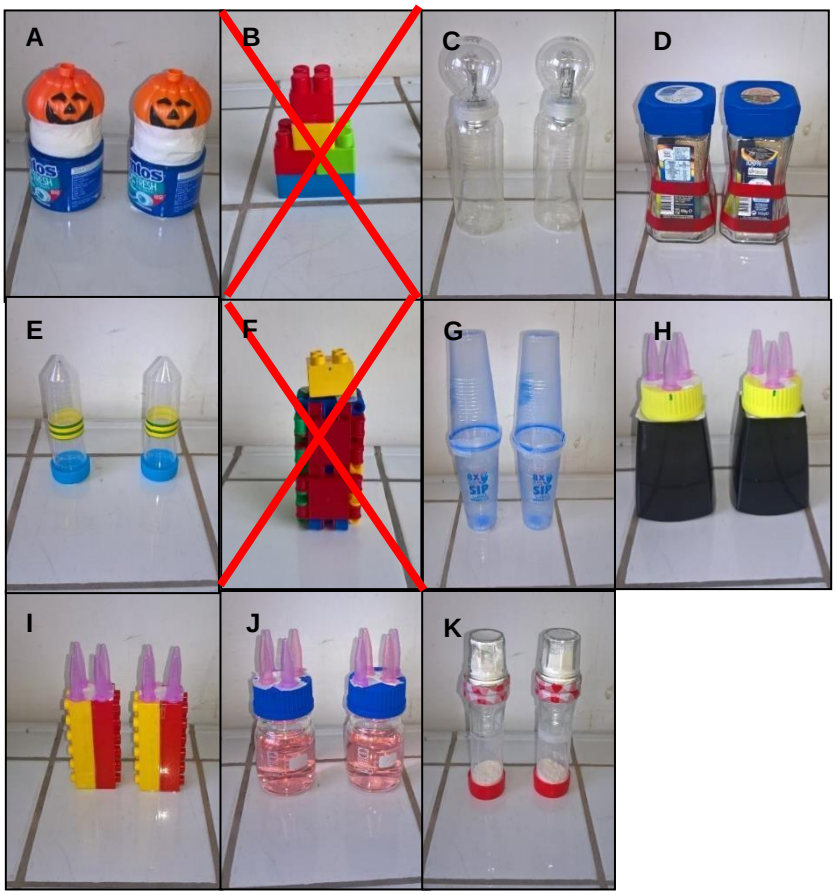


Fig VI: **Objects of OPT A** = pumpkin ; **B** = blocks ; **C** = lamp; **D** = coffee ; **E** = tube ; **F**= sticks; **G** = cups ; **H** = penholder ; **I** = lego ; **J** = pink water ; **K** = jam. The objects that were excluded from the NOL are indicated with a red cross.

3.3 RESULTS OFT+ NOL

3.3.1 Effect of hM4Di-activation on behavioural tests

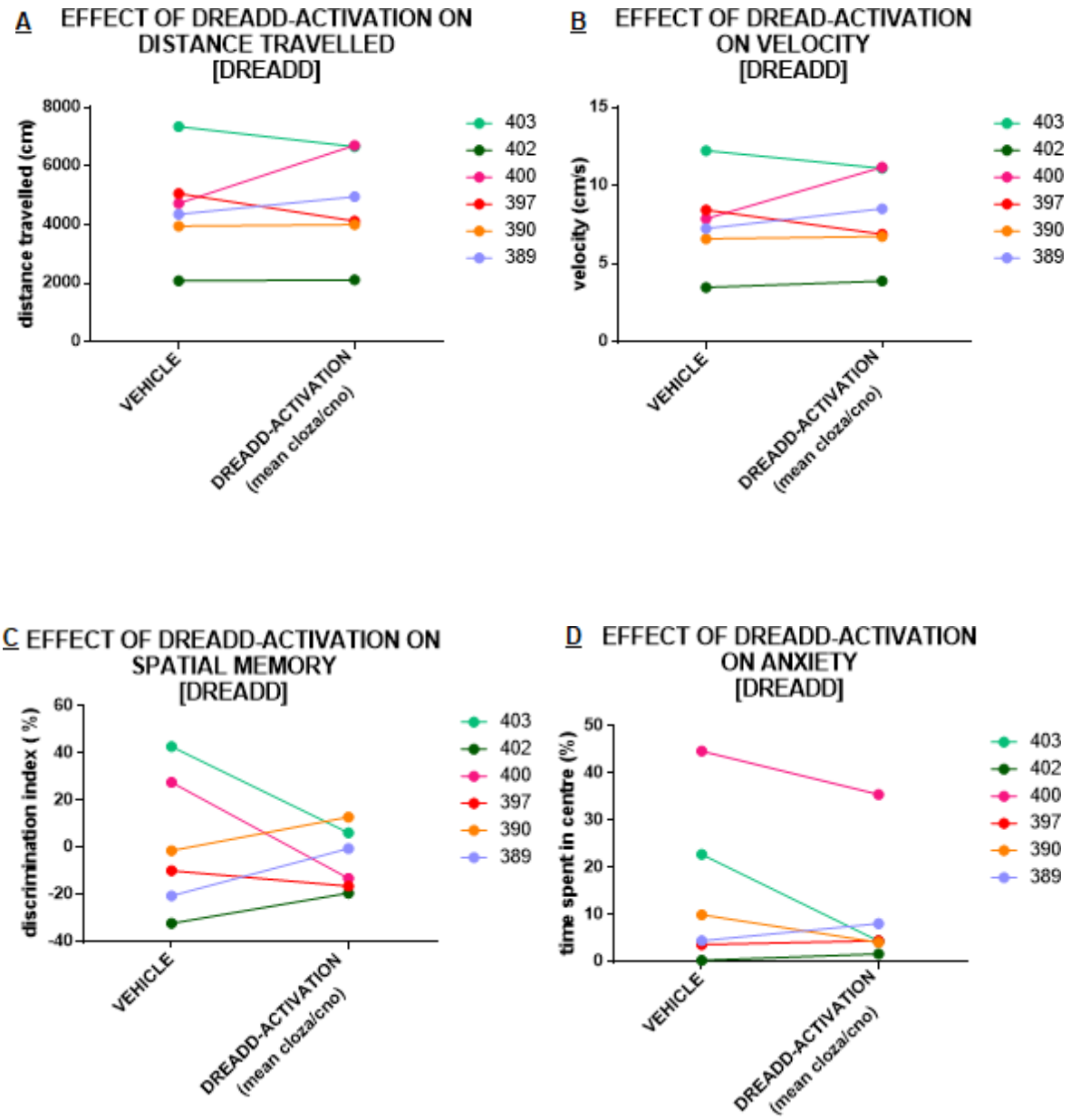


Fig VII: **Effect of hM4Di-activation on behavioural tests. A: Effect of mean DREADD activation on distance travelled. B: Effect of mean DREADD activation on average velocity. C: Effect of mean DREADD activation on spatial memory. D: Effect of mean DREADD-activation on anxiety.** The results of vehicle-injection are compared to the mean results of clozapine and CNO for each individual DREADD-animal.

Table VI: **Effect of hM4Di-activation on behavioural tests.** Statistical analysis was executed with the Wilcoxon matched-paired signed rank (two-sided) test. P-values are shown in table (* $p<0.05$ is considered significant. The mean values ± S.E.M. are provided in the table below.

	Distance travelled (cm) p = 0.8438	Velocity (cm/s) p = 0.6875	Spatial memory (%D.I.) p = 0.8438	Anxiety (% time in centre) p = 0.4375
Vehicle	4589 ± 697.6	7.651 ± 1.162	1.152 ± 11.76	14.36 ± 6.88
Mean DREADD-activation	4763 ± 717.3	8.057 ± 1.150	-4.960 ± 5.388	9.750 ± 5.22

3.3.3.1 DESCRIPTIVE STATISTICS

A. EFFECT OF TREATMENT ON DISTANCE TRAVELLED

Table VII: **Descriptive results of total distance travelled during 10 min of OFT.** Mean distance travelled (cm) ± SEM.

	Vehicle	Clozapine	CNO
DREADD	4589 ± 697.6	4880 ± 745.3	4647 ± 762.5
SHAM	5662 ± 454.0	5785 ± 543.6	5349±616.1

B. EFFECT OF TREATMENT ON VELOCITY

Table VIII: **Descriptive results of velocity during 10 min of OFT.** Mean velocity (cm/s) ± SEM.

	Vehicle	Clozapine	CNO
DREADD	7.651 ± 1.162	8.145 ± 1.239	7.968 ± 1.202
SHAM	9.464 ± 0.7714	9.641 ± 0.9059	8.922 ± 1.029

C. EFFECT OF TREATMENT ON ANXIETY

Table IX: **Descriptive results of percentage of time spent in the centre during the first 5 min of OFT.** Mean time spent in the centre (%) ± SEM.

	Vehicle	Clozapine	CNO
DREADD	14.36 ± 6.880	8.776 ± 4.561	10.72 ± 6.034
SHAM	12.74 ± 1.593	10.69 ± 2.819	11.32 1.32

D. EFFECT OF TREATMENT ON SPATIAL MEMORY

Table X: **Descriptive results of D.I during NOL.** Mean D.I.(%) ± SEM.

	Vehicle	Clozapine	CNO
DREADD	1.152 ± 11.76	-7.853 ± 4.348	-2.066 ± 6.894
SHAM	8.394 ± 6.624	-5.811 ± 8.709	6.985 ± 8.621

E. VALIDATION OF SOFTWARE

Table XI: **Descriptive results of software validation.** Mean values ± SEM are provided for each behaviour assessed with Optimouse, Optimouse corrected and Ethovision

	Optimouse	Optimouse corrected	Ethovision
Time spent in centre (%)	16.27 ± 3.536	16.27 ± 3.536	17.94 ± 3.877
Distance travelled (cm)	5776 ± 544.4	5774 ± 545.3	5025 ± 466.9
Velocity (cm/s)	9.602 ± 0.9042	9.542 ± 0.9019	8.393 ± 0.7857
D.I. of NOL-training(%)	-8.035 ± 5.445	-4.637 ± 5.161	1.093 ± 3.436

3.3.2 EFFECT OF hM4Di- EXPRESSION ON BEHAVIOURAL TESTS

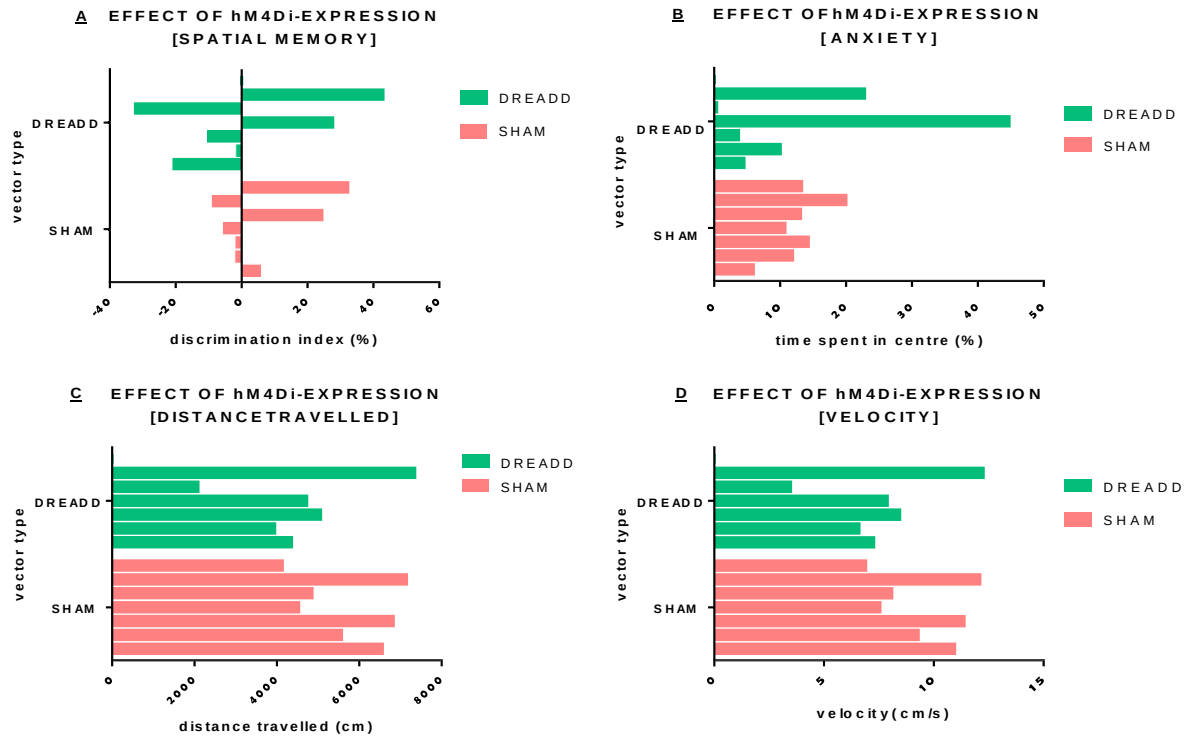
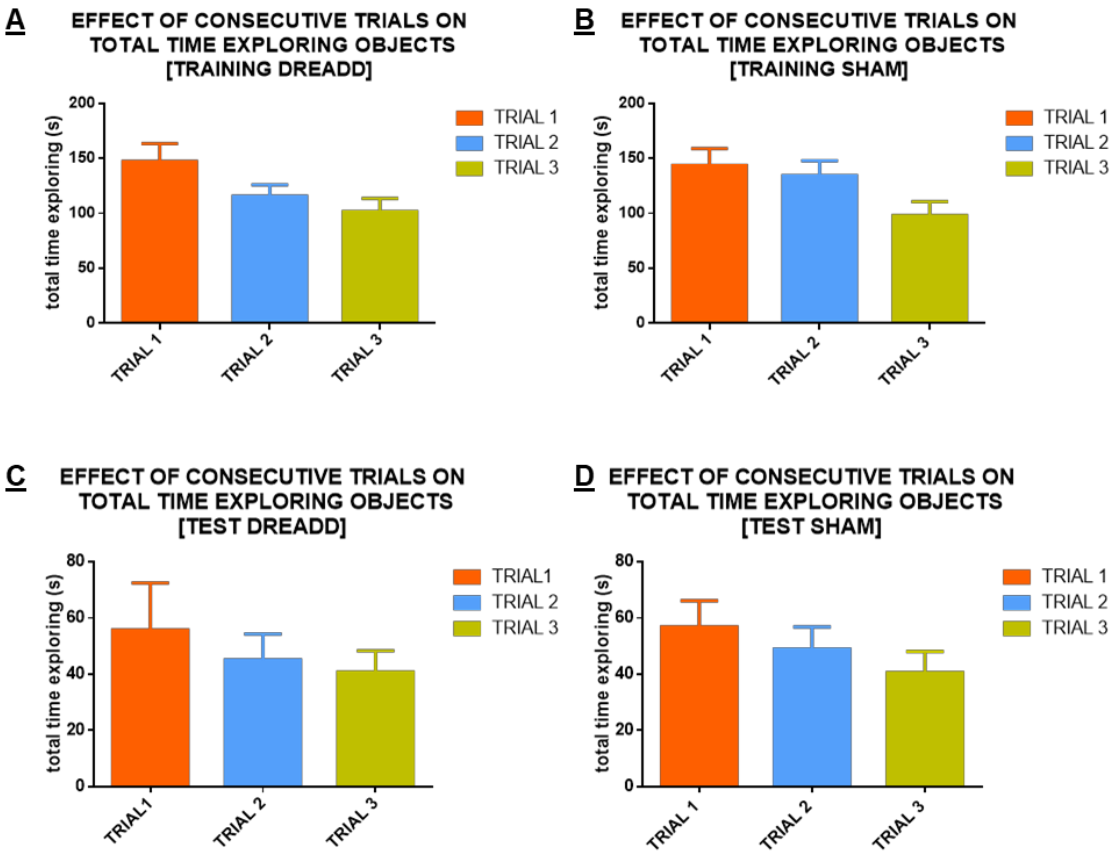


Fig VIII: **Effect of hM4Di-expression on behavioural tests. A: Effect on spatial memory. B: Effect on anxiety. C: Effect on distance travelled. D: Effect on velocity.** Individual results are shown of vehicle-treated DREADD- and SHAM- animals.

Table XII: **Descriptive results of software validation.** Mean values ± SEM are provided for each behaviour assessed with Optimouse, Optimouse corrected and Ethovision

	P-value	DREADD- animals	SHAM- animals
Spatial memory	p = 0.6037	1.152 ± 11.76	6.468 ± 5.921
Anxiety	p = 0.4312	14.36 ± 6.81	12.74 ± 1.593
Distance	p = 0.2890	4589 ± 697.5	5662 ± 454
Velocity	p = 0.2890	7.651 ± 1.162	9.464 ± 0.7714

3.3.3 EFFECT OF CONSECUTVE TRIALS ON BEHAVIOURAL TESTS



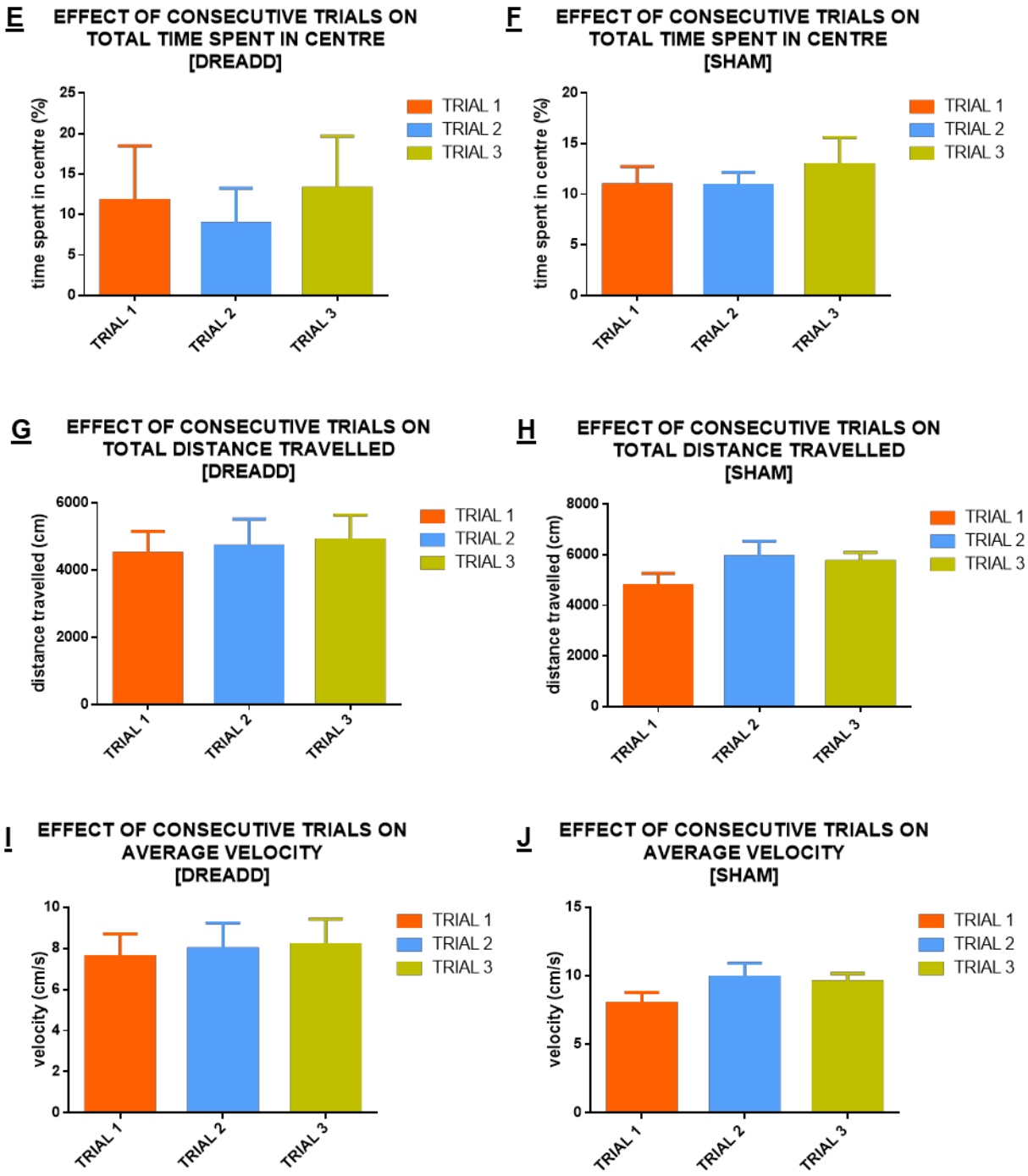


Fig IX: **Effect of consecutive trials on behavioural tests. A: Exploration during training in DREADD-mice. B: Exploration during training in SHAM- mice. C: Exploration during testing in DREADD-mice. D: Exploration during testing in SHAM-mice. E: Time spent in centre in DREADD-mice. F: Time spent in centre in SHAM-mice. G: Total distance travelled in DREADD-mice. H: Total distance travelled in SHAM-mice. I: Average velocity in DREADD-mice. J: Average velocity in sham-mice.** Mean values are shown in bar charts. Error bars indicate the S.E.M. Significant differences are marked(* $p<0.05$).

Table XIII: **Descriptive results of the effect of consecutive trials on behavioural tests.** Mean values ± SEM are provided for each behaviour assessed in trial 1,2, and 3 for both DREADD - and SHAM - animals

	Trial 1 DREADD	Trial 2 DREADD	Trial 3 DREADD	Trial 1 SHAM	Trial 2 SHAM	Trial 3 SHAM
Explor. Training (s)	147.2 ± 16.10	115.6 ± 10.32	101.8 ± 11.8	145.5 ± 15.42	134.2 ± 13.7	97.94 ± 12.65
Explor. Testing (s)	55.79 ± 16.73	45.16 ± 9.202	40.75 ± 7.613	56.91 ± 9.26	48.99 ± 7.936	40.6 ± 7.502
Time spent in centre (%)	11.73 ± 6.728	8.893 ± 4.359	13.23 ± 6.445	10.94 ± 1.804	10.88 ± 1.315	12.93 ± 2.675
Distance (cm)	4504 ± 649.8	4717 ± 803.1	4898 ± 741.8	4792 ± 476.4	5939 ± 605.5	5738 ± 359.7
Velocity (cm/s)	7.592 ± 1.115	7.989 ± 1.246	8.183 ± 1.234	7.989 ± 0.7938	9.898 ± 1.009	9.569 ± 0.6036

3.3.4 SPECIFICATIONS FOR NOL

3.3.4.1 Time spent exploring objects during training

Table XIV: **Time spent exploring objects during training.** O= object; T= trial.
Mice who did not succeed to explore more than 38s are underlined. ↑indicates the death of an animal. [1]The training of 1 animal took 20 minutes as the first 10 minutes were not recorded. The animal did not exceeded the prolonged 38s of exploration during the last 10 minutes. Presumably, the animal has spent more than 38s exploring the objects during the full 20 minutes of training.

ID	O1 T1	O2 T1	O1 T2	O2 T2	O1 T3	O2 T3
387	101,132	99,2657	97,499	51,2995	38,8329	44,0329
389	88,8658	72,3326	40,6996	47,7995	51,8995	44,9329
390	55,6328	67,8327	38,5329	46,1662	27,7997[1]	21,1331[1]
391	105,799	62,3994	55,2994	56,9328	42,7996	40,3996
393	67,8327	63,066	64,0327	73,5326	70,466	65,5993
394	61,4994	47,5329	48,1995	41,6329	↑	↑
397	79,1992	76,4992	69,866	57,8661	73,1659	58,4994
398	66,5993	84,2325	36,8663	41,9662	28,4997	31,4997
400	105,266	88,1325	66,6993	54,3995	51,7328	69,7993
402	37,6996	43,4996	49,9995	69,7326	53,8995	47,4662
403	105,999	62,4994	67,466	84,3658	54,5995	55,9661
407	49,0995	65,6993	55,1661	101,666	70,166	65,2327
410	35,9996	60,0994	66,7327	104,099	46,8329	43,2996

3.3.4.2 Place preference

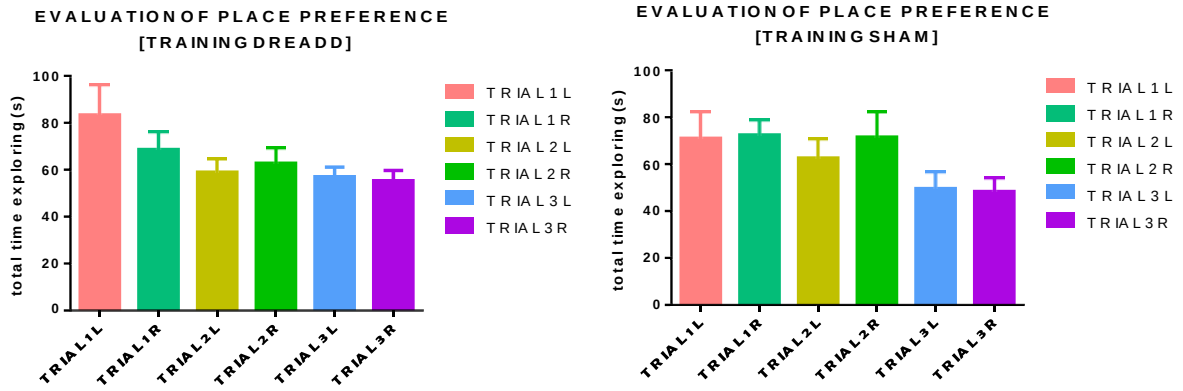


Fig X: **Evaluation of place preference during training. A: Place preference in DREADD-animals. B: Place preference in SHAM-animals.** L = left ; R = Right. Mean values are shown in bar charts. Error bars indicate the S.E.M. Significant differences are marked(* p<0.05).

Table XV: **P-values and descriptive results of the evaluation of place-preference** L = left; R = right. P-values for the DREADD- and SHAM- group were calculated with the Friedman-test and are provided in the table below. Mean values for total exploration time (s) ± S.E.M. are shown for DREADD- and SHAM- animals.

	Trial 1 L	Trial 1 R	Trial 2 L	Trial 2 R	Trial 3 L	Trial 3 R
DREADD (p= 0.3627)	83.4 ± 12.50	68.59 ± 7.5	58.95 ± 5.76	62.83 ± 6.456	57.06 ± 4.065	55 ± 4.414
SHAM (p= 0.1562	71.08 ± 11.33	72.46 ± 6.442	62.6 ± 8.181	71.58 ± 10.75	49.6 ± 7.008	48.34 ± 5.692

3.3.4.3 Effect of treatment on total exploration time

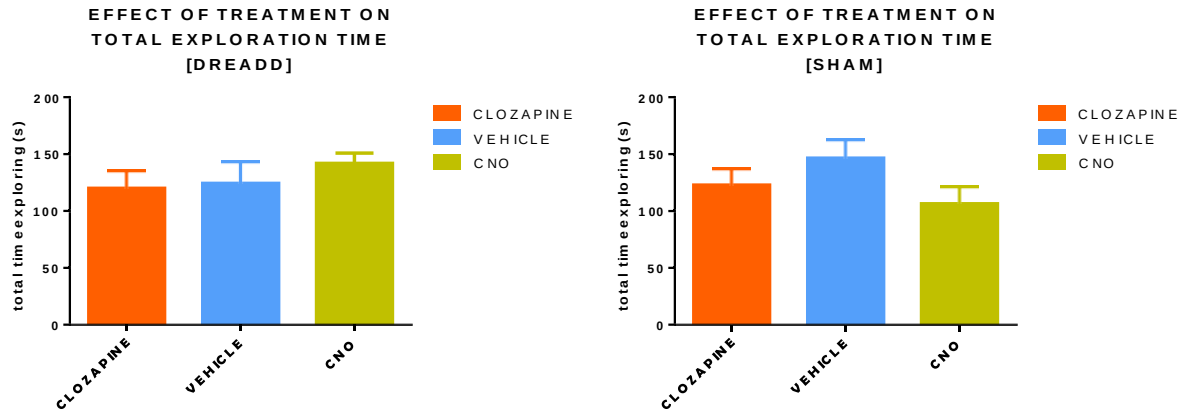


Fig XI: **Effect of treatment on total exploration time during training. A: Total exploration time during training for each treatment in DREADD-animals. B: Total exploration time during training for each treatment in SHAM-animals**. Mean values for total exploration time (s) are shown in bar charts for each treatment. Error bars indicate the S.E.M. Significant differences are marked(* p<0.05).

Table XVI: **P-values and descriptive results of the effect of treatment on total exploration time during training.** P-values for the DREADD- and SHAM- group were calculated with the Friedman-test and are provided in the table below. Mean values of total exploration time (s) ± S.E.M. are shown for DREADD- and SHAM- animals for each treatment.

	DREADD (p= 0.3673)	SHAM (p= 0.4297)
Clozapine	119.9 ± 15.05	122.8 ± 14.12
Vehicle	124.3 ± 18.42	146.4 ± 16.04
CNO	142.0 ± 8.833	106.5 ± 14.13