Properties of glutamate receptor subunit GluN2B antagonists and effects of prenatal opioid exposure – studies in chicken and rat

Thesis for the degree of Philosophicae Doctor
Acknowledgements

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Oslo, September 2019
Marthe Fredheim Fjelldal
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Summary of thesis

N-methyl-D-aspartate receptors (NMDARs) are important for brain development and function. They are involved in learning, memory and the formation of long term potentiation (LTP) and long term depression (LTD). Changes in the GluN2B receptor subtype distribution and expression are associated with several neurodegenerative pathologies such as Parkinson’s disease, Alzheimer’s disease and ischemic brain injury. This has also been observed in psychological disorders, such as schizophrenia and major depression. NMDARs have been linked to development of opioid tolerance and addiction, a research area of particular interest as the opioid crisis continues unabated in the US. Despite the clear potential, no drugs or probes targeting the GluN2B subunit are available for clinical use today. The aim of this thesis was to expand the knowledge of NMDAR pharmacology, concentrating on GluN2B specific antagonists. We established the chicken embryo forebrain calcium (Ca^{2+}) influx assay and utilised it to investigate the inhibition potency of the known GluN2B specific antagonists Ro 25-6981, ifenprodil, eliprodil, EVT-101 and Ro 04-5595. Chicken embryos are a desirable alternative to rodent models because they require less animal facilities and are not considered animals until embryonal day (E) 14, contributing to the 3R principle. The GluN2B expression in forebrain and cerebellum during development was found to be comparable to the expression in rat and human. The resulting IC_{50}-values were compared to predicted binding properties obtained with in silico modelling. The interesting compound Ro 04-5595 was predicted to bind the recently discovered EVT-101 binding site, prompting us to establish an order of Ca^{2+} inhibition potency of a selection of new potential GluN2B specific antagonists derived from Ro 04-5595. The Ca^{2+} influx assay was successfully transferred to chicken cerebellar cultures, where it was utilised to examine the effects of short-term in ovo opioid exposure on NMDAR function. It was found that both methadone and morphine increased NMDA/glycine stimulated Ca^{2+} influx. GluN2B expression in the opioid-exposed cerebella was examined, but no change was detectable. However, in rat pups exposed to buprenorphine or methadone during gestation, a significant decrease in GluN2B expression was observed in buprenorphine-exposed rat pups at postnatal day (PND) 14. The thesis concludes that the chicken model can be used to obtain vital information about GluN2B specific antagonists in combination with in silico techniques, and be utilised to assess opioid effects on cerebellar development.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AAT</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ABD</td>
<td>Agonist-binding domain</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxyethylmethyl</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
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<tr>
<td>CaM</td>
<td>Calmodulin</td>
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<tr>
<td>CAM</td>
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<td>CaMKs</td>
<td>Ca$^{2+}$/CaM-dependent protein kinases</td>
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<td>cCGC</td>
<td>Chicken cerebellar granule cell</td>
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<tr>
<td>cEFC</td>
<td>Chicken embryo forebrain cell</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
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<td>Carboxyl-terminal domain</td>
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<td>E</td>
<td>Embryonal day</td>
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<td>Excitatory amino acid transporters</td>
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<td>External granule layer</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>GC</td>
<td>Granule cell</td>
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<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthase</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granule layer</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
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<tr>
<td>MD</td>
<td>Molecular dynamic</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>MM-GBSA</td>
<td>Molecular Mechanics/Generalised-Born Surface Area</td>
</tr>
<tr>
<td>NAS</td>
<td>Neonatal abstinence syndrome</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NTD</td>
<td>N-terminal domain</td>
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<tr>
<td>OR</td>
<td>Opioid receptor</td>
</tr>
<tr>
<td>PCL</td>
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<tr>
<td>PKC</td>
<td>Phosphokinase C</td>
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<tr>
<td>PND</td>
<td>Postnatal day</td>
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<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
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<td>RyR</td>
<td>Ryanodine receptors</td>
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<tr>
<td>SERCA</td>
<td>Sarcoplasmic reticulum Ca$^{2+}$ ATPase</td>
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<tr>
<td>STIM</td>
<td>Stromal interaction molecule</td>
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List of Publications

Paper I

Exploring the overlapping binding sites of ifenprodil and EVT-101 in GluN2B containing NMDA receptors using novel chicken embryo forebrain cultures and molecular modelling.


Paper II

Functional analysis of the EVT-101 binding site using new GluN2B non-competitive antagonists.

Marthe Fredheim Fjelldal, Jimmy E. Jakobsson, Ragnhild Paulsen.

*Unpublished manuscript.*

Paper III

Opioid receptor-mediated changes in the NMDA receptor in developing rat and chicken.

Marthe Fredheim Fjelldal, Mussie Ghezu Hadera, Mette Kongstorp, Lars Peter Engeset Austdal, Ana Šulović, Jannike Mørch Andersen, Ragnhild Elisabeth Paulsen

1. Introduction

Glutamate is a major neurotransmitter in the CNS (1.1), activating NMDARs, among other receptors (1.2). NMDAR expression and function has been associated with several neurodegenerative diseases, making it a prioritised target for probe and drug development (1.3). Activation of NMDARs causes calcium (Ca^{2+}) influx, leading to intracellular signalling and potential propagation of nerve signals (1.4). Opioid receptors (ORs) have been shown to interact with NMDARs (1.5) and in light of the ongoing opioid crisis in the US and increased opioid abuse in the rest of the world (1.6), it is important to investigate potential harmful effects and the mechanisms driving addiction and drug tolerance (1.7-1.8). The development of the cerebellum is dependent on normal NMDAR and OR expression, and is therefore a plausible target for harmful defects related maternal drug abuse (1.9-1.10).

1.1 Neurotransmitters in the CNS

To be defined as a neurotransmitter a compound must be synthesised in the neuron, be present at the presynaptic terminal and have a specific mechanism to remove it after signal transduction is completed, according to Dales’ principles. The neurotransmitter needs to elicit a defined effect on the postsynaptic neuron and this effect must be reproducible when administering the compound exogenously. Two classes of neurotransmitters exists in the central nervous system (CNS): Small-molecule transmitters (e.g. glutamate) and neuroactive peptides (e.g. endogenous opioid peptides) (1).

The non-essential amino acid L-glutamate belongs to the former category, and is packed in small synaptic vesicles before exocytosis into the synapse. L-glutamate is produced in the neurons from α-ketoglutarate, a constituent in the carboxylic acid cycle (1). It is taken up from the synapse by high-affinity excitatory amino acid transporters (EAATs), primarily into astrocytes. In the astrocytes the L-glutamate can either be directly converted to glutamine by the glial enzyme glutamine synthase (GS) or transformed to α-ketoglutarate by glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT). The α-ketoglutarate enters the tricarboxylic acid (TCA)-cycle and is oxidatively metabolised. Glutamine can be released extracellularly where it is taken up by neurons and deaminated to L-glutamate by phosphate-activated glutaminase (PAG) (figure 1). However, the importance of the glutamine-L-glutamate cycle for L-glutamate synthesis is still debated (2).
Disrupted glutamate homeostasis in the synaptic cleft has been associated with neurological diseases such as stroke, epilepsy and amyotrophic lateral sclerosis (ALS). The glutamate concentration is therefore tightly regulated by a fine balance of release and clearance of the neurotransmitter (3). It is removed from the synaptic cleft by EEATs, which are located in astroglial cells and nerve terminals (4). To date, five such transporters have been identified (EEAT1-5) and the EEAT2 is dominant in the CNS (3).

Figure 1: Schematic overview of the glutamate-glutamine cycle. Glutamate is released from the presynaptic neuron and taken up into the astrocyte. In the astrocyte it is converted to either glutamine or α-ketoglutarate. The glutamine is shuttled back to the neuron where it is transformed back to glutamate, while the α-ketoglutarate enters the TCA-cycle (figure reprinted with permission from (5)).
For example, excitotoxicity, stemming from impaired glutamate transport is thought to contribute to the clinical picture of ALS. Therefore, substantial research effort has been invested in increasing EEAT2 activity, aiming to remove excess glutamate from synapses (6).

1.2  *N*-methyl-α-aspartate receptors

The ionotropic NMDAR family consists of three subunits: GluN1, GluN2 and GluN3 in a tetrameric assembly. Eight different splice variants of GluN1 exist, originating from one gene. There are four variants of GluN2-subunits (A-D), encoded by four genes, and two GluN3 types (A-B) encoded by one gene each (7). The receptors can be heterodimeric or heterotrimeric, but must always contain two GluN1 subunits. Crystal structures of complete NMDA receptors have shown that the subunits are arranged in an alternating pattern. The subunits consist of four domains: The N-terminal domain (NTD) and the agonist-binding domain (ABD), both located extracellularly, while the transmembrane domain (TMD) crosses the cell membrane three times. The carboxyl-terminal domain (CTD) is situated intracellularly and may interact with PSD-95, calmodulin and neurofilament subunit NF-1 (8), depending on which subunit the CTD belongs to. The CTDs can also be targeted by kinases and phosphatases (9), and is the domain with largest sequence variance and length between the subunits (7).

The ionotropic L-glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is transiently activated by L-glutamate, leading to influx of sodium and Ca$^{2+}$ that depolarises the membrane (10). This depolarisation leads to the removal of the endogenous voltage-dependent NMDAR pore blocker magnesium (Mg$^{2+}$) and enables L-glutamate to activate the NMDARs. The binding site for L-glutamate resides in the GluN2 subunit. Although the activation of NMDARs is slow, the amount of ion influx can be substantial due to the longevity of the open channel state (11).

The mandatory subunit, GluN1, contains the small hydrophobic binding site for glycine or D-serine, which is located within the ABD. Glycine is thought to be released both from astroglial cells, and glutaminergic axon terminals (12), while D-serine originates from glial cells (13). These co-agonists are present extracellularly in relatively stable concentrations, compared to L-glutamate, which is released as a response to a nerve signal and taken up by L-glutamate transporters after the signal has receded. A study found D-serine to be the primary co-agonist for NMDARs located in the synapse, while glycine dominated in NMDARs located
extrasynaptically (14) although these conclusions are not universally agreed upon. It has been shown that GluN2A-containing NMDARs have a higher affinity for D-serine, while GluN2B-containing NMDARs have a higher affinity for glycine (15) (16). Glycine and D-serine are present in non-saturating concentrations extracellularly, which adds another level of receptor activation control (figure 2) (14).

The expression of the different subunits varies during development, with the exception of the mandatory GluN1 subunit. GluN2A can be detected shortly after birth in the rat, and steadily progresses to be expressed at high levels in all CNS areas. GluN2B and GluN2D are the only

Figure 2: The structure of the NMDA receptor, showing the location of the N-terminal domain (NTD), agonist-binding domains (ABD), pore domains and C-terminal domains (CTD). The binding sites for glycine/D-serine, glutamate and pore blockers are indicated on the figure (Modified with permission from (17)).
subunits found in the embryonic brain. The amount of Glu2B peaks postnatally in the forebrain and then declines slightly until it reaches a stable level in adulthood (7), while in the cerebellum it peaks simultaneously with the granule cell (GC) migration around PND14. When the migration is completed, GluN2B is gradually replaced by GluN2C, which is continuously expressed from that time point (18). GluN2C is otherwise found in the adult olfactory bulb, while GluN2D is restricted to the mesencephalon and diencephalon (figure 3). GluN3A is only found prenatally, while GluN3B is highly expressed in motor neurons (7).

![Figure 3: The expression of NMDAR subunit mRNA in the rat brain during development. GluN1 is constitutively expressed in the whole CNS. GluN2B is transiently expressed in the cerebellum, while gradually increasing in the forebrain up until adulthood. The expression of GluN2A gradually increases from birth, in both forebrain and cerebellum. The expression of GluN2C starts in the cerebellum between PND7 and PND11, while GluN2D is strongly expressed in the midbrain before the expression gradually declines (Modified with permission from (18)). Most NMDARs are found in the post-synaptic area of neurons, but they can move laterally in the membrane and have also been demonstrated presynaptically. The receptors with GluN2B subunits are particularly prone to change their location between synaptic and extrasynaptic sites while GluN2A-containing receptors are thought to be associated with the postsynaptic density (PSD) in the synapse of mature neurons (19). Receptors with GluN2C and GluN3 subunits can also be found in both astrocytes and in oligodendrocytes (7).]
The GluN2B receptor subtype is thought to be involved in excitotoxic pathways following excessive glutamate release, especially when located extrasynaptically. Toxic insults, or brain trauma such as stroke or epilepsy are associated with over-activation of NMDA receptors and a rise in extracellular glutamate. NMDA receptor-mediated Ca\(^{2+}\) entry leads to excessive generation of reactive nitrogen species, dysregulation of calcium dependent proteases in the cytosol, mitochondrial depolarisation with release of apoptotic factors and reactive oxygen species formation. The GluN2B receptor subtype in particular has been implicated in this mechanism (20, 21) although this issue has not been resolved yet. The NMDA receptor-mediated calcium disruption of mitochondrial metabolism is also implicated in several chronic neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease and ischemic brain injury. Changes in the distribution and expression of GluN2B are also associated with psychiatric disorders, such as schizophrenia and major depression (22). Mutations in the GRIN1 and GRIN2A/B genes (coding for GluN1 and GluN2A/B subunits, respectively), have been associated with epilepsy and schizophrenia in humans (23, 24). As such, NMDARs are very interesting targets for drugs and diagnostic probes.

1.3 NMDAR modulators

Several NMDAR inhibitors and subunit-specific allosteric antagonists are commercially available. Among them are MK-801 (non-specific inhibitor, also known as dizocilpine), ifenprodil and Ro 25-6981 (GluN2B-specific allosteric antagonists). The binding site of the NMDAR blocker MK-801 was recently located inside the channel vestibule, and MK-801 is considered to bind the open, activated channel (25). The compound has anticonvulsant and anaesthetic properties, and is neuroprotective in ischemia (26). However, due to its psychomimetic effects it is precluded from clinical use (27, 28). These side effects are probably caused by the total NMDA channel block by an inhibitor with high affinity, and its longevity of binding. Thus, it appears that the NMDA receptors must retain at least partial function to ensure normal brain function. This can be obtained by using low-affinity inhibitors, such as the Alzheimer drug memantine (25), or subunit-specific antagonists.

Ifenprodil was one of the first GluN2B subunit-specific allosteric antagonists, which binds to the polyamine site in the NTD (29). Ifenprodil gave rise to several other GluN2B specific antagonists, among them Ro 25-6981, which shares binding site with ifenprodil. This
antagonist displays less unspecific binding and more potency than ifenprodil (30). Due to a mixed population of NMDA receptor subunit composition in the brain, the Ca\(^{2+}\) block inferred by these antagonists will never be complete (31), which makes them more relevant in terms of clinical use. Competitive antagonists also exists, such as CGP-39551 that binds to the glutamate binding site (32), and 1-thioxo-1,2-dihydro-[1,2,4]triazolo[4,3-a]quinoxalin-4(5)hone (TK40) that binds to the glycine binding site (33).

Mg\(^{2+}\) and zinc (Zn\(^{2+}\)) are two well-known endogenous NMDAR antagonists, albeit with quite different inhibition mechanisms. Mg\(^{2+}\) binds in the NMDAR channel at the same site as memantine and blocks Ca\(^{2+}\) influx (34). The blockage is voltage dependent, so at resting potential the block is close to complete, while an action potential will release Mg\(^{2+}\) and allow Ca\(^{2+}\) influx. This gives NMDARs a coincidence detection property, the receptor needs to be activated by glutamate and glycine/D-serine at the same time as the action potential lifts the Mg\(^{2+}\) inhibition, features that are thought to be important for long-term potentiation (LTP) formation (reviewed in (35)).

Zn\(^{2+}\) on the other hand, only inhibits Ca\(^{2+}\) influx in GluN2A and GluN2B subunit containing NMDARs. GluN2A is more sensitive to Zn\(^{2+}\) than GluN2B, as GluN2A is affected by nanomolar concentration while GluN2B requires micromolar concentrations of Zn\(^{2+}\) to be inhibited. The binding site of Zn\(^{2+}\) is located in the NTD in both subunits, and in GluN2B it has been shown to overlap the binding site of ifenprodil. The effect of Zn\(^{2+}\) in GluN2A is strongly dependent on pH, with higher maximal inhibition at higher pH. However, changes in pH does not affect Zn\(^{2+}\) inhibition effect on the GluN2B subunit (36).

Positive receptor modulators also exist. The polyamine compound spermine is an endogenous potentiator of the GluN1/GluN2B receptors. Its binding site is predicted to be located in the lower lobes of the ATDs of GluN1/GluN2B subunits, interacting with acidic residues and overlapping with the ifenprodil binding site. Thus, both of these positive and negative modulators bind within the polyamine site (37). When bound, spermine potentiates agonist-induced currents and increases glycine affinity, possibly by stabilising the lobes in an open conformation (38) (figure 4).
Figure 4: Crystal structure of GluN1/GluN2B NMDA receptor. The CTD is omitted, and the binding sites for allosteric antagonists, channel blockers and glutamate/glycine competitive antagonists are indicated in the figure (modified with permission from (39)).

1.4 Calcium as a signal molecule in the CNS

Calcium ions are important signal molecules in the CNS. Intercellular Ca$^{2+}$-dependent signalling can be divided into gap junction signal communication and paracrine signal communication. Gap junctions are ubiquitous in the CNS and enable cell-to-cell transfer of chemical and electrical signal. However, transmission is slow because cytoplasmic calcium buffers hinder Ca$^{2+}$ diffusion. The Ca$^{2+}$-mobilising messenger inositol trisphosphate (InsP$_3$) can also diffuse through the junctions and indirectly lead to increased intracellular Ca$^{2+}$ concentration in neighbouring cells (40).

Paracrine signal communication is characterised by cell communication mediated by an extracellular messenger, resulting in elevated cytosolic Ca$^{2+}$ concentration in a neighbouring cell. Examples of these messengers are glutamate, nitric oxide (NO) and adenosine triphosphate (ATP). Thus, glutamatergic neurons communicate by paracrine signalling, translating electrical currents to chemical signals. An action potential in the presynaptic cell activates voltage-gated channels, including Na$^+$, K$^+$, and Ca$^{2+}$ channels. Of these, Ca$^{2+}$ channel activation is involved in transmitter release. The extracellular Ca$^{2+}$ concentration can be up to
four orders of magnitude higher than the intracellular concentration, which ensures a rapid and large influx when the channels open. In turn, this leads to transmitter vesicle release within 0.2 ms after Ca$^{2+}$ entry. The amount of released transmitter is proportional with the duration of the action potential (41).

To uphold the concentration gradient over the plasma membrane, the concentration of intracellular free Ca$^{2+}$ in neurons is tightly regulated. This is very important because Ca$^{2+}$ acts as a signal molecule, not only in nervous signalling, but also secretion, gene expression, apoptosis and metabolism. In a resting cell the gradient is kept stable by the plasma membrane Ca$^{2+}$ transporter ATPase (PMCA), and the Na$^+$ Ca$^{2+}$ exchanger. At elevated Ca$^{2+}$ levels, the excess ions are transported by the sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) into the Ca$^{2+}$ storage unit, the endoplasmic reticulum. Ca$^{2+}$ is also stored in the mitochondria, transported there by the mitochondrial Ca$^{2+}$ uniporter (mtCU). The transporters are activated by elevated cytosolic levels of Ca$^{2+}$ (reviewed in (42)).

The Ca$^{2+}$ stored in the endoplasmic reticulum (ER) is important in intracellular signalling. Activation of G-coupled receptors located in the plasma membrane (such as the group I metabotropic glutamate receptors (43)), in turn activates phospholipase C (PLC). PLC hydrolyses 4,5-biphosphate (PIP$_2$) phosphatidylinositol into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ activates the IP$_3$ receptor, which is located in the ER membrane. Upon activation, it opens and releases Ca$^{2+}$ into the cytosol (44). The Ca$^{2+}$ stored inside the ER can also be released by Ca$^{2+}$-mediated activation of the ryanodine receptors (RyR), located in the ER membrane (figure 5) (reviewed in (45)).
Figure 5: Schematic overview of Ca\textsuperscript{2+} mobilisation from ER storage. An activated G-protein coupled receptor (GPCR) activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP\textsubscript{3}). IP\textsubscript{3} activates the IP\textsubscript{3} receptor that releases Ca\textsuperscript{2+} into the cytosol. The ryanodine receptor (RyR) is activated by Ca\textsuperscript{2+} and also releases Ca\textsuperscript{2+} into the cytosol.

Under resting condition the SERCA is sufficient to maintain the Ca\textsuperscript{2+} homeostasis in the ER, but the Ca\textsuperscript{2+} level in the ER can be depleted by intensive Ca\textsuperscript{2+} signalling. The store-operated calcium channels (SOCs) are activated by Ca\textsuperscript{2+} dissociation from the ER transmembrane stromal interaction molecule (STIM) 1 or STIM2 proteins, leading to the release of the store-operated Ca\textsuperscript{2+} entry (SOCE)-associated regulatory factor (SARAF) from STIM1/2. This leads to translocation of STIM1/2 to ER-plasma membrane junctions where it activates the Ca\textsuperscript{2+} channel Orai, located in the plasma membrane. When activated, it restores ER Ca\textsuperscript{2+} levels (42).

Ca\textsuperscript{2+} flows into the cell during action potentials, through activated AMPA and NMDA receptors (among others, such as the serotonin 5-HT\textsubscript{3} receptors (46)) and it elicits intracellular signalling through secondary messengers in the cytosol. Calmodulin (CaM) is considered the most important regulator of intracellular Ca\textsuperscript{2+}-mediated signalling (47), with hundreds of target proteins that contain CaM binding sites. It has been described as an adaptor protein, which by the formation of the Ca\textsuperscript{2+}/CaM complex amplifies the ion signal enough to affect proteins (48). When complexed with Ca\textsuperscript{2+}, CaM has a wide range of tasks: Remodelling of targets,
relieving auto-inhibition and dimerization of proteins are relevant examples (49). Two examples of Ca\textsuperscript{2+}/CAM dependent targets relevant to NMDARs are described below.

The Ca\textsuperscript{2+}/CaM-dependent protein kinases (CaMKs) are regulated by the Ca\textsuperscript{2+}/CaM complex. The CaMKs phosphorylate the Ser/Thr residues in their targets, and multifunctional CaMKs that phosphorylate and regulate many protein substrates are important for brain function. There are four members in the CaMK enzyme class, and CaMKII has been linked to NMDAR regulation. When CaMKII is activated by Ca\textsuperscript{2+}/CAM, it autophosphorylates its Thr268 residue, causing it to bind the PSD, often initiated by Ca\textsuperscript{2+} influx through the NMDARs (reviewed in (50)). It has also been shown to interact directly with the NMDAR subunit GluN2B, locking CaMKII in an active state. This is presumed to be necessary for the formation of LTP, involving NMDARs, Ca\textsuperscript{2+}/CAM and CaMKII in memory formation and learning processes (reviewed in (51)).

Calpain is a Ca\textsuperscript{2+} dependent protease that is involved in neuron differentiation, neurite outgrowth and synaptic remodelling, among many other functions. It has been shown to cleave GluN2A and GluN2B subunits in vitro (52), and has been noted to selectively cleave GluN2B in hippocampal cultures (53). Calpain is thought to be implicated in both NMDAR turnover and regulation, as the cleaved subunit sometimes remains active instead of being degraded (54).

1.5 Opioid receptors

ORs were discovered in the mid-1960s and are members of the metabotropic G-protein coupled receptors. Three classical opioid receptor subtypes have been identified, the mu- (MOR), kappa- (KOR) and delta-receptors (DOR). They are widely expressed in the brain and spinal cord, regulating ionic homeostasis. The endogenous agonists to ORs are endorphins, enkephalins and dynorphins. Binding of an OR agonist leads to inhibition of adenylyl cyclase (AC), which causes a reduction in cyclic adenosine monophosphate levels (cAMP). A synthetic OR antagonist, naloxone, was discovered in the 1980s (55). The overall effect of OR activation appears to be inhibitive, by reducing Ca\textsuperscript{2+} entry into the neuron by closing the N-type calcium channel (reviewed in (56)) and promote K\textsuperscript{+} transport out of the cell (57). However, some studies have shown an increase in intracellular Ca\textsuperscript{2+} concentration during chronic OR activation, leading to increased protein kinase C (PKC) activity. PKC is able to phosphorylate
NMDARs and enhance their function, causing Ca\textsuperscript{2+} influx (58). PKC has been detected in the forebrain of chicken embryos at E6, and it was shown to be activated by opioids in forebrain culture (59).

Another possible pathway is OR-coupled activation of CaM, which in turn activates the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII). This kinase is also able to phosphorylate the NMDAR. CaMKII is widely expressed in the avian brain (60). In both PKC and CaMKII activation, the resulting increased Ca\textsuperscript{2+} concentration can activate PKC and CaM, creating a positive feedback loop (reviewed in (61)), resulting in chronically elevated intracellular Ca\textsuperscript{2+} level as a result of OR activation (figure 6).

![Figure 6: Schematic overview of selected intracellular signalling pathways after OR activation. K\textsuperscript{+}-channels are activated and N-type Ca\textsuperscript{2+}-channels are inhibited, while the NMDARs can be phosphorylated by CaMKII and/or PKC, leading to Ca\textsuperscript{2+}-influx. For simplicity, OR inhibition of AC has been omitted. Some studies have shown that increased cytosolic Ca\textsuperscript{2+} concentration reduce OR agonist affinity (section 1.8).](image)

1.6 History of opioid use

The earliest reference to opium poppy cultivation for medicinal and recreational use originates from Mesopotamia, around 3400 B.C. The plant was spread to areas connected to the Silk Road, among them Afghanistan, the largest producer of illicit opium in the world in the present day (62). Many Asian rulers prohibited opium consumption and trade, due to the detrimental effects of drug abuse. Regulations were put into effect from approximately 1360
to the 1700s, predating the awareness of the negative effects of drug use in the West by centuries. Underscoring this statement is Britain’s two wars with China for the right to sell opium, resulting in a legalisation of opium in China in 1858 (63).

In Europe and the US, opioids were used recreationally and as medication for anxiety, menstrual pain and cough. The pharmaceutical company Bayer AG has received some media attention for promoting administration of heroin to children, when the company made a fortune on opioids and aspirin in the late 1890s (figure 7) (64).

Figure 7: Example of heroin sold as a pharmaceutical drug in the late 1890s, by Bayer AG (64).

The first drug regulation law in Europe was passed in England and named the Pharmacy Act of 1868. The Act sought to control the distribution of poisons and drugs, as doctors felt they should administer such compounds. In addition, overdose deaths started to increase in prevalence. Because of the law, the number of people dying from overdose were reduced, but increased again towards the 1900s. However, the number of children dying of opiate use diminished permanently (65). In the US a rapid increase in medicinal and recreational drug use took place in the mid- to late 19th century. Though initially viewed as acceptable, increased knowledge about addiction and dangerous side effects led to the passage of the Harrison Anti-Narcotic Act of 1914. This legislation formed the foundation of the strict drug policies marshalled by the authorities in the US up until today (66).
A rise in recreational drug use in USA during the 1960s led President Nixon to name drug abuse “public enemy number one”, and his administration declared a “war on drugs”. The initiative led to harsh law enforcement, criminalising distributors and the users themselves (67). In the 1980s, President Reagan and the First Lady launched the “Just say No” campaign, aiming at educating young people about the dangers of drug use. While raising awareness of the issue, the campaign did not reduce drug abuse significantly, and the Global Commission on Drug Policy declared the war on drugs a lost cause in 2011. The Commission stated that drug policies should be based on scientific evidence, humane treatment of addicts, consideration of national differences and that drug control must integrate both penal systems, social and public health programs (68).

Through the 1990s, the therapeutic use of opiate analgesics increased in the US, with little attention to the addiction issues that inevitably followed. The drugs were gradually administered to more and more patient groups, catering to a growing expectancy of pain relief from both patients and caregivers (69). Pharmaceutical companies contributed heavily to this development, often by using paid physicians as consultants, undercommunicating the risks involved with opioid use (70). New drugs, such as OxyContin were introduced to patients, offering extended release of the opioid oxycodone. The number of prescriptions of this drug rose incredibly fast in the beginning of the 00s (71). As of January 2019, of patients prescribed opioids due to chronic pain in the US, slightly less than 30% misuse them and approximately 10% develop dependence (72). According to the National Vital Statistics System 130 persons die of opioid overdoses every day and the rates of overdose deaths has risen steeply all over the US from 2016 to 2017 (73). Today, the US experience an opioid crisis and the pharmaceutical company behind OxyContin faces lawsuits from 45 states, accused of deliberate deceptive marketing of the drug (74).

1.7 Foetal damage caused by opioid use

Opioids are readily transferred from the mother to the foetus (75) and neonatal withdrawal symptoms and birth defects caused by maternal opioid use has been recorded since the late 1800s (66). As the prevalence of opioid use rises, the number children born addicted to opioids will inevitably increase as well. Indeed, in the period from 2000 to 2012 the number of children born with neonatal abstinence syndrome (NAS) increased from 1.19 to 5.63 per
1000 births in the US (76). NAS involves the autonomic, gastrointestinal and neurological system and manifests with symptoms ranging from weight loss, seizures and excessive crying (77). The occurrence and severity of NAS is currently not predictable, and may depend on genetic factors, type of opioid exposure and polysubstance use (reviewed in (78)). The ongoing opioid crisis calls for a deeper understanding of what causes NAS and how it best can be treated and if possible, avoided.

A study from 2011 found a significant relationship between analgesic opioid use early in pregnancy and birth defects, such as congenital heart disease, spina bifida and glaucoma. Some of these birth defects have been observed in animal studies, but the authors call for more research on the mechanisms causing them (79, 80). A small Norwegian study found a higher prevalence of attention deficit hyperactivity disorder (ADHD) and increased prefrontal activation when solving demanding cognitive tasks in children exposed to opioids during gestation, implying that the exposed children struggle more to solve tasks than control children (81). A larger study on children born to mothers using methadone during pregnancy, suggested that these children have poorer inhibitory control and learning impairments, supporting the Norwegian study (82).

Animal studies targeting behavioural effects showed that rat pups exposed to methadone, morphine or buprenorphine during gestation displayed impaired recognition memory function and elevated anxiety behaviour compared to controls. For some of the tests the results were different for male and female rats, suggesting that female offspring displayed an increased sensitivity towards opioids later in life (83, 84). However, the gender specific responses are ambiguous as some studies only showed changed behaviour in male offspring after gestational morphine exposure. These discrepancies reflect that dosage, timing and type of drug affects the outcomes for offspring born to opioid treated mothers (85).

1.7.1  **Morphine**

Morphine is an alkaloid extracted from the poppy, *Papaver somniferum*. It is a full agonist of the μ- opioid receptor, with limited activation of κ- and δ- opioid receptors. Morphine has an analgesic effect, by mimicking the effect of the endogenous opioids (endorphins and enkephalins) and reducing nociceptive transmission of nerve signals from the periphery to the thalamus in addition to stimulating inhibitory pathways. This leads to reduced consciousness
and airway reflexes, which are advantageous qualities during anaesthesia. It also produces a feeling of euphoria, making it a coveted substance of abuse. Side effects include constipation, nausea, muscular rigidity and miosis. Overdose can lead to fatally reduced respiratory rate (86). Morphine is metabolised to morphine-3-glucoronide and the active compound morphine-6-glucoronide in the intestinal mucosa and the liver by the enzyme UGT2B7. It has 40-60 % bioavailability when ingested orally.

1.7.2 Methadone
Methadone is a synthetic opioid with high bioavailability, limited first pass metabolism and long half-life. It elicits limited euphoria and leads to rapid OR internalisation, unlike morphine. It is a full µ-agonist and has inhibitive effect on the NMDA receptor. Ebert et al (1995) showed that methadone displaced the unspecific NMDA channel blocker MK-801. One study showed that giving D-methadone concomitantly with morphine to rats attenuated the development of tolerance to the analgesic effect of morphine. As D-methadone has low affinity to the opioid receptors, this effect is attributed to its NMDA antagonist properties (87). Methadone has been used in opioid maintenance therapy (OMT) since the 1960s, and in pregnant women it improves foetal outcome, compared to uncontrolled opioid abuse (88).

1.7.3 Buprenorphine
Buprenorphine is a semi-synthetic opioid with partial agonist effect on the µ-receptor. The analgesic effect of buprenorphine is not complete and it reaches a plateau where administering more drug has no effect, making buprenorphine a safer choice in terms of overdose. This plateau effect has been explained by partial µ-receptor binding and possible activation of the Nociceptin Opioid Receptor (NOP), where the latter is thought to reduce the antinociceptive effect of µ-receptor binding (89). Buprenorphine is also used in OMT of pregnant women, and one comparative study found similar outcome for the mother but improved outcome for the foetus compared to methadone treatment (88).

1.8 Tolerance and dependence
Chronic opioid use leads to tolerance, meaning that the user has to increase the dose gradually to obtain the effect she or he experienced the first time. A 10-fold increase in dose during treatment is not uncommon in a clinical setting, and opioid addicts can need hundred-fold higher doses to obtain the desired effect. This is due to homeostatic mechanisms that
strive to uphold normal cell function in spite of opioid influence, such as the loss of G-protein coupling that effectively disrupts the intracellular signalling pathway. Another defence mechanism is internalisation of the ORs, but the ability to induce internalisation varies among the opioids. Internalisation may also lead to a reduced number of ORs, as some undergo lysosomal degradation.

Chronic use of opioids enhances NMDAR function and leads to increased cytosolic Ca\(^{2+}\), possibly mediated by OR-related activation of PKC and/or CaMKII (described in section 1.5). This association has been linked to the development of opioid tolerance, as studies have shown that administering low doses of non-competitive NMDA antagonists with high and low affinity inhibit the development of sensitisation and tolerance to morphine (90). Similar results have been obtained with a competitive NMDA inhibitor, suggesting that the diverse behavioural side effects of the antagonists cannot be confounded with the ability to inhibit sensitisation and tolerance to morphine. In addition, the unspecific NMDA channel blocker MK-801, has been shown to inhibit sensitisation to methadone and morphine as well, indicating a common pathway of sensitisation development that includes NMDA receptors for all three opioids (87). CaMKII and PKC inhibitors have also been shown to reverse opioid tolerance (reviewed in (61)). Increased Ca\(^{2+}\) levels have in itself been shown to reduce the antinociceptive effect of opioids (91), which likely exacerbates the need to increase opioid use.

Dependence is defined by three manifestations: Drug taking becomes compulsory, physical and psychological changes occur due to abstinence and one loses control over amount taken (55). Chronical opiate use leads to increased norepinephrine and corticotrophin-releasing factor (CRF) signalling, interfering with the brain stress systems and causing anxiety during abstinence. Compensatory upregulation of AC and protein kinase A (PKA) has been reported in the locus coeruleus, allowing the neurons to fire at normal rates. During abstinence, the LC neurons become overactive because they are no longer inhibited by opioids. These factors contribute to withdrawal symptoms such as nausea, anxiety and stress (92).

A recent paper reported that rats prenatally exposed to heroin displayed a sensitised behavioural response to heroin postnatally (84), a result supported by several other studies on prenatal opioid exposure and postnatal sensitisation to drugs (85, 93). This may suggest a
vicious circle, where children of opioid-addicted mothers are more likely to abuse drugs later in life.

1.9 Overview of the cerebellum

Proper NMDAR function is essential for a key step in cerebellar development, the GC migration (94) that occurs postnatally in mammals and in ovo in the chicken (95). The GluN2B receptor subunits is temporally upregulated during the migration (96), simultaneously with the OR (97), which may indicate that these two receptor systems co-operate in the process of guiding the GCs from the external granule layer (EGL) to the internal granule layer (IGL). This putative interaction makes the cerebellum a probable target for maternal opioid abuse effects.

In adult humans, the cerebellum represents 10.5 % of the total brain weight. It is located above the brainstem and is built up by the anterior, posterior and flocculonodular lobes. The lobes consist of lobules, built up by folia that give the cerebellum its characteristically striped appearance. In mammals, the cerebellum is divided vertically by the vermis.

Three layers compose the cerebellar cortex and from the surface they are the molecular layer (ML), the Purkinje cell layer (PCL) and the GC layer. The ML consists of stellate cells, basket cells, and Golgi and Purkinje cell (PC) dendrites. The stellate cells are located in the outer portion of the ML with the basket cells positioned below. Both use γ-aminobutyric acid (GABA) as neurotransmitter and receive input from parallel fibres of GCs. The stellate cells form synaptic contacts with Purkinje dendrites, while basket cells make inhibitory connections to the Purkinje cell bodies. The Purkinje cell layer consists of PC bodies and candelabrum cells, both of which are GABAergic neurons. While the PCs project to the cerebellar nuclei, little is known about the function of candelabrum cells. GCs, Golgi cells, Lugaro cells and mossy fibres originating from neurons located in brain stem and spinal cord build up the granular layer. Of these, the glutamatergic GCs are the most numerous in the cerebellum and they connect to the PCs, determining much of the PC activity. The GC also receive excitatory input from unipolar brush cells and inhibitory input from Golgi cell axons. In addition to the PCs, they make excitatory synapses to basket cells, stellate cells and Golgi cells (98). The GABAergic Golgi neurons receive input from the parallel fibre and provide inhibition of the dendrites of the GCs. Unipolar brush cells use glutamate as a neurotransmitter and are
linked to mossy fibres, GCs and other unipolar brush cells. The Lugaro cells are associated with Golgi cells and thought to be the target of serotoninergic fibres.

Below the three layers of the cerebellar cortex, the cerebellar nuclei reside. The white matter is mainly composed of afferent and efferent fibres that relay information to and from the cerebellum. The deep nuclei are also embedded in the white matter, receiving input from the cerebellar cortex via PCs. These nuclei project to the thalamus, red nucleus, reticular formation and vestibular nuclei, to name a few (figure 8) (99, 100).

![Figure 8: Schematic overview of the cell layers in the adult cerebellum. Stellate (S) and basket (B) cells constitute the molecular layer, together with the dendrites of Golgi and Purkinje cells. The Purkinje cell (P) layer is found below, followed by the GC layer. Here the GCs, unipolar brush cells (U), Golgi cell bodies (Go), Candelabrum (Ca) and Lugaro cells (L) are located. The core of the cerebellum is built up by deep nuclei (DN), climbing (CF), mossy (Mo) and serotoninergic (ser) fibres (figure from 101), reprinted with permission.]

### 1.10 Development of the cerebellum

In mice, the development of the cerebellum starts at E9, when the neural tube in closing. The cerebellar cells originate from the alar plate of the brainstem, rostral to the isthmus. The glutamatergic neurons have been shown to stem from the rhombic lip, while GABAergic neurons stem from the ventricular zone (102).
The GCs start to proliferate at E10 and gradually spread across the cerebellar anlage surface, forming the EGL, which is completed at E15 (103). Postnatally, the layer expands until a thickness of 6-8 cells is obtained (104). The GCs then extend two uneven processes parallel to the cerebellar surface, and begin to migrate tangentially in the direction of the larger cell extension (98). The speed of migration doubles from PND7 to PND13, but the ML expands to double thickness during this time, so the speed of migration appears constant (105).

When the cell body of a GC enters the PCL, it changes shape from elongated to spherical. At the end of the leading process a motile lamellodopia develops, which enters the IGL. After a stationary phase in the PCL the GC bodies elongate once again and migrate towards the IGL bottom, now disengaged from the Bergmann glia, which have been guiding the GC migration through the ML and PCL (94). When they reach their target at the IGL-white matter border the cell returns to spherical shape (105). In mice, the average migration time is 51 hours. During that time the GCs covers a distance of 220 µM tangentially in the EGL, and 250 µM radially across the ML and PCL (106). Upon arrival in the IGL each GC forms a synapse with one afferent mossy fiber, thereby processing and relaying sensory signals from the pons to the PCs (107) (figure 9).

![Figure 9: Overview of granule cell (GC) migration, from PND3 to PND15 in rat. The GCs move tangentially in the EGL before they migrate radially across the ML and PCL, along the Bergmann glia (BG). After passing the Purkinje cells (PC) the GCs detach from the BCs and form the IGL (reprinted with permission from (108)).](image-url)
Ca²⁺ is important for GC migration. At the beginning of the translocation across the ML the GCs start to express N-type Ca²⁺ channels, and the number of channels increases during the migration. If an N-type Ca²⁺ channel-inhibitor blocks the channels, the cell speed is reduced. NMDARs are also important for GC migration (109). The receptors display high spontaneous activity in the ML (110), and the cell speed is correlated to both frequency and amplitude of intracellular Ca²⁺ concentration spikes (106). While the cell is migrating, the NMDARs are composed of GluN1/GluN2B subunits (111). Upon arrival in the IGL the GluN2B is replaced by GluN2C. This replacement may be connected to the loss of intracellular transient Ca²⁺ fluxes, which appear to be required for IGL settlement (112).

ORs were reported to be upregulated in developing human and rat cerebella (97, 113), while they are expressed from E4 in the chicken embryo brain (114). Since developmental processes are shared between species, several animal models are available to investigate NMDAR and OR function and development.

It is important to consider the differences in neurogenesis when translating results across species, especially when using a non-mammal as a model animal. A general model of neural development has been established in order to compare neurodevelopmental stages in a selection of mammalian animal models (115), and while no such tool exists for birds, the neurodevelopment of chicken is well characterised (116). This enables the use of histology, imaging techniques and biomarkers, such as GluN2 subunit levels, to assess the developmental stage of brain structures in chicken.

Studies have found that the development and morphology of the cerebellum are surprisingly similar between mammals and birds, regardless of the temporal differences of the cerebellar development (see section 4.1.4) (95, 116, 117). The chicken forebrain is morphologically different from the mammalian forebrain, despite sharing many of the transcription factors, axon projections and neocortical neuron subtypes with mammals (see section 4.1.3). Thus, to ensure the transferability of the mechanisms elucidated using chicken cerebellum and forebrain as model systems, the results should be verified in other animal models.

Many aspects of NMDARs are yet to be resolved. The functional properties of triheteromeric receptors, such as their mode of trafficking, regulation and sensitivity to allosteric modulators remain elusive (118). The therapeutic effect of NMDAR antagonists ketamine and memantine
on depression and Alzheimer’s disease, respectively, has not been attributed to a specific NMDAR subunit at the time of writing (119, 120). NMDARs are clearly involved in the development of addiction (see section 1.5), but the mechanism has not been universally agreed upon. It also remains to be seen if NMDARs are directly involved in the birth defects, NAS and later symptoms observed in children born to mothers using opioids.

These examples illustrate that NMDARs represent a virtually inexhaustible field of research, in need of new subtype specific antagonists, knowledge about receptor mechanisms and its involvement in diseases and addiction.
2. **Aims of the thesis**

The NMDARs are vital for normal brain function, and detailed knowledge about their complicated pharmacology is essential in order to understand neurological function. In the healthy brain, NMDARs are involved in memory formation, nerve signalling, neuronal development and synaptic plasticity, among many other processes. However, they are also associated with Alzheimer's disease, excitotoxicity, Parkinson's disease and stroke. Despite the obvious potential, only one NMDAR-targeted drug, memantine, is currently used in the clinic.

The general aims of the study were therefore to focus on the NMDAR subunit GluN2B and investigate the properties of GluN2B specific antagonists *in vitro*, combined with developing the chicken embryo as a NMDAR research animal model. We also utilised *in silico* modelling of the ligand-receptor complexes to predict binding affinities and binding residues.

Specific aims were:

1. Validate the chicken embryo forebrain as a model for NMDAR research, with focus on known GluN2B subunit antagonists (paper I).
2. Explain the differences in Ca$^{2+}$ influx inhibition by *in silico* modelling, describing the partially overlapping GluN1/GluN2B binding site for ifenprodil and EVT-101 (paper I).
3. Evaluate the structure-activity relationships of new antagonists derived from the known antagonist Ro 04-5595 (paper II).
4. Utilise the chicken cerebellar model to assess effects of short-term prenatal opioid exposure on the GluN2B expression in the cerebellum and function in cerebellar culture, compared with the effects of long-term prenatal exposure on the GluN2B expression in rat cerebellum up to PND21 (paper III).
3. Summary of papers

Paper I: Exploring the overlapping binding sites of ifenprodil and EVT-101 in GluN2B containing NMDA receptors using novel chicken embryo forebrain cultures and molecular modelling.


NMDARs are important Ca$^{2+}$ channels, expressed all over the CNS. They are potential drug targets, the GluN2B subunit in particular, as it has been associated with Alzheimer’s disease, depression and excitotoxic pathways. The goal of this paper was to compare the differences in Ca$^{2+}$ inhibition potency of known GluN2B specific antagonists and explain these with *in silico* modelling to elucidate their binding properties. We established the chicken embryo forebrain assay to evaluate the inhibitors, and found that it provided fast and reproducible access to native NMDARs containing GluN2B subunits. The antagonists were ranked as follows: EVT-101 ($EC_{50}$ 22 ± 8 nmol/L) < Ro 25-6981 ($EC_{50}$ 60 ± 30 nmol/L) < ifenprodil ($EC_{50}$ 100 ± 40 nmol/L) < eliprodil ($EC_{50}$ 1300 ± 700 nmol/L), reflecting observations in rat cortical cultures and cell lines overexpressing chimeric receptors. The less explored Ro 04-5595 had an $EC_{50}$ of 186 ± 32 nmol/L. The computer simulations predicted that this compound bound the recently discovered EVT-101 site, rather than the ifenprodil site and that the difference in potency may be attributed to the difference in their binding residues. Eliprodil, on the other hand, appeared to fail to bind important amino acids and that might explain its high $EC_{50}$-value.


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*Unpublished manuscript.*

Despite its involvement in brain development and neurological diseases, no probes or drugs targeting the GluN2B subunit of the NMDARs are in clinical use today. A range of GluN2B
subunit specific antagonists derived from the structure of the commercially available GluN2B specific inhibitor Ro 04-5595 have recently been published, and in the present paper we have evaluated these in terms of Ca\textsuperscript{2+} influx inhibition potency. Ro 04-5595 displays less unspecific binding than other GluN2B specific antagonists, and our recent work (paper I) has predicted that it binds to the recently discovered EVT-101 binding pocket. The aim was to discover a more potent antagonist than Ro 04-5595, and to that end we utilised the chicken embryo forebrain culture model to test the inhibition potency of 14 novel NMDA receptor GluN2B subunit specific antagonists. Functional influx data was obtained by ratiometric measuring of intracellular Ca\textsuperscript{2+} in forebrain cultures by the Fura-2 plate reader assay and through this work the chicken forebrain cell culture assay has been validated as an accessible and reliable screening tool for neuro-active compounds. Several GluN2B antagonists of potential clinical relevance were discovered and their structure-activity relationships were evaluated. However, none displayed higher potency than Ro 04-5595.

**Paper III: Opioid receptor-mediated changes in the NMDA receptor in developing rat and chicken.**

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The US is currently experiencing a rampant opioid abuse crisis. It is therefore important to understand the molecular mechanisms behind the developmental problems observed in children born to mothers using opioids during pregnancy. In this paper we sought to investigate if opioids affected GluN2B expression and NMDAR function in the cerebellum. To that end, we administered opioids to chicken (injection of 20 mg/kg morphine or methadone at E13 and E14 *in ovo*) and rat (methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day) throughout gestation), to investigate the effects of short-term and long-term opioid exposure. We performed western blotting to assess GluN2B expression in both rat and chicken cerebellar tissue, while Ca\textsuperscript{2+} influx assay on chicken cerebellar cultures and [\textsuperscript{3}H]-ifenprodil binding were done on chicken cerebellar tissue. It was found that 14 days old rat pups exposed to the OMT drug buprenorphine showed significantly lower level of the GluN2B subunit, while the cerebellar GluN2B expression in chicken embryo exposed to methadone or
morphine remained unaffected at embryonic day 17. Despite this, a significantly higher level of NMDA-induced Ca\textsuperscript{2+} influx was detected in chicken cerebellar cultures from embryos exposed to morphine or methadone, when tested at DIV1. Methadone is an OR agonist and NMDAR inhibitor, and since there were no difference in effect observed between pure OR agonists and methadone, we conclude that the effects were OR-mediated.
4. **Methodological considerations**

4.1 **The chicken as an animal model**

4.1.1 *Background*

Historically, the chicken embryo has been an important model for the understanding of embryonic development. The origin of the neural crest, nerve growth factor and the first cellular oncogene were all discovered in the chicken (121-123). There is 60% single gene orthologues between chicken and human, and 75% median amino acid similarity in those orthologue pairs (124). As such, the chicken is phylogenetically closer to mammals than zebrafish or nematodes and offers more genetic variation than the inbred murine model. Also, the chicken embryo is inexpensive, readily accessible and it is a nutritionally self-sufficient model that does not require extensive animal facilities. The hen is not included in the experiments, the number of live embryos is predictable and the embryo is not considered an animal before E14. Thus, the use of chicken embryos contributes to the 3R principles (116).

However, drug administration to chicken embryos cannot be directly compared to the route of administration in humans. In terms of animal welfare it is an advantage that the mother is left out from the experiments, but it follows that embryonal exposure of the chicken embryo is not comparable to mammalian embryos. As such, maternal drug metabolism, the placental barrier and excretion of drugs are absent from studies using the chicken embryo as a model system (125). Even so, the chicken embryo model can provide supplementary data in an early non-clinical drug screening process. The model is especially relevant in the less prioritised field of paediatric and obstetric pharmacological research (116).

4.1.2 *Drug exposure*

The chorioallantoic membrane (CAM) is a highly vascularised membrane that envelops the chicken embryo and is in direct contact with the eggshell. By E13 the CAM is fully developed. It mediates gas exchange, electrolyte transport, stores excretions and is connected to the embryonic circulation by two allantoic arteries and one allantoic vein (126). The CAM has been extensively studied in order to understand vascular development and to study tumour development (reviewed in (127)), and has been compared to the placenta in mammals, despite having a much simpler organisation (128).
Drugs can be applied topically through a hole in the eggshell or injected into the allantois, and both routes of administration ensure a quick uptake in the embryo (126). In paper III in the current work we injected methadone or morphine into the allantois (figure 10), and LC-MS studies have proven that methadone reaches the brain in concentrations comparable to those obtained in rat (129). Our group has published studies on exposure of drugs and chemicals in chicken embryos following the same procedure, with apparent effects on the embryo (95, 130, 131). Compared to humans who have a well-developed blood-brain-barrier (BBB) at the time of birth, the chicken has a less developed BBB at the time of exposure and hatching (132, 133). This must be taken into consideration in drug exposure studies on chicken embryos.

Figure 10: Trans-illumination of egg, to avoid blood vessels (left) and injection of egg (right) (photo by Denis Zosen, printed with permission).

4.1.3 Comparison of avian and mammalian forebrain structure

Birds were thought to display an instinctive and inflexible behaviour, largely orchestrated by hypertrophied basal ganglia, as opposed to the complex cognitive behaviour and layered cortex cerebri attributed to mammals. In later years however, both the understanding of the cognitive abilities and brain structure of birds have needed revision. The Avian Brain Nomenclature Forum, held at Duke University in 2002, reviewed the classical view of avian forebrain organisation. Despite that the last common ancestor of birds and mammals existed 286 million years ago, the avian telencephalon is functionally comparable to mammalian neocortex, claustrum and pallial amygdala. The structure of the cortex is different, with a
semi-layered nuclear pallium in birds, while mammals have defined a six-layered organisation of the cortex. The avian nuclear organisation also prevents the folding of the cortex that exists in mammals (reviewed in (134)).

Furthermore, the pallial, striatal and pallidal domains are homologous in fish, reptiles, amphibians, mammals and birds. The striatal and pallidal domains are similarly organised in birds and mammals, with the exception of the dorsal pallidum. In mammals, two classes of neuron phenotypes are divided in two different segments, while in birds these phenotypes appear to be mixed (135). The avian pallial domain is divided into four domains in birds, and contains the hippocampus, olfactory cortex, olfactory bulb and amygdaloidal complex, all homologous among vertebrates (134).

4.1.4 Comparison of avian and mammalian cerebellar development
Virtually all vertebrates have a cerebellum, and while their blood circuitry is surprisingly similar, the shape and size vary greatly between species (136). The foliation of the cerebellum is correlated with the complexity of the animal. Both birds and mammals have highly foliated cerebella (137), and near identical histological organisation of the cortex. The most pronounced difference between avian and mammal cerebella is that birds do not have a distinct vermis, separating the paired hemispheres (138).

The developmental stages are also comparable between birds and mammals, with the exception that many cerebellar events take place prenatally in birds, as opposed to postnatally in mammals. The chicken (Gallus gallus) has a thick EGL composed of proliferating GCs between E12 to E17 (95), corresponding to gestational week 25 to the second postnatal month in human (139). In the rat, the EGL is thickest between postnatal days (PNDs) 8-9 (140). The thickness of the ML in chicken expands from E16-20, while in human this event takes place during the first year after birth (141) and in rat the ML matures around PND20-21. Common for all species is an inversely proportional relationship of the EGL and ML size, which is plausible, considering the migration of postmitotic GCs from the EGL to the IGL through the ML (140). Consequently, the cell density of the IGL increases from E16 to E21 in chicken (95), reaches adult levels after two years in human (142) and transcends PND30 in rats and mice (140). Of these stages, formation of the IGL deviates the most between chicken and human, as the EGL in humans has an extended growth phase compared to chickens (95). A more
extensive discussion of cerebellar development can be found in the PhD thesis of L. Austdal (143).

4.2 Chicken embryo brain cultures

The chicken embryo forebrain cell cultures were established as described in Paper I, and will be discussed in section 5.1.

4.2.1 Chicken cerebellar granule cell culture

The chicken cerebellar granule cell (cCGC) culture was first presented by our group as a reliable alternative to murine cerebellar GC cultures in 2006. As opposed to rat culture, the cCGC culture is not dependent on a depolarising culture conditions (25 mM K+) and nerve growth factor supplement to survive in culture. In addition, the cCGC culture develops NMDA-mediated toxicity much faster than the rat culture. Thus, it is possible to study glutamate toxicity without addition of the chemotherapy medication AraC, usually added to repress division of non-neuronal cells in murine cerebellar cultures (144).

Earlier work has shown that the GCs are migrating from the EGL to the IGL at the time of chicken cerebellar tissue isolation (E17) (95), and that the resulting cerebellar culture contains approximately 80% GCs at this time point (144). This is comparable, but somewhat lower than the neuron percentage found in rat cerebellar cultures (approximately 95%), isolated at PND8 (145). However, this reflects that the GCs constitutes a large homogenous neuronal cell population in both species.

Primary cells have a much shorter life span than cell lines. This necessitates the sacrifice of new chicken embryos for each culture preparation, which introduces batch-related variability. However, primary cell cultures reflect the in vivo properties better than cell lines (146). We found that the cCGC cultures tended to be overgrown by astrocytes by DIV7 in the absence of AraC, and the cells also dissociated from the plate surface at this time point. Our experiments were executed at DIV1, as we were dependent on a firmly attached cCGC culture due to many washing steps in the Ca²⁺ influx measurement procedure.

An earlier study in rats has shown that only GCs from the EGL survives tissue isolation, and that these mature and differentiate into IGL-like GCs over time in vitro (147). Consequently, the cCGCs are most likely EGL- like at the time point of our experiments. Due to the low
number of PCs and difficulties associated with maintaining them in culture (148), it is assumed that the GCs form synapses with each other. This does not reflect the situation in vivo where the GCs predominantly form synapses with PCs (149).

In vivo, the NMDARs change composition from primarily GluN1-GluN2B subunits upon arrival in the IGL, to GluN1-GluN2C, possibly via GluN1-GluN2B-GluN2A triheteromeric receptors (see section 1.5) (150). A similar development has been observed in murine cerebellar GC cultures, possibly driven by NMDAR stimulation (151) (152). The cCGC cultures were used at DIV 1 in our experiments and the Ca\(^{2+}\) influx could be 70 % reduced by GluN2B specific inhibitors. Therefore, we strongly assume that the majority of the NMDARs in the cCGC cultures contained GluN2B subunits.

4.3 Antibody specificity

The reproducibility of experiments based on the use of antibodies have been debated. Cross-reactivity, batch variability and variable experiment conditions has been listed as common reasons for problems related to irreproducible research (153). We have tried to address these issues by validating antibodies as described below.

The selection of chicken specific antibodies is very limited, and in the present work we wanted to use antibodies to assess the amount of GluN2A and GluN2B protein in chicken brain samples. For this purpose we tested many candidates, for the most part raised against rat epitopes. The antibodies were chosen on the basis of rat and chicken amino acid sequence similarity, retrieved from UniProt (154) and aligned using BlastP (155). However, a few non-similar amino acids proved to be enough for the antibodies to fail to recognize the chicken epitope, leaving us with a limited number of alternatives for further testing.

HEK-293 cells do not express NMDARs endogenously (156), and with the aim of testing antibody specificity, we transfected HEK-293 with GluN2B, CMV (non-coding plasmid) and GluN2A-encoding plasmids. This set-up (including chicken, rat and mouse brain samples) allowed us to test whether the GluN2A and GluN2B antibodies displayed any cross-reactivity. Sadly, we were not able to find a suitable GluN2A specific antibody. However, we identified a reliable GluN2B specific antibody (# ab65981, Abcam, Cambridge, UK) that was able to detect chicken, rat and mouse GluN2B.
In the present work, we utilised antibodies for neuronal detection (Anti-NeuN, immunocytochemistry in paper I) and GluN2B detection and quantification (western blotting, paper I and III). In the quantification experiments we normalized the GluN2B-signal against the housekeeping protein β-actin to reduce variability, a procedure with several pitfalls. The expression of β-actin can be affected by age, disease (157) and also opioid treatment (158).

However, the raw data (not normalised against β-actin) displayed similar trends as the normalised values, albeit with higher variation. To counteract possible confounding bias related to β-actin-normalisation, the same amounts of total protein were loaded carefully, the intensity of the Ponceau-stained membrane was compared to the immunostained membrane and the intensity of bands were visually inspected. After membrane analysis, outlier intensity values were removed by GraphPad (Robust regression and Outlier removal, Q=1%).

4.4 Calcium influx measurements

The relative cytosolic free Ca$^{2+}$ level was estimated with the ratiometric Ca$^{2+}$ indicator Fura-2 acetoxyethyl (AM) ester in the present work. The AM ester allows passive loading into cells, due to its hydrophobic properties, and intracellular esterases hydrolyse the molecule, leading to entrapment and accumulation of negatively charged Fura-2. An earlier study has shown that loading and retention of Fura-2 in primary chicken brain cell cultures was adequate without addition of the detergent pluronic F-127 and the multidrug resistant transporter inhibitor probenecid, respectively (159). The excitation wavelength of the indicator shifts from 380 to 340 nm upon Ca$^{2+}$ binding and it has a Ca$^{2+}$ sensitivity ranging from 100 nM to 100 µM (160). The emission is measured at 510 nm, and the relative change in the $\frac{F_{340}}{F_{380}}$ ratio was used to estimate IC$_{50}$-values in the present work. This ratio is independent of cell thickness, probe loading or leakage and sensitivity of the detection instrument (161). However, a study has showed that the fluorescence intensity of Fura-2 is affected by both temperature and pH (162). The experiments in the present work were executed at a constant temperature and the solutions were buffered to keep the pH at 7.4.

Primary cells were plated in 96-well plates. Before measuring basal Ca$^{2+}$ level with a CLARIOstar® plate reader (BMG Labtech, Ortenberg, Germany), the voltage-dependent NMDA channel blocker Mg$^{2+}$ (1 mM) was added to prevent Ca$^{2+}$-influx. The Mg$^{2+}$ was removed and treatments were added (depending on the experiment), before a second measurement was
performed. The basal Ca\(^{2+}\) level was subtracted from the second measurement Ca\(^{2+}\) level. A certain amount of batch variation was expected, due to the primary nature the cell culture, tentatively reduced by a strict egg handling and tissue isolation protocol. Also, as the present work focused on the GluN2B subunit, which changes expression over time \textit{in vivo} and \textit{in vitro}, a similar level of GluN2B expression could not be guaranteed in every batch of brain tissue. The Ca\(^{2+}\) influx that Ro 04-5595 was not able to inhibit, was assumed to stem from NMDARs without GluN2B subunits. In paper II, we chose to subtract the maximum inhibition ratio induced by the GluN2B specific antagonist Ro 04-5595 (10 µM) from all inhibition curves with the aim of alleviating the differences in GluN2B expression between egg batches.

4.5 Molecular modelling

Molecular modelling is a collection of computational tools that can be applied to mimic the behaviour of biomolecules in nature. In the present work, the molecular modelling platform Schrödinger was utilised, using the Glide (163) and Desmond (164) software, to perform docking procedures and molecular dynamics (MD) simulations, respectively. The docking procedure can be applied to align small molecules (ligands) within a defined binding pocket in a 3D structure of a molecule, often a protein. MD simulation can thus be applied to study the natural behaviour of a system over time. Molecular modelling was utilised in paper I and II to investigate binding properties and predict binding affinities of ligands using the available x-ray structure (experimental structure) of the GluN1/GluN2B NTD.

Compared to the large amount of sequenced proteins, few protein 3D structures have been solved experimentally. Thus, computational protein 3D structure prediction can be a useful technique to obtain information about proteins that have not been resolved yet, for example by x-ray crystallography. The 3D structure of a protein is largely predicted by its amino acid sequence, which is not always similar between species. However, even though the amino acid sequence deviates, the 3D structure remains conserved. This makes it possible to use a resolved protein as a template to model a non-resolved protein (target), despite the evolutionary changes in amino acid sequence. Very briefly, the steps in homology modelling can be described as follows: Identify a suitable template and align it with the target sequence (165). A minimum of 30 % sequence similarity is needed to create a homology model (personal comment, Prof. I. Sylte) and there are several commercially available computer
programmes that can build the protein model after alignment. The loops in the protein structures are less evolutionarily conserved, and due to the difficulties in predicting their conformation, they need to be modelled subsequently to the strands and helices in the protein. Amino acid side chains are also modelled after the main structure is finished and the hydrogen bond network of the model must be optimised to improve its quality. Lastly, the model must be validated to evaluate potential steric clashes, for example by Ramachandran plotting.

The docking process can result in many ligand poses, and the Glide scoring function estimates the binding free energy of each pose. This “pose” includes information about the ligand position and orientation in relation to the protein. The binding poses with the lowest estimated binding free energies are minimised, a process that relaxes strained bonds, angles and clashes between atoms. The most likely poses are then presented with a corresponding Glide score. The Glide score is measured in kcal/mole, which indicates the predicted affinity of the ligand and allows rank ordering of active to non-active compounds. Glide score is an empirical scoring function based on the physics of electrostatic and van der Waals forces, hydrogen bonds and lipophilic interactions. The Glide score provides predictions of which binding pose is most energetically favourable (has the lowest predicted binding free energy) and thus, most likely represents the native binding mode (166).

The Glide docking program has several levels of accuracy called docking algorithms, ranging from high-throughput virtual screening (HTVS), standard precision (SP) to extra precision (XP) (166, 167). The SP algorithms enables fewer false negatives, while the XP modality penalises physiochemical violations to a larger degree, and is consequently more time-consuming. We therefore chose the SP mode in our experiments, as it is a suitable modality for the virtual screening of tens to thousands of ligands (168).

However, as SP docking is tailored to screen many ligands efficiently, many simplifications are necessary to speed up the process. Most docking programs do not include water as a solvent, which may affect the prediction of H-bond formation and the energy estimations. In Glide SP docking, the ligand is flexible, while the target protein is rigid. Obviously, this does not reflect the real situation, as proteins frequently change conformation in vivo. The calculations for the
estimation of protein-ligand interactions are also simplified, due to computational demands and time efficiency (168).

To address these issues and improve the affinity predictions, we implemented Molecular Mechanics combined with Generalised-Born Surface Area (MM-GBSA) rescoring on the binding poses that obtained the best Glide scores (169). This process combines molecular mechanics calculations with continuum solvation models, and thus improves the accuracy by also considering the free unbound ligand and receptor. The result is an approximated binding free energy (170) (169). To investigate the binding properties of the ligands further, we also included molecular dynamic (MD) simulation of the most energetically favourable binding pose of each ligand (paper I). This entails a simulation of the receptor and ligand movement, and bonds formed between the ligand and specified amino acids over a defined time period, giving a detailed and dynamic overview of the protein-ligand interactions. As MD simulations are computationally costly, the protein motions are studied for short time periods ranging from nanoseconds to microseconds. MD simulations consider both the ligand and the target protein as flexible structures, and due to the time-consumption, it is currently not well suited for fast virtual screening (168). Instead, it gives detailed information about temporal ligand-protein interactions. To some extent, the MD results obtained in paper I could be related to the IC50-values of the ligands, established by \textit{in vitro} Ca\textsuperscript{2+}-influx measurements.
5. Discussion of the results

5.1 Establishing chicken forebrain as a research tool (paper I)

The chicken embryo is not considered as a research animal before 2/3 of the gestation is completed (EU Directive 2010/63/EU). The cortical tissue in the present work was harvested at E10 and the use of embryos was therefore not subject to the restrictions enforced by the authorities (Mattilsynet, in Norway). This is an advantage of using chicken forebrain, in addition to those mentioned in section 4.2.1., which reduces the number of animals used in research. This corresponds with the 3R-principles, substantiated by the nomination of our group to the Norecopa 3R price in 2019.

The harvesting and culturing of chicken embryo forebrain cell (cEFC) was performed as described in the master thesis of A. Ziegler (159), and we observed comparable viability, attachment and gradual glial overgrowth in cEFC cultures and chicken cerebellar cultures. It can also be observed that the GCs in the cerebellar cultures tend to form aggregates to a larger degree than the neurons in the cEFC cultures over time in vitro, indicated by red arrows (figure 11). The cEFC cultures appeared to give less variation in Ca\(^{2+}\) influx experiments than the cCGC culture. This was surprising, as the cortical cultures consist of a more heterologous cell population than the cerebellar cultures (see section 4.2.1). In paper I, we showed that the fraction of neurons at a certain level of maturity in the cEFC culture was approximately 40 %, indicating the presence of other cell types and immature neurons. In later experiments, it would be interesting to immunostain for doublecortin, a marker of less mature neurons (171). Adding the antineoplastic agent AraC to cEFC harvested at E10 has been shown to induce cell death (172). These findings support that the remaining 60 % of the cell population consists of proliferating glial cells and immature neurons.
Mammalian forebrain development is highly conserved across species, and recent research has shown that avian forebrain also expresses many of the transcription factors regulating the mammalian forebrain development (173). Despite the lack of a layered neocortex in birds, many of the neocortical neuron subtypes are conserved in the avian pallium. With the exception of the corpus callosum found in placental mammals, the axon projection patterns and interconnectivity are quite comparable in birds and mammals (reviewed in (174)). These common features of avian and mammalian forebrain development makes avian forebrain a suitable animal model, despite the differences in spatial organisation between birds and mammals.
In paper I, we reported that the chicken forebrain tissue contained the NMDAR subunit GluN2B. The GluN2B NTD subunit amino acid sequence is predicted to be 95% similar to human and rat, which reflects the comparable IC_{50}-values of the GluN2B-specific antagonists eliprodil, ifenprodil, Ro 25-6981 and EVT-101 in chicken and rat (175). The Ca^{2+} influx of chicken NMDAR has also been shown to be stimulated by NMDA and glycine in the same order of magnitude as rat NMDARs, regardless of cerebellar or forebrain origin (159) (176, 177). Likewise, the NMDAR blocker MK-801 inhibits 90% of NMDA stimulated Ca^{2+} influx in cEFC cultures (175). These features make the cEFC culture suitable for GluN2B specific antagonist research.

The whole chicken GluN2B subunit is 93% similar to the human subunit. While this is a high percentage of amino acid sequence similarity, most of the non-matching amino acids are located in the CTD (88% similarity between chicken and human GluN2B CTD). This portion of the receptor subunit is located intracellularly and connects the NMDAR to the secondary messenger system. For instance, CaMKII has been reported to control the NMDAR composition, by casein kinase 2-mediated phosphorylation of the GluN2B CTD (178). Structural differences between chicken and mammals in this domain may indicate that the chicken is less suitable as a model for research on NMDAR-related downstream signalling.

However, PKC and CaMKII have been reported to phosphorylate mouse GluN2B CTD amino acids Ser1303, Ser1323 and Ser1480 in vitro. Phosphorylation of the first two serine residues leads to enhanced NMDAR activation and the last disrupts the PDZ binding, causing increased mobility (179). These amino acids are conserved in chicken, according to alignment of human, mouse and chicken GluN2B sequences (154, 155). This strengthens the chicken as a model for research that includes PKC and CaMKII phosphorylation of NMDARs.

5.2 Using in silico modelling to evaluate ligand affinity in terms of Ca^{2+} influx inhibition potency (paper I)

It is important to investigate the interactions between ligands and proteins to elucidate the cellular mechanisms and binding site properties. Detailed knowledge about molecular processes in living systems can help developing drugs, understand mechanism behind disease and expand current knowledge about cellular processes.
In both paper I and II, the *in vitro* screening established an order of Ca\(^{2+}\)-influx inhibition potency of the ligands, which we ventured to explain by investigating their binding properties by *in silico* predictions of their affinity and binding residues. We utilised the Glide docking program (163), with the SP method to dock the ligands in the chicken homology model of the GluN1/GluN2B NTD domain. As stated in Paper I, the docking poses of all ligands were sufficiently similar with the ligand poses observed in the crystal structures (PDB id: 5EWJ and 5EWM respectively) and the chicken homology model, making it possible to use the crystal structures in further docking and molecular modelling structures. This accentuates the similarity of the GluN1/GluN2B NTD in chicken, rat and human. The use of experimentally resolved 3D structures also provide more accurate predictions than using homology models which is a constructed 3D model based on similar resolved structures (165).

The SP docking of ligands gave several poses per enantiomer of the molecule, and the score of each pose was calculated using the scoring function of the software. The scores of the ligands did not correlate well with the IC\(_{50}\)-values obtained in the *in vitro* screening, meaning that the predicted affinity did not match with the Ca\(^{2+}\) influx inhibition potency of the ligands that were established by the *in vitro* assay. This may be due to the many approximations introduced by the SP method, as accuracy is necessarily traded for speed to obtain high throughput screening. The method also keeps the binding pocket rigid, while the ligand is considered flexible. In reality both affect in each other’s conformation upon binding. The three poses with the best affinity scores per enantiomer were subjected to a final MM-GBSA rescoring, providing a more accurate prediction of their binding energies (180).

When a ligand is docked *in silico*, all of its enantiomers are docked independently, predicting a set of binding affinities per enantiomer. In the *in vitro* assay, we only had access to the racemic mixture of the compounds. Thus, the IC\(_{50}\)-values obtained *in vitro* were not directly comparable to the predicted binding affinities. Jakobsson *et al* reported that the R-enantiomer of Ro 04-5595 was considerably more biologically active than the S-enantiomer (181). This was also predicted by our *in silico* modelling, supporting our selection of enantiomers for MD simulation (paper I). Nevertheless, the software (Ligprep, (182)) predicted that two chiral centres existed in the Ro 04-5595 molecule at physiological pH. This gives four enantiomers, while Jakobsson *et al* only considered two enantiomers in their work. However, using *in silico* modelling to predict which enantiomer is biologically active is a
very complicated matter, as they have very similar properties (183). Considering this, it might be of interest to test enantiomerically pure ligands the \textit{in vitro} assay as well.

The pose of each enantiomer with the best MM-GBSA score were further used in MD simulation (paper 1), which gave detailed information about bond types, binding residues and ligand and receptor movement over a period of 100 ns. The information obtained from this simulation was possible to relate to \textit{in vitro} results, as we could compare the IC$_{50}$-values of the ligands to which residues they bound during simulation, and assess which residues were important in order for the ligand to bind, to obtain Ca$^{2+}$-influx inhibition. Admittedly, ligands could also bind with great affinity without eliciting any Ca$^{2+}$ inhibition, which would not be detected in the \textit{in vitro} assay. We sought to address this issue by the rather crude method of adding 10 µM of each compound to an inhibition curve measurement of Ro 04-5595 (paper I and II). The active compounds were shown to shift the curve to the left, while the non-active compounds left the inhibition curve unaffected. We theorised that a non-active compound that displayed strong affinity to the binding site would shift the curve to the right, but no such effect was observed for any of the compounds that were tested.

The molecular modelling performed in paper I did not take individual water molecules into account, and thus treated water as an implicit solvent. This may have affected the ability to correctly predict ligand binding affinities, as water molecules can act as bridges in hydrogen bonds and be structurally conserved in the binding site (184). Stroebel \textit{et al} lists 290 and 339 crystallographic water molecules in the crystal structures complexed with EVT-101 and ifenprodil, respectively (185). Most likely, some are conserved water molecules, potentially involved in the protein-ligand interaction if located in the binding pocket.

The experimental structure for the GluN1/GluN2B NTD employed in docking and MD simulations in paper I was co-crystallised with ifenprodil (PDB id: SEWJ). We also tried using the crystal structure co-crystallised with EVT-101 (PDB id: SEWM), but found that the rotamers of GluN1-I133 and GluN1-L135 obstructed the ifenprodil binding pocket when EVT-101 was bound. To allow access to both binding pockets it was therefore decided to use the experimental structure accommodating ifenprodil. However, this conformation represented only one of a virtually infinite number of conformations, a limitation that is important to keep in mind when assessing the results.
Considering the limitations associated with molecular modelling, it is still necessary to include reliable \textit{in vitro} screening of compounds to assess the quality of the \textit{in silico} predictions of binding affinity.

5.2.1 \textit{Evaluation of the binding sites of known GluN2B specific antagonists (paper I)}

In paper I, we described two overlapping GluN2B specific antagonist binding sites, the ifenprodil-site and the EVT-101 site, using a combination of \textit{in vitro} screening in chicken forebrain culture and \textit{in silico} prediction of binding properties. Our results confirmed earlier research, predicting that ifenprodil, Ro 25-6981 and eliprodil bound to the ifenprodil site (185-187). The less investigated compound Ro 04-5595 was predicted to bind to the relatively recently discovered EVT-101 binding site. This is of interest because a very similar molecule, HON0001, is orally available and produces an analgesic effect in rats (188). Ro 04-5595 also displays less unspecific binding than ifenprodil (189), and $^{11}$C Ro 04-5595 has been established as a GluN2B specific PET probe in rats (181). Ligands that bind to new binding pockets can be useful research tools, or even potential drug candidates.

For the ifenprodil binding pocket, the ligands were structurally similar. The least potent inhibitor, eliprodil, failed to bind the residues GluN1Phe113, GluN1Ser132, GluN2BPhe114, GluN2BMet207 and GluN2BGlu236, but displayed a more stable bond to GluN2BPhe176 than ifenprodil and Ro 25-6981. These properties may cause eliprodil to be a less potent inhibitor, but to be certain it is necessary to perform \textit{in vitro} mutant studies, similar to the functional mutant studies performed by Stroebel \textit{et al} (185). EVT-101 and Ro 04-5595 are not structurally similar molecules and even though they shared some residue interactions in the binding pocket, the difference in inhibitor potency appeared to stem from more diverse binding residues. The ability of the binding pocket to accommodate more structurally varied ligands that interacts with different amino acids, but still elicits Ca$^{2+}$ influx inhibition, may be interesting in terms of drug development.

5.3 \textit{Assessing new potential GluN2B-specific antagonists (paper II)}

In paper II we established IC$_{50}$-values for 14 new molecules derived from the Ro 04-5595 structure, synthesised by Jakobsson \textit{et al} (181). We found that the changes introduced to the molecule made it significantly less potent in terms of Ca$^{2+}$ influx inhibition, in ascending order:
Removal of a methoxy group < Adding a methoxy group < Removal of a methyl group on the N-atom < Opening the ring conformation < Addition of a double bond.

We performed preliminary in silico experiments for all new molecules (SP docking and MM-GBSA rescoring), but as observed in paper I, there was little compliance between the in vitro results and in silico affinity predictions. SP docking and scoring has been shown to predict inaccurate binding affinities in enantiomer pairs and even also for molecules of different molecular weight (183). As our ligands were structurally very similar, it may have been difficult for the software to discover the differences in their binding properties. We can speculate that the adding and removal of oxygen and carbon atoms disrupted hydrogen bonds to important residues, and that opening the molecule probably rendered it incapable of fitting into the binding pocket. Introducing a double bond restricted the torsional flexibility of the ligand (190), which may have affected its binding properties as well.

5.4 Prenatal opioid exposure of chicken and rat (paper III)

In light of the ongoing opioid crisis in the US and an increasing number of opioid abusers worldwide, it is important to investigate the mechanisms behind the long-term effects on children born to mothers using opioids during pregnancy. In the present work we have focused on the effects of prenatal exposure of morphine, methadone and buprenorphine on GluN2B subunit expression and NMDAR function in rat pups and chicken embryos.

In paper III, we exposed rat pups to methadone or buprenorphine throughout the gestation period, and measured the cerebellar GluN2B expression level at PND1, 7, 14 and 21. It was found that the buprenorphine exposed pups displayed a significantly lower GluN2B expression level at PND 14, compared to control. There was no significant difference between buprenorphine or methadone exposed rat pups. We also exposed chicken embryos to morphine and methadone at E13 and E14, before harvesting the cerebella at E17. At this time point the GCs are migrating from the EGL to the IGL, a process that is finished approximately at E21 (95). These cultures displayed increased Ca$^{2+}$ influx when stimulated with NMDA/glycine, at a time point where no opioids were present. The GluN2B expression in opioid exposed chicken cerebella was unperturbed, however. This indicated that a lasting opioid-related change had occurred. Both in chicken and rat the results pointed towards an
OR-related effect rather than a result of NMDAR inhibition, as the response to the non-competitive NMDAR antagonist methadone was not different from the other opioids (191).

Acute methadone or morphine treatment (10 µM) of cerebellar cultures without NMDA/glycine stimuli appeared to decrease and increase the intracellular Ca\(^{2+}\) level compared to non-treated control, respectively (not shown). A slight activation of the NMDARs caused by plate movement and buffer replacement between measurements must be expected, as discussed in paper III. This would account for the inhibitive effect of methadone compared to non-treated control, which was also observed to a greater degree with NMDA/glycine stimulation (Figure 4C, paper III). The possible potentiating effect of morphine may be explained by opioid-mediated activation of PKC or opioid-related CaMKII activation, which in turn enhance NMDA-induced Ca\(^{2+}\) influx (58, 61) (see section 1.5). This hypothesis could be tested in further studies, by adding a PKC or CaMKII inhibitor in the in vitro acute opioid exposure experiments. However, a potentiating effect of acute morphine treatment could not be observed in the presence of NMDA/glycine stimulation (figure 4D, paper III), and earlier studies have reported contradictory effects of acute morphine treatment on the intracellular Ca\(^{2+}\) level in neuron cultures (192, 193).

In cerebellar cultures from chicken embryos treated with methadone or morphine in ovo, increased intracellular levels of Ca\(^{2+}\) after NMDA/glycine stimulation was observed. We also reported decreased GluN2B expression level at PND14 in prenatally opioid exposed rat pups (paper III). Increased intracellular Ca\(^{2+}\) levels are associated with accelerated GC migration (94) and the effect observed in the chicken embryo cerebellar cultures may be long-lasting and similar for rat. If so, it may lead to accelerated GC migration in opioid-treated rat pups and premature arrival of GCs in the IGL. This would explain the tendency towards elevated GluN2B expression at PND1 and PND7 during premature migration and significantly lower GluN2B expression at PND14 in the buprenorphine treated samples.

5.5 Future perspectives

To further develop the chicken embryo forebrain model it would be of interest to characterise the cell population at E10 and how it changes over time in ovo. Regarding NMDAR expression during forebrain development, an investigation of the gradually increasing GluN2A expression and possible formation of GluN1/GluN2A/GluN2B triheteromers would be beneficial,
especially in terms of comparison with murine models and human development. If the NMDAR development in the forebrain reflects the cerebellar development, the increase in GluN2A expression should occur before hatching.

Considering the computer power, time needed and many limitations associated with molecular modelling, it is important to compare the obtained predictions with reliable in vitro screening results. Optimally, molecular dynamic modelling with explicit water solvent can be combined with functional validation using structure-guided mutagenesis. It would be interesting to use this technique, and compare with in vitro screening of pure enantiomeres of the compounds described in paper II.

To complete the comparison of opioid exposure in rat and chicken, cerebellar cultures could be made from rat pups with the aim of detecting increased Ca\(^{2+}\) influx like we observed in chicken. It could also be examined how long the opioid-related enhanced Ca\(^{2+}\) influx-effect lasts, and include chicken samples from after hatching as well. It is also possible to expose chicken embryos throughout incubation, by implanting a gel that releases controlled amounts of opioids.

Then it could be investigated if opioid exposure affects GC migration temporally in both chicken embryos and rat pups. Another prospective study could focus on the molecular mechanisms behind the drug sensitisation later in life, associated with prenatal exposure to opioids with the aim of detecting a potential involvement of NMDARs.

It would also be interesting to use the GluN2B specific PET probe \(^{11}\)C Ro 04-5595 to assess developmental effects on GluN2B expression, both in chicken and rats. The prospective of using chicken embryos in PET scanning is especially interesting, as the embryo is confined within the egg. This might make it possible to omit sedation, a possible confounder of brain research.
6. **Main conclusions**

- Chicken embryo forebrain cultures are a reliable model for research on NMDAR function and GluN2B specific antagonists
- The Ro 04-5595 molecule is predicted to bind the recently discovered EVT-101 binding pocket, located at the GluN1/GluN2B interface
- *In silico* modelling can provide insights into binding properties, but the high-throughput modalities are not accurate enough to be related to *in vitro* results at this time point
- Molecular dynamic simulation of the ligand-receptor complex predicts bond formation, movement and binding residues, which to some extent can be related to *in vitro* results
- Short-term opioid exposure of chicken embryos does not affect cerebellar GluN2B expression, but promotes increased NMDA stimulated Ca\(^{2+}\) influx in cerebellar cultures
- Long-term opioid exposure during rat gestation leads to lower GluN2B expression in buprenorphine-exposed pups on PND 14, but not at PND 1, 7 and 21.
- The downregulation of GluN2B and increased Ca\(^{2+}\) influx are likely mediated by the OR as there were no significant difference between methadone (NMDAR antagonist and OR agonist) and buprenorphine or morphine (OR-agonists).
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Scientific papers I-III
PAPER I
Exploring the overlapping binding sites of ifenprodil and EVT-101 in GluN2B-containing NMDA receptors using novel chicken embryo forebrain cultures and molecular modeling

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Abstract
N-methyl-D-aspartate receptors (NMDAR) are widely expressed in the brain. GluN2B subunit-containing NMDARs has recently attracted significant attention as potential pharmacological targets, with emphasis on the functional properties of allosteric antagonists. We used primary cultures from chicken embryo forebrain (E10), expressing native GluN2B-containing NMDA receptors as a novel model system. Comparing the inhibition of calcium influx by well-known GluN2B subunit-specific allosteric antagonists, the following rank order of potency was found: EVT-101 (EC50 22 ± 8 nmol/L) > Ro 25-6981 (EC50 60 ± 30 nmol/L) > ifenprodil (EC50 100 ± 40 nmol/L) > eliprodil (EC50 1300 ± 700 nmol/L), similar to previous observations in rat cortical cultures and cell lines overexpressing chimeric receptors. The less explored Ro 04-5595 had an EC50 of 186 ± 32 nmol/L. Venturing to explain the differences in potency, binding properties were further studied by in silico docking and molecular dynamics simulations using x-ray crystal structures of GluN1/GluN2B amino terminal domain. We found that Ro 04-5595 was predicted to bind the recently discovered EVT-101 binding site, not the ifenprodil-binding site. The EVT-101 binding pocket appears to accommodate more structurally different ligands than the ifenprodil-binding site, and contains residues essential in ligand interactions necessary for calcium influx inhibition. For the ifenprodil site, the less effective antagonist (eliprodil) fails to interact with key residues, while in the EVT-101 pocket, difference in potency might be explained by differences in ligand-receptor interaction patterns.

KEYWORDS
chicken embryo model, EVT-101, GluN2B antagonists, ifenprodil, in silico modeling

Abbreviations: ATD, amino terminal domain; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MSV, multiple Sequence Viewer; NMDAR, N-methyl-D-aspartate receptors; PDB, Protein Data Bank; TBS-T, Tween-Tris-buffered saline solution; TGX, Tris-Glycine-extended.
1 | INTRODUCTION

The N-methyl-d-aspartate (NMDA) receptors are found in all brain regions and are involved in synaptic plasticity, learning, and memory. They belong to a subfamily of excitatory glutamate receptors that are ligand- and voltage-gated channels with permeability predominantly for Ca\textsuperscript{2+}, but also for Na\textsuperscript{+} and K\textsuperscript{+}. The NMDA receptors consist of heteromeric tetramers built up by the subunits GluN1, GluN2, and GluN3. Two GluN1 and two GluN2 or GluN3 subunits must be present to enable ligand binding. There are four variants of GluN2: GluN2A, GluN2B, GluN2C, and GluN2D, and the structure can be homo- or heterotetrameric with respect to the different GluN2 subunits. The receptor distribution and composition are dynamic and change during development and in response to sensory input.

Neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and epilepsy are associated with pathological changes in the assembly and location of NMDA receptors. Changes in these receptors are also observed in psychiatric disorders such as schizophrenia and depression. Memantine, a low affinity antagonist that alleviates symptoms of Alzheimer’s disease, is one of the very few commercially available drugs targeting NMDA receptors. The severe psychotomimetic side effects observed when administering unspecific total channel blockers such as MK-801 and ketamine to humans and animal models, indicate a need for new partial and/or subtype-specific antagonists. To develop new drug candidates, it is essential to understand the receptor binding mechanisms and explore the conformational space of the receptor binding sites.

The amino terminal domain (ATD) of the subunits contains binding sites for allosteric compounds, such as the synthetic GluN2B-specific antagonists ifenprodil, Ro-25-6981, and ifenprodil. Recently, an x-ray crystal structure of the GluN1 and GluN2B ATD dimer in complex with ifenprodil was resolved. However, ifenprodil displays unspecific binding to sigma opioid receptors and monoamine receptors, causes behavioral toxicity, and it is readily inactivated by hepatic metabolism. Based on the structural features of ifenprodil, several new GluN2B-specific antagonists such as Ro 04-5595, which has been shown to displace Ro 25-6981, have been investigated. X-ray crystal structure complexes showed that EVT-101 (another GluN2B antagonist) binds at the same GluN1/GluN2B interface as ifenprodil, but occupies an overlapping and less explored binding site. The GluN2B-specific allosteric antagonist HON00001 (structurally similar to Ro 04-5595) has also been shown to have a potent dose-dependent oral analgesic effect in rats, with less side effects and higher receptor specificity than ifenprodil and it has been predicted to interact with the EVT-101 binding site. In this paper, ligands predicted to bind the ifenprodil-binding site are denoted A-ligands, while those predicted to bind the EVT site are named B-ligands.

The NMDA receptor is evolutionarily conserved across species. Many studies have therefore used overexpressed chimeric NMDA receptors with constituents from Rattus norvegicus (R. norvegicus), Xenopus laevis (X. laevis), or Homo sapiens (H. sapiens). In vitro cultured neurons from the developing chicken brain was recently suggested as a suitable model for nonclinical drug testing. Chicken forebrain culture expresses native, functional NMDA receptors with a high proportion of GluN2B subunits, features that make them suited for the present study.

In this paper, we utilized chicken embryo primary forebrain culture and a functional calcium influx assay to investigate the potency of GluN2B-specific allosteric antagonists. Their binding mode was investigated by docking studies and molecular dynamics simulations using experimental structures of GluN1/GluN2B ATD, and the predicted binding data were compared to functional results. We also investigated amino acids critical for ligand binding by in silico mutation studies and found that the residues that differentiate the EVT-binding site from the ifenprodil site are predicted to be located in the GluN2B subunit. EVT-101 was significantly more potent than Ro 04-5595 in terms of calcium influx inhibition, which may be explained by the interaction of EVT-101 with GluN2B/ATD and GluN2B/Ala135. When comparing ligands that are predicted to bind to the ifenprodil site, it appeared that the less potent allosteric antagonist eliprodil failed to interact with residues GluN1/Ser132, GluN2B/Tyr175 and GluN2B/Met207, all of which display interaction with the stronger inhibitors Ro 25-6981 and ifenprodil. Among the compounds tested, the ligands proven (ifenprodil and Ro 25-6981) and predicted (eliprodil) to be located in the ifenprodil-binding pocket are structurally similar, while the EVT-101 binding site appears to accommodate more structurally diverse ligands and binding poses, which is supported by earlier work.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin (Pen-Strep 100X), N-2 supplement (100X), GlutaMAX™ supplement, and L-glutamine were purchased from Gibco™ (part of ThermoFisher Scientific, Waltham, MA). Fetal bovine serum (FBS) and trypsin-EDTA were acquired from BioWhittaker® (Lonza, Switzerland). Leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, sodium orthovanadate, formaldehyde, and anti-β-actin antibody were purchased from Sigma-Aldrich® (now part of Merck, USA). Triton™ X-100, Fura-2 AM cell permeant, and ProLong™ Gold Antifade Mountant with DAPI were obtained from ThermoFisher™ (USA), while anti-GluN2B came from Abcam (Cambridge, UK). Donkey anti-rabbit IgG-HRP was obtained from Santa Cruz Incorporated (Santa Cruz, CA), while Luminata Crescendo and Classic Western HRP substrate and anti-NeuN antibody came from Merck Millipore (Temecula, CA). FITC A109, anti-mouse originated from Chemicon International Inc. (later acquired by Merck Millipore), and goat anti-mouse IgG-HRP was bought from Biorad (Hercules, CA).

2.2 | Animals

Fertilized eggs (Gallus gallus) from different hatches were purchased from Nortura Samvirkekylling (Våler, Norway). The eggs were incubated
at 37.5°C and 45% relative humidity in an OvaEasy 380 Advance EXII Incubator (Brinsa, Weston-super-Mare, UK). The viability of the embryos was checked with trans-illumination using a LED lamp (Brinsa) by observing spontaneous movement. Embryos were sacrificed at embryonic day 10 (E10), and sex determination was not performed. Animals were handled in accordance with the Norwegian Animal Welfare Act and the EU Directive 2010/63/EU. However, chicken embryos are not regarded as research animals before E14 (2010/63/EU; EU, 2010). They have a short incubation time, do not require animal housing and elicit fewer allergies than murine animal models. It is also easier to predict the number of embryos obtained compared to rat or mice, and the hen is exempted from experiments. Thus, their use is in accordance with the 3Rs principles of animal research.

2.3 | Chicken embryo forebrain cultures

The eggs were submerged in crushed ice for 7 minutes to anesthetize the embryos before decapitation. The forebrain was surgically removed, and the meninges were discarded. The tissue was homogenized by chopping with a scalpel before trypsinization in buffered solutions as previously described. Cells were suspended in DMEM supplemented with 1% N-2, 100 U/mL penicillin and 0.1 mg/ml streptomycin (Pen-Strep), 10% fetal bovine serum (FBS), and 0.75% GlutaMAX™. Cells were seeded (1.7 × 10^6 cells/mL) on 35 mm Petri dishes or in 96-well plates (Corning® CellBIND® 96 well plates; Merck) precoated with poly-£-lysine, and incubated at 37°C, with 5% CO₂. These cultures contain an abundance of functional GluN2B receptors on DIV1 (A. Ring, pers. commun.).

2.4 | Transfection of control HEK-293 cells

Human embryonic kidney cells (HEK-293 cells, CRL-1573™ from ATCC®, USA) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin and 4 mmol/L l-glutamine. Experiments were performed after passage number 3 was reached. The HEK-293 cells were transfected with K2 Transfection System® (Biontex Laboratories, Munich, Germany), according to the manufacturer’s protocol. Briefly, the cells were transfected in 35 mm cell culture dishes at 80% confluency, with 1.42 µg DNA/dish and 4.26 µL K2 solution. The cells were incubated at 37°C and 5% CO₂ for 24 hours. The transfection efficiency was estimated to be ≥70% by fluorescence microscopy of pEGFP-N1 (Clontech, USA) transfected HEK-293 cells. The GluN2A and GluN2B plasmids were kind gifts from Professor S. Vicini (Georgetown University, School of Medicine, Washington, DC), and Dr Luo (Zhejiang University, School of Medicine, China), respectively. An empty vector plasmid containing the cytomegalovirus promoter (CMV plasmid) was a gift from J. Milbrandt (Washington School of Medicine, St. Louis, MO).

2.5 | Western blotting

Chicken embryo forebrain cultures (harvested at day in vitro 1 [DIV1]) and HEK-293 cell cultures were washed twice with ice-cold PBS (4°C) and harvested in 2% SDS (in PBS) added the following protease inhibitors: 5 µg/µL leupeptin, 1 µg/µL pepstatin A, 300 µmol/L phenylmethylsulfonyl fluoride, and 100 µmol/L of the phosphatase inhibitor sodium orthovanadate.

Isolated tissues from chicken forebrain (E7-18) and mouse cerebellum (postnatal day 21) were frozen in N₂ (~196°C) before long-term storage at –20°C. To prepare for western blotting analysis, tissue was homogenized as previously described. In short, samples were kept on ice, added tris-EDTA (TE) buffer containing the same protease inhibitors as described above, and homogenized using a motorized pellet pestle. TE with SDS (final concentration 2%) was added to the sample before further homogenization by syringe (25 G) and heat inactivation of proteases (95°C, 5 min). Protein concentration was determined with Pierce™ BCA Protein Assay Kit (ThermoFisher™, USA). Each sample (25 µg) was mixed with Laemmli buffer with 5% mercaptoethanol and then applied to a precast 10-well polyacrylamide Mini-Protein Tris-Glycine-extended (TGX™) gel (BioRad, Germany). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (TransBlot®Turbo™; BioRad, Germany) which was blocked with 5% dry skimmed milk in 1% Tween-Tris-buffered saline solution (TBS-T) for 1 hour at room temperature (RT). The primary GluN2B antibody was diluted in 5% dry skimmed milk in TBS-T to a concentration of 1:1000 and added to the membranes which were then incubated for 24 hours at 4°C. The membranes were rinsed three times with TBS-T and incubated for 1 hour at RT with anti-rabbit secondary antibody (1:10 000 in TBS-T with 5% dry skimmed milk) before a further rinse cycle with TBS-T. Bands were detected using chemiluminescence with HRP substrates in the bio-imaging system ChemiGenius 2 with GeneSnap software (both by Syngene, UK). The amount of internal standard was assessed by immunostaining with β-actin antibody and anti-mouse secondary antibody. The data were analyzed using ImageJ software, and the amount of GluN2B was normalized against the amount of β-actin protein.

2.6 | Immunocytochemistry

The cell culture was grown in poly-£-lysine-coated petri dishes with glass bottom (MatTek Corporation, USA). The cell medium was aspirated. Dishes were added 1 mL of PBS with 3.7% formaldehyde and left at RT for 10 minutes before washing twice with PBS (4°C). The cell membranes were permeabilized with 0.1% Triton-X in PBS before blocking with 5% dry skimmed milk in 1%TBS/Tween for 30 minutes at RT. After washing twice with cold PBS, the neuronal marker antibody NeuN was diluted in PBS (1:100) and 100 µL was added to the dishes and incubated at 4°C for 12 hours. The dishes were washed three times with cold PBS before 400 µL of secondary FITC antibody diluted in 5% dry skimmed milk in 1%TBS/Tween was added at a concentration of 1:250 and left to incubate in the dark for 1 hour at RT. The cells were mounted with the nuclear marker DAPI and visualized with fluorescence microscopy (Eclipse TE300; Nikon, Japan).
2.7 | Calcium influx measurement

The procedure was similar to that previously described by Ring et al. Cells were plated in poly-L-lysine coated 96-well black plates with clear glass bottom (Corning® CelBIND®) and each well was incubated with 4 μmol/L fluorescent calcium (Ca^{2+}) indicator Fura-2 at 37°C, 5% CO₂ for 45 minutes. The medium was then replaced with a standard buffer (140 mmol/L NaCl, 3.5 mmol/L KCl, 15 mmol/L Tris (pH 7.4), 1.2 mmol/L Na₂HPO₄ -NaH₂PO₄ (pH 7.4), 5 mmol/L glucose, and 2 mmol/L CaCl₂ in distilled water) with 1 mmol/L MgCl₂ (wash buffer) and further incubated for 15 minutes in the dark for de-esterification of Fura-2. Fura-2 fluorescence was measured using CLARIOstar® plate reader (BMG Labtech, Germany). Intracellular Ca^{2+} changes were expressed as changes in 340/380 nm fluorescence emission ratio. The wash buffer was then carefully replaced with test compound (20 mmol/L to 10 μmol/L) in standard buffer (n = 4 per concentration, two compounds per 96-well plate). NMDA receptor-mediated Ca^{2+} influx was induced by NMDA (0.2 mmol/L) and glycine (0.1 mmol/L) in each well. The resulting rise in intracellular Ca^{2+} was expressed as a change in the 340/380 emission ratio by subtracting the initial Ca^{2+} level from the NMDA stimulated Ca^{2+} responses. For the compound EVT-101, additional experiments with a lower dose range (2 mmol/L to 1 μmol/L) were included due to the low IC₅₀ value. Inhibition curves for each compound were established by dose response experiments (n ≥ 5). For Ro 04-5595, four representative IC₅₀ values were chosen to make Figure 2A, while 13 experiments around the median were chosen for Figure 2C.

2.8 | Sequence analysis and homology modeling

The Schrödinger Suite version 2018-1 was employed to perform the homology modeling and the docking procedures. Several chimeric x-ray structures of the ATD domain of the NMDA receptor are available in the Protein Data Bank (PDB). Among them are X. laevis/H. sapiens complex with ifenprodil and EVT-101 (PDB id: 5EWW and 5EWM, respectively) and X. laevis/R. norvegicus in complex with Ro 25-6981 (PDB id: 3QEM). The experimental structures contain two dimers with each dimer consisting of the GluN1 from X. laevis (chain A) and the GluN2B from H. sapiens or R. norvegicus (chain B). Two different conformations of EVT-101 binding pose can be observed in the crystal structure, depending on what dimer is considered. In this paper, the A dimer was selected for molecular modeling studies. The experimental structures (5EWW, 5EWM, and 3QEM) were prepared in Protein Preparation Wizard feature in Maestro by assigning bond orders, adding hydrogen atoms, creating zero-order bonds to metal and disulfide bonds and building missing loops <20 amino acids (GluN1: amino acid 97-101, GluN2B: amino acid 53-62 and 54-59 for H. sapiens and R. norvegicus, respectively) using Prime. The large missing loop (186-209 located in GluN1) was not modeled as it was far from the ligand binding pocket and was therefore not considered to have any impact on the binding pocket. Crystal structure water molecules were retained, and the ionization state of the heteroatoms was handled with a pH of 7.4 ± 0.2. The protonation state of the different residues and the optimization of the hydrogen bonds network were performed with PROPKA at pH = 7.4 ± 0.2 with sampling of the crystal water molecules before a final restrained minimization of heavy atoms.

The chicken GluN1 sequence was retrieved from UniProt (ID: Q4KXTT1) while the chicken GluN2B sequence was retrieved from the predicted target sequence with BLAST (Basic Local Alignment Search Tool, XP_015144845.2, NIH, USA). The retrieved sequences were aligned with the sequences from chain A and B of the x-ray crystal structures, using the Multiple Sequence Viewer (MSV) tool. The chicken GluN1 ATD (1-400 residues) sequence is 91% similar to the GluN1 ATD sequence from X. laevis, while the chicken GluN2B ATD has a 95% sequence identity with the human and rat GluN2B ATD. A sequence alignment between human, rat, and chicken GluN2B subunits can be found in supplementary data (Figure S1), made with the Clustal Omega multiple sequence alignment program available at Uniprot's webpage. The total rat and human GluN2B amino acid sequence is 93% similar to the chicken GluN2B sequence, while rat and human GluN2B sequences are 98% similar to each other. The homology model building tool included in MSV was used to construct homology models of each subchain based on the alignment with default settings. Each subunit was merged into a dimer of chicken GluN1 and GluN2B called chicken_NMDA_5EWM and chicken_NMDA_5EWM, respectively. Finally, the entire model was refined and prepared for docking using the Protein Preparation workflow, which ensured structural accuracy by correcting protein and peptide bond orders, tautomeric and ionization states, and restrained minimization.

The only difference close to the allosteric binding pockets of ifenprodil and EVT-101 (14.4 and 13.1 Å, respectively), between the chimeric experimental structures and the chicken NMDA receptor, is a valine at position 107 in the chicken GluN1 sequence compared to an isoleucine in position 107 in the X. laevis GluN1 sequence. The allosteric binding pocket of the chicken NMDA receptor (chicken_5EWW and chicken_5EWM) was created by mutating the isoleucine residue in position 107 to valine in the chimeric X. laevis/H. sapiens and X. laevis/R. norvegicus NMDA receptor crystal structure (PDB id: 5EWW and 5EWM, respectively). The comparison of the docking poses of the co-crystallized ligands in the allosteric binding pocket of the chicken vs their binding pose in their respective crystal structure did not reveal any relevant differences (Figure S1A, in supplemental data). Furthermore, the two conformations of EVT-101 binding pose observed in the crystal structure could be predicted by docking with similar docking scores (Figure S1B). It was therefore decided to use the crystal structures in further docking studies and molecular dynamics simulations.

2.9 | Ligand preparation and docking studies

The docking procedures were performed in Schrödinger’s Glide software. Receptor grid maps were generated for both crystal structures in complex with ifenprodil and EVT-101 (PDB id: 5EWW and 5EWM, respectively) using default settings and co-crystallized
ligands as the centroid of the map. Two overlapping allosteric binding sites have been described for the GluN1/GluN2B subunits: the ifenprodil and the EVT-101 binding pockets. In order to study the ligand-protein interactions of the ligands used in vitro, the complexes GluN1/GluN2B: ifenprodil, GluN1/GluN2B: Ro 25-6981, and GluN1/GluN2B: EVT-101 were taken from the PDB while GluN1/GluN2B: eliprodil and GluN1/GluN2B: Ro 04-5595 were generated through docking.

The structure of eliprodil and Ro 04-5595 was drawn with the software Maestro and prepared using Ligprep (Schrödinger Release 2018-1: LigPrep, Schrödinger, LLC, New York, NY, 2018). Enantiomers and protonation states at a target pH = 7.4 ± 0.2 were generated. Stroebel et al. reported that the GluN1 residues leucine 135 and isoleucine 133 rotate to fill the empty space of the ifenprodil-binding pocket when EVT-101 is co-crystallized with the NMDA receptor, obstructing the binding pocket of ifenprodil. Hence, eliprodil and Ro 04-5595 were docked into both chimeric X. laevis/H. sapiens NMDA x-ray crystal structures using the virtual screening workflow. Standard precision was employed with retention of three docking poses per enantiomer for a final MM-GBSA calculation. For each ligand, the complex protein-docking pose with the best MM-GBSA score was chosen as input for the MD simulation.

Due to its structural similarity with Ro 04-5595 and interesting pharmacological properties, HON0001 was compared to Ro 04-5595 using the MOLPRINT2D fingerprint and Tanimoto similarity metrics in the Canvas software (Schrödinger Release 2017-3: Canvas, Schrödinger, LLC, New York, NY, 2017), obtaining a Tanimoto score (similarity) of 0.750.

2.10 Molecular dynamics simulations

Molecular dynamics simulations were performed with the Desmond program. The selected complexes were set up in an orthorhombic simulation box with periodic boundary condition, the OPLS3 force field TIP3 water model was employed for the solvation of the system before it was neutralized and 0.15 mol/L NaCl was added. The generated systems were relaxed using the Desmond default protocol and run for 100 ns on a GPU. The NPT ensemble was selected with a $P = 1.01325$ bar and $T = 300$ K using the Martynas-Tobias-Klein barostat method (relaxation time of 2 ps and isotropic coupling style) and the Nose-Hoover Chain thermostat method (relaxation time of 1 ps and one group for temperature), respectively. The RESP integrator was selected and the bonded and close nonbonded interactions were handled with a timestep of 2 fs while for far nonbonded interactions the timestep was set to 6 fs. A cut-off of 9 Å was used for the short-range columbic interactions. The trajectories and energies were recorded every 10 ps giving a total of 10,000 frames. Root Mean Square Deviations of protein and ligand can be observed in Figure S3. The last 10 ns (90-100 ns corresponding to the last 1000 frames) were considered for analysis of the protein-ligand interactions, the generation of average ligand–receptor complexes, and alanine scanning calculation utilizing the residue scanning tool from BioLuminate.

All residues within 5 Å of the ligand in the averaged complexes were mutated into alanine and their contribution to the free energy of binding (ΔG) was analyzed by calculating the difference in ΔG before and after mutation for each residue. The averaged conformation of eliprodil and EVT-101’s receptor-ligand complex required additional minimization before alanine mutation scanning could be performed. This was done by the minimization panel featured in the MacroModel software, with OPLS3 force field, water as solvent and extended cut-off.

2.11 Analysis and statistics

Outlier values were tested for by the built-in feature in GraphPad (Robust regression and Outlier removal, Q = 1%) and normality was checked with the D’Agostino-Pearson omnibus normality test. Statistically significant differences were evaluated by Kruskal-Wallis’ test or Mann-Whitney’s test depending on the number of samples. Dunn’s multiple comparison test was included as post hoc-test when appropriate.

3 RESULTS

3.1 Cultures from chicken forebrain express GluN2B

Since chicken primary forebrain neuron cultures have not been described before, we immunostained them with NeuN, a marker of most neurons that have reached a certain level of maturity. The fraction of NeuN-positive cells was estimated to be 40% relative to the overall cell number (DAPI-stained nuclei) at DIV1 (Figure 1A,B).

The presence of GluN2B was confirmed with western blotting. Specificity of the GluN2B antibody was assessed in transfected HEK-293 cells overexpressing the GluN2B subunit, shown as a strong band at 166 kDa [consistent with the expected $M_w$ of GluN2B] (Figure 1C). No band in this range was detected in the negative control samples (HEK-293 cells transfected with control vector CMV or GluN2A subunit plasmid). Both chicken embryo forebrain tissue (E7-E8), mouse cerebellar tissue (postnatal day [P] 21), and cultures from chicken forebrain (DIV1) expressed GluN2B. The relative level of GluN2B protein in the chicken forebrain increased rapidly from E12 and reached a plateau at E15 (Figure 1D).

3.2 Functional properties of chicken NMDA receptors resemble their human and rat counterparts

Functional properties of the NMDA receptor in chicken forebrain culture were tested with the calcium influx assay as described previously. It was shown that the receptor was activated by standard protocol concentrations of NMDA and glycine. The NMDA receptors in the cultures were assumed to contain a significant fraction of GluN2B subunits as approximately 70% of the calcium influx could be blocked by the GluN2B-specific allosteric antagonists Ro 25-6981,
ifenprodil, eliprodil, and EVT-101, at concentrations shown to elicit similar responses in rat and human NMDA receptors (Table 1 and Figure 2A) and that the calcium influx was reduced by 90% by 10 μmol/L of the unspecific NMDA receptor inhibitor MK-801. The less-explored antagonist Ro 04-5595 showed an IC$_{50}$ value of <200 nmol/L. The differences in IC$_{50}$ values between eliprodil and the other A-ligands were statistically significant: Eliprodil vs ifenprodil and eliprodil vs Ro 25-6981 (*P ≤ 0.05, and **P ≤ 0.001, respectively, shown in Figure 2B). B-ligands EVT-101 and Ro 04-5595 gave significantly different IC$_{50}$ values when tested experimentally in the chicken forebrain primary culture calcium influx assay (**P < 0.0001, Figure 2C).

### 3.3 Computer modeling reveals conserved tertiary structure of chicken GluN1/GluN2B ATD

The high percentage of amino acid sequence similarity between chicken GluN1/GluN2B ATD and X. laevis/H. sapiens GluN1/GluN2B
Our docking studies and molecular dynamic simulations supported interactions between ligands and binding site residues. EVT-101 binding site in the crystal structures into the corresponding amino acid in the chicken, GluN1Ile107 to GluN1Val107 in chicken, did not affect the binding poses and properties of ifenprodil or EVT-101 compared with the x-ray complexes (supplemental data, Figure S2). These similarities enabled the use of experimental x-ray structures instead of the chicken homology model for studying the dynamics of ligand interactions. X-ray structures are regarded as both structurally and energetically more stable than homology models and more reliable predictions are expected.

3.4 Molecular dynamics simulations predict interactions between ligands and binding site residues

Our docking studies and molecular dynamic simulations supported that Ro 25-6981 and ifenprodil shared the ifenprodil-binding site and showed that eliprodil interacted with the ATD domain via the ifenprodil-binding site. This is supported by earlier findings. Elliprodil gave a Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) score of −93.65 kcal/mol when docked in the ifenprodil-binding pocket vs a −70.75 kcal/mol MM-GBSA score when docked in the EVT-101 binding pocket. The calculations also predicted that EVT-101 and Ro 04-5595 bound to the less explored EVT-101 binding site, sharing a hydrophobic pocket with the ifenprodil-binding site. The best MM-GBSA score for Ro 04-5595 was −71.23 kcal/mol in the EVT-101 binding pocket vs −61.84 kcal/mol in the ifenprodil pocket. An overview of the overlapping binding poses and residue interactions of Ro 25-6981, ifenprodil, eliprodil, EVT-101, and Ro 04-5595 are shown in Figure 3 and Table 2, respectively. Individual binding poses and selected interactions are shown in Figure 3B-F.

3.4.1 The common hydrophobic pocket

Docking indicated that a part of the binding pocket is common for all compounds. This region of the receptor is hydrophobic and includes Tyr109 and Phe113 in GluN1, as well as Ile111 and Phe114 in GluN2B which all accommodate an aromatic ring, or the interface toward the linker region of the ligands. In addition, all ligands were predicted to interact with GluN1Ile133 (Ro 25-6981 interacted sporadically), and all except Ro 04-5595 were predicted to interact with GluN2Bphe176. Molecular dynamics simulations suggested that all ligands formed nontransient interactions with GluN1Tyr109, but B-ligands were predicted to have the most stable interaction with this residue (Figure 4A). Mutating Tyr109 into alanine and calculating the change in binding free energy did indeed predict a more substantial drop in affinities for B-ligands than for A-ligands. The B-ligands were also predicted to interact more strongly with GluN1Phe113. Despite that, alanine mutation scan of GluN1Phe113 predicted quite similar changes in affinity for Ro 25-6981, eliprodil and Ro 04-5595, while ifenprodil and EVT-101 had a lesser decrease in affinity compared to the other ligands. GluN2Bile111 was predicted to have quite similar interaction with all ligands, reflected by the uniform effect of the alanine mutation on ∆G values. Of all, Ro 04-5595 was predicted to have the most stable interaction with GluN2Bphe114, followed by ifenprodil and Ro 25-6981. This was supported by alanine mutation scanning data, predicting the largest change in ∆G for Ro 04-5595 when mutating GluN2Bphe114 into alanine in silico.

Ifenprodil, EVT-101, and Ro 04-5595 were predicted to bind GluN1Ile133 equally firmly, but with different bonding patterns. Ifenprodil interacted with GluN1Ile133 through a water bridge (Figure 4A), while eliprodil displayed less stable interaction than the others with GluN1Ile133. However, all ligands except Ro 25-6981 received a comparable reduction in affinity when GluN1Ile133 was mutated into alanine. GluN2Bphe176 on the other hand was predicted to interact with Ro 25-6981, ifenprodil and EVT-101 in a fairly similar manner, while eliprodil displayed a very stable interaction to the residue. Despite that, the affinities of Ro 25-6981 and eliprodil were lowered similarly by alanine mutation, while the affinities of ifenprodil and EVT-101 were affected to a lesser degree. The affinity of Ro 04-5595 was not affected, as expected (Figure 4B).
3.4.2 The ifenprodil-binding site

The predicted common residues for the A-ligands Ro 25-6981, ifenprodil and eliprodil were Arg115, Leu135, Ser132 (GluN1), Gln110, and Glu236 (GluN2B). Mutating GluN1Arg115 did not noticeably affect the affinity for any of the ligands. For ifenprodil, this was quite surprising, as it was predicted to have a stable interaction with the residue. This may be explained by the distance between ifenprodil and the positive charge of the residue, which exceeds 5 Å. Both Ro 25-6981 and ifenprodil were predicted to bind firmly to GluN1Ser132, while eliprodil was only predicted to be loosely associated with this residue (Figure 4C). Alanine scanning mutation showed minimal difference in affinity for all three ligands, as they interact primarily with the amino acid backbone. GluN1Leu135 displayed the largest amount of interaction with eliprodil and slightly less with ifenprodil, while Ro 25-6981 was predicted to bind GluN1Leu135 to a lesser extent. However, the predicted affinities were similarly affected for all ligands when mutating the residue into alanine. Molecular dynamics simulations predicted comparable interaction properties for all ligands toward GluN2BGlu110. Alanine mutation scanning supported this result giving quite similar ΔG values, but with the largest decrease in affinity for eliprodil. Eliprodil was anticipated to interact the
FIGURE 3 Representation of the average binding mode of each ligand with display of important binding site residues in their respective average protein structure. The bright red band indicates the position of the divergent residue in the chicken NMDA receptor. N1 and 2B prefix denote GluN1 and GluN2B, respectively. Yellow dashes represent hydrogen bonds and magenta dashes π-stacking/α-cations. A-ligands are blue, B-ligands are red. A, Overview of the binding poses of Ro 25-6981, ifenprodil, eliprodil, Ro 04-5595, and EVT-101 combined. B, Ro 25-6981 C, Ifenprodil, D, Eliprodil, E, EVT-101 F, Ro 04-5595
least with GluN2BGln236, while both Ro 25-6981 and ifenprodil displayed stable bonding patterns. Despite that, affinities of Ro 25-6981 and eliprodil were lowered quite equally when mutating the residue in silico, though the affinity of ifenprodil was more affected.

Both Ro 25-6981 and ifenprodil were predicted to bind GluN2B Tyr175 and GluN2B Met207, although ifenprodil displayed a more stable interaction. Both ligands were predicted to have comparable loss of affinity when mutating GluN2B Tyr175 into alanine, while mutating GluN2B Met207 into alanine only slightly decreased the affinity of ifenprodil. Eliprodil and ifenprodil were predicted to both have a stable binding to GluN2B Glu106. Affinity of ifenprodil to the binding site was predicted to be severely decreased by mutating the residue to alanine, while the affinity of eliprodil was less affected, suggested to be caused by its bond type (π-π stack vs water bridge). According to the molecular dynamics simulations Ro 25-6981 interacted with two amino acid residues on its own: GluN1Leu131

### TABLE 2

Overview of predicted binding residues

<table>
<thead>
<tr>
<th>Ifenprodil binding site</th>
<th>Residues shared by all ligands</th>
<th>EVT-101 binding site</th>
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<tbody>
<tr>
<td>Ro 25-6981</td>
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<tr>
<td>ifenprodil</td>
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<td>Eliprodil</td>
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<td>EVT-101</td>
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A schematic overview of the shared binding residues, binding residues in each binding pocket and specified to ligands within each binding pocket. Prefix N1 denotes that the residue is located in the GluN1 subunit, while 2B indicates the GluN2B subunit. Residues shared between ligands are shown in black: 2BPro78 is shared between Ro 25-6981 and EVT-101, 2BPhe176 is common for ifenprodil, eliprodil, and EVT-101, while 2BAla107 is predicted to bind both Ro 25-6981 and eliprodil.
FIGURE 4 Observed interactions between the ligands and the receptor during molecular dynamics simulations and comparison of the free energy of binding $\Delta G$ (kcal/mole) before and after alanine mutation scanning. The percentage of frames (complexes) showing interactions between the ligands and their binding amino acids during the last 10 ns of the molecular dynamics simulations (1000 frames). The interactions comprise H-bonds, $\pi$-cation interaction, $\pi$-$\pi$ stacking, other hydrophobic interactions, ionic bonds, and water bridges. One residue can have several interactions, which is why some values exceeded 100%. Prefix N1 denotes that the residue is located in the GluN1 subunit, while 2B indicates the GluN2B subunit. A, The interactions between Ro 25-6981, ifenprodil, eliprodil, EVT-101, Ro 04-5595, and the residues shared by all ligands: N1Tyr109, N1Phe113, N2Bile111, and N2BPhe114. N1lle133 was shared by all except Ro 25-6981 and 2BPhe176 was shared by all except Ro 04-5595. B, Differences in the free energy of binding ($\Delta G$) (kcal/mole) for Ro 25-6981, ifenprodil, eliprodil, EVT-101 and Ro 04-5595 when mutating residues shown in 4A into alanine. C, The interactions between Ro 25-6981, ifenprodil, eliprodil, and their shared residues located in the ifenprodil-binding site: N1Arg115, N1Ser132, N1Leu135, 2BGLN110 and 2BGlu236. D, $\Delta$ affinity (kcal/mole) for Ro 25-6981, ifenprodil and eliprodil when mutating residues shown in 4C into alanine. E, The interactions between EVT-101 and Ro 04-5595 and their shared residues located in the EVT-101-binding site: 2BAsp113, 2BAsp136, and 2BPro177. F, $\Delta$ affinity (kcal/mole) for EVT-101 and Ro 04-5595 when mutating residues shown in 4E into alanine.
and GluN2BLeu205. Both were weak hydrogen bond interactions. Mutation of both residues into alanine was not predicted to have an extensive effect on the affinity of Ro 25-6981 to the binding site (Figure 4D).

3.4.3 | The EVT-101 binding site

The amino acid residues Asp113, Asp136, and Pro177 (GluN2B) were predicted to interact with both B-ligands Ro 04-5595 and EVT-101. GluN2BAsp113 presumably displays a stable interaction to Ro 04-5595 and a weaker connection to EVT-101. Introducing in silico mutations of asparagine to alanine had an opposite effect on the affinity of the two ligands. The affinity of Ro 04-5595 was lowered, while EVT-101 was predicted to bind the mutant stronger. Both predicted interactions with GluN2BAsp136 appeared in <5% of the last 10 ns of simulation time, reflected by the relatively low impact on affinity when mutating the residue into alanine. GluN2BPro177 was predicted to have interactions with both EVT-101 and Ro 04-5595, and the effect of changing the residue to alanine was relatively similar for both ligands. EVT-101 appears to have a hydrophobic interaction with GluN2BMet134 and a stable H-bond with the backbone of GluN2B Ala135 and the effect on the affinity of the ligand when mutating the methionine residue was noticeable. Ro 04-5595 was predicted to interact weakly with GluN2BLys137 and GluN2BAsp138, mainly through water bridges. Alanine mutation scanning indicated an increase in ligand affinity when mutating both residues to alanine. The residues that were predicted to interact with both EVT-101 and Ro 04-5595 are shown in Figure 4E and the corresponding alanine mutation scanning results are shown in Figure 4F.

Some of the residues that did not interact with the ligands still affected their affinities when running an alanine mutation scan, probably due to local conformational changes within the binding cavities or indirect effects. The residues are summarized in Figure S4 in supplementary data, and we found that Ro 25-6981, ifenprodil, and eliprodil shared some of them. The only residue shared by all ligands is GluN1Thr110, for which an alanine mutation is predicted to be especially critical for the affinity of EVT-101, but enhances the affinity of Ro 04-5595. Overviews of all predicted interactions and the ΔG differences (kcal/mole) for all residues and ligands predicted by alanine mutation scanning are included in the supplementary data (Figure S5).

4 | DISCUSSION

In the present study, we have used primary cultures from chicken embryo forebrain as a model to study potencies of different GluN2B polyamine site antagonists to reduce calcium influx. To support the experimental data, computational methods were applied to predict binding to amino acids in the two overlapping ifenprodil and EVT-101 sites.

The chicken embryo forebrain cell culture expresses GluN2B, established by using a specific antibody raised against a rat GluN2B epitope. Compared to human and rat, the expression pattern of GluN2B in developing chicken forebrain follows a similar trajectory. However, the decline in GluN2B protein expression appears to take place prenatally in chicken, as opposed to postnatally in human and rat. This may reflect a higher degree of relative maturity of the cortex in newly hatched chickens compared to new-born rats or humans. This is an advantage when considering chicken embryos as an animal model for NMDA receptor development, as it enables easy access to study developmental processes occurring postnatally in other research animals, while the chicken is still contained within the egg.

Since expression studies confirmed the presence of GluN2B-containing receptors, we wanted to confirm that these were functional in vitro. This was done by studying NMDA- and glycine-induced calcium influx. However, it is important to note that the chicken forebrain cell culture contains different cell types, with approximately 40% mature neurons (NeuN positive). As the NMDA-induced Ca$^{2+}$ influx could be inhibited 70% by GluN2B-specific antagonists and 90% with the nonspecific NMDA receptor blocker MK-801, we assume that some of the Ca$^{2+}$ influx originated from NMDA channels with a different subunit composition. Naturally, this contributes to the larger standard deviations we observed in our experiments, compared to that observed in pure, transfected GluN1/GluN2B receptors. Still, chicken E10 embryo primary forebrain cultures proved to be an effective and reproducible way of accessing native, functional GluN2B-containing NMDA receptors. The difference in the chicken CTD compared to human (88% similarity) may reduce the validity of the chicken model in experiments regarding downstream NMDA receptor signaling, but as we obtained similar IC$_{50}$ values for known GluN2B allosteric antagonists in chicken that have previously been described for rat and human$^{48,52}$ it is most likely that the human, rat, and chicken receptors share similar functional properties.

The significant differences in IC$_{50}$ values between the antagonists tested suggest different binding properties and these were investigated in silico by docking studies and molecular dynamics simulations, predicting temporal information on the interactions between ligands and binding residues as well as providing details on bond types. As no experimental structures of NMDA receptor in complex with eliprodil and Ro 04-5595 were available at the time of the writing, the molecular modeling approach gave new knowledge about the binding properties of these compounds. The only non-identical amino acid (GluN1Val107 in the chicken, vs Glun11Lei107 in the X. laevis/H. sapiens crystal structure) close to both binding sites in the chicken homology model did not have any effect on the docking pose of the antagonists ifenprodil and EVT-101 compared to that of the x-ray structures, because valine and isoleucine rotamers were predicted to point away from the binding pockets, which implies less probability of influence on the binding. None of the ligands were predicted to interact with Glun11Lei107 so the residue does not appear to be important for either the ifenprodil or EVT-101 binding pocket.
Of the three ligands that bind the ifenprodil-binding site, Ro 25-6981 and ifenprodil were the most effective GluN2B subunit-specific Ca\textsuperscript{2+} influx inhibitors when tested in the chicken forebrain primary cell culture assay, supported by the work of Hedegaard et al.\(^2\) in rat. Our in vitro experiments showed a significant difference in IC\textsubscript{50} values between Ro 25-6981 and ifenprodil vs eliprodil, which was thus addressed in the in silico studies with supporting findings: Eliprodil was predicted to interact less with GluN1Phe113, GluN1Ser132, GluN2BPhe114, GluN2BMet207 and GluN2BGlue236, but more with GluN2BPhe176 than the rest of the ligands. Of these, GluN1Ser132, GluN2BMet207, and GluN2BGlue236 have been cited as important binding residues for known GluN2B-specific allosteric inhibitors binding the ifenprodil-binding pocket in earlier publications.\(^13,17,30,51\) Mutel et al.\(^18\) found Ro 25-6981 to have larger affinity to the binding site than both ifenprodil and eliprodil and it might be suggested that the predicted interactions with GluN1Lys131, GluN2BPro78 and GluN2BLeu205 granted the Ro 25-6981 a better ability to inhibit Ca\textsuperscript{2+} influx than the other A-ligands. Computational mutation of these residues showed that of these, only mutation of GluN2BPro78 into alanine was predicted to have larger effect on the affinity of Ro 25-6981 than the other A-ligands. However, the weak bonds with GluN1Lys131 and GluN2BLeu205 may still be involved in the antagonistic effect of the ligand.

In silico docking of the less-explored GluN2B-specific allosteric antagonist Ro 04-5595 predicted that it bound to the recently discovered EVT-101 binding pocket. This conclusion is supported by docking studies of the structurally similar compound HON0001\(^24\) which predicted that HON0001 would bind to the EVT-101 site as well.\(^17\) The analgesic effect of orally administered HON0001 encourages further investigations of Ro 04-5595 as a potential research tool or drug.

The IC\textsubscript{50} values of EVT-101 and Ro 04-5595 were significantly different, with EVT-101 as the most effective antagonist. Compared to EVT-101, Ro 04-5595 was predicted to interact more strongly with GluN2BPhe114 and much less with GluN2BPh176, and this is supported by the alanine scanning results. It appears that EVT-101 and Ro 04-5595 are predicted to interact with different residues to a larger degree than in the ifenprodil site, rather than Ro 04-5595 failing to interact with important residues, as might be the case with eliprodil. This is supported by the work of Stroebel et al.\(^17\) who predicted more diverse binding poses of the ligands docked in the EVT-101 binding pocket, compared to the ligands docked in the ifenprodil-binding pocket. EVT-101 was predicted to interact with GluN2BMet134, GluN2BAla135 and GluN2BPhe176 (shared with the A-ligands) on its own, and has a much stronger interaction with GluN2BPro177 than Ro 04-5595. Of these, in silico alanine mutation of GluN2BMet134 decreased the ligand affinity noticeably. Ro 04-5595 supposedly interacts with GluN1Leu135 (shared with all the A-ligands), GluN2BPro78 (shared with Ro 25-6981), GluN2BLeys137 and GluN2BAsp138 alone, where the interaction with the last two mentioned may be less favorable in terms of causing an inhibiting effect on Ca\textsuperscript{2+} influx. All of these interactions had corresponding alanine mutation scanning results. Earlier mutagenesis experiments changing GluN2BAla135 to proline, GluN2BPhe176 to alanine, and GluN2BPro177 to cysteine did indeed increase the IC\textsubscript{50} value of EVT-101.\(^17\)

Stroebel et al.\(^17\) have analyzed three in vitro alanine mutations. They observed that in vitro mutation of GluN1Leu133 led to a lower IC\textsubscript{50} value for ifenprodil, and a higher value for EVT-101. This corresponded with our in silico observation of higher loss of affinity for EVT-101 than for ifenprodil. However, mutation of GluN1Leu135 to alanine in vitro, which gave small changes in IC\textsubscript{50} values, did not correspond with our in silico alanine mutation scan results, which predicted a reduction in both ifenprodil and EVT-101 affinities. Also, in vitro alanine mutation of GluN2BPh176 increased the IC\textsubscript{50} values of both ligands drastically. Interestingly, only the least efficient antagonist, eliprodil, was predicted to have a stable interaction with this residue, and ifenprodil and EVT-101 affinities were predicted to be less affected by this mutation. These discrepancies underscore the importance of comparing in silico data with experimental data.

In conclusion, we have established the chicken primary forebrain culture as a useful, reliable and convenient model to study functional properties of native GluN2B-containing NMDA receptors, giving experimental support to in silico binding studies. The less investigated GluN2B-specific allosteric antagonist Ro 04-5595 was predicted to interact with the novel EVT-101 binding site, an interesting pharmaceutical target as it mediates a high degree of calcium influx inhibition when bound. The EVT-101 binding pocket accommodates more structurally diverse ligands compared to the well-known ifenprodil site and contains interesting binding residues such as GluN2BMe134 and GluN2BAla135. Eliprodil was predicted to interact less with the ifenprodil-binding site than Ro 25-6981 and ifenprodil, supporting our in vitro experiments where it presented as the least potent antagonist.

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DISCLOSURES

None declared.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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PAPER III
Opioid receptor-mediated changes in the NMDA receptor in developing rat and chicken

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\textbf{ABSTRACT}

The use of opioids during pregnancy has been associated with neurodevelopmental toxicity in exposed children, leading to cognitive and behavioural deficits later in life. The N-methyl-D-aspartate receptor (NMDAR) subunit GluN2B plays critical roles in cerebellar development, and methadone has been shown to possess NMDAR antagonist effect. Consequently, we wanted to explore if prenatal opioid exposure affected GluN2B subunit expression and NMDAR function in rat and chicken cerebellum. Pregnant rats were exposed to methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day) for the whole period of gestation, using an osmotic minipump. To further examine potential effects of prenatal opioid exposure in a limited time window, chicken embryos were exposed to a 20 mg/kg dose of methadone or morphine on embryonic days 13 and 14. Western blot analysis of cerebella isolated from 14 days old rat pups exposed to buprenorphine showed significantly lower level of the GluN2B subunit, while the opioid exposed chicken embryo cerebellar GluN2B expression remained unaffected at embryonic day 17. However, we observed increased NMDA/glycine-induced calcium influx in cerebellar granule neurone cultures from opioid exposed chicken embryos. We conclude that prenatal opioid exposure leads to opioid receptor-dependent reduction in the postnatal expression of GluN2B in rat cerebella, and increase in NMDA-induced calcium influx in chicken embryo cerebella.

1. Introduction

The prevalence of both clinical use and personal abuse of opioids during pregnancy has increased dramatically in recent years (Committee on Obstetric P, 2017). This has been associated with increased incidence of premature birth, and neonatal abstinence syndrome (NAS), in addition to reduced foetus size and head circumference in children prenatally exposed to opioids (Winklbaur et al., 2008). Potential deficits in cognitive, motor and behavioural development have also been suggested (Ornoy et al., 2001). Opioid maintenance therapy (OMT) with the full Mu-opioid receptor (MOR) agonist, methadone, or the partial MOR agonist buprenorphine, remarkably improves neonatal outcomes (Zedler et al., 2016). However, some reports indicate that OMT with methadone is associated with higher frequency and severity of NAS, even compared with heroin use (Binder and Vavrinkova, 2008). How the opioids cause neurodevelopmental toxicity has not been revealed, and confounding factors in epidemiological studies and lack of mechanistic understanding call for animal model studies to elucidate the underlying mechanisms. Chicken and rat are both well-established animal models for opioid exposure (Newby-Schmidt and Norton, 1981; Sun et al., 2010; Ebert et al., 1995). Comparative anatomy studies have shown that the cerebellum is a well-conserved structure between species (Sultan and Glickstein, 2007) and the ontogeny of the opioid system in both developing chicken and rat brain, including the cerebellum, has been well characterised (Vernadakis et al., 1990; Spain et al., 1985). The migration of granule neurones from the external granule layer (EGL) to the internal granule layer (IGL) occurs before hatching in the chicken (Austdal et al., 2016), while it is completed by two weeks after birth in the rat (Hager et al., 1995). The migration is guided by N-methyl-D-
aspartate receptor (NMDAR)-mediated calcium ion (Ca\(^{2+}\)) influx, among many other signals (Komuro and Rakic, 1993). Because of its perinatal development, cerebellum is a probable late gestation target of opioids.

The NMDARs belong to a subfamily in the group of excitatory ionotropic glutamate receptors. They are ligand-gated cation channels with a high permeability for Ca\(^{2+}\) and functional NMDARs are composed of four subunits, with two NR1 and two NR2 or NR3 subunits (Paolotti and Neyton, 2007). NMDARs play critical roles in brain development, such as neurite outgrowth (Pearce et al., 1987), neuronal migration (Komuro and Rakic, 1993), apoptotic (Gupta et al., 2013) and excitotoxic neuronal death (Ikonomidou et al., 1999). The composition and localization of NMDAR subunits are dynamic and vary across the CNS, particularly during development. GluN2B is widely expressed in the rat CNS before birth, whereas expression of GluN2A begins perinatally. GluN2B is then gradually restricted to the forebrain after postnatal week 1. In higher brain structures of the adult brain, GluN2A and GluN2B predominate, and this may reflect that they are primarily associated with synaptic function and plasticity (Paolotti et al., 2013). In avian forebrain, a gradual increase in GluN2B was observed between embryonic day 7 and 15, followed by a plateau (Fjelldal et al., 2019). In the rat cerebellum, the expression of GluN2B reaches a peak around PND14, before GluN2A and GluN2C increasingly replace it towards adulthood (Monyer et al., 1994). Thus, the expression of different NMDA receptor subunits, in particular GluN2B, is important for the developmental plasticity in the cerebellum.

Some opioids, including methadone, are also non-competitive antagonists of NMDARs (Ebert et al., 1995) and chronic binding of antagonists to NMDARs during development has been associated with changes in receptor composition (Neal et al., 2011). Supporting this, an Adverse Outcome Pathway (AOP) has been established for the correlation between NMDAR antagonists and their effects on learning and memory, with ID-number 13 (Sachana et al., 2018). Co-localisation of MOR and NMDARs and close interactions in their downstream signalling have been observed during anti-nociceptive tolerance to opioids (Commons et al., 1999; Gracy et al., 1997; Rodríguez-Munox et al., 2012). In a related manner, non-nociceptive doses of NMDA antagonists tend to prevent or reverse nociceptive tolerance against morphine (Marek et al., 1991). The GluN2B subunit has been implicated in the rewarding effect of morphine, and its expression in CNS has been demonstrated to change after repeated morphine injections (Narita et al., 2000).

The hypothesis of the present study was that prenatal opioid treatment disturbs cerebellar development. Because of the importance of NMDARs during neurodevelopment, this was addressed by measuring the expression level of the GluN2B subunit in the developing rat cerebellum and NMDA-induced calcium influx in isolated cerebellar neurones from chicken embryos previously exposed to opioids.

2. Materials and methods

2.1. Reagents

Basal Eagle’s medium (BME), penicillin-streptomycin (PenStrep) (100 Units/ml Penicillin; 100 μg/ml Streptomycin), Pierce BCA Protein Assay Kit, Glutam-ate™ and chicken serum were purchased from ThermoFisher Scientific (Waltham, MA, US). L-glutamine originated from Lonza BioWhittaker (Verviers, Belgium). Fura-2 was purchased from Invitrogen™ Molecular Probes (Eugene, OR, US). Luminata Forte™‐HRP, nitrocellulose membrane and acrylamide were bought from Bio‐Rad (Hercules, CA, US). Goat anti-rabbit immunoglobulin (Ig) G-horseradish peroxidase (HRP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, US). Goat anti-mouse IgG-HRP, nitrocellulose membrane and acrylamide were bought from Bio-Rad (Hercules, CA, US). Anti-NMDA2B antibody (ab65783) and anti-MOR antibody (ab177898) were from Abcam (Cambridge, UK). Methadone–HCl (MW 345.91) and anti-β-actin antibody (#A5316) were bought from Sigma (now Merck, Darmstadt, Germany), buprenorphine–HCl (MW 504.11) from Chiron (Trondheim, Norway), morphine–HCl (MW 321.84) from Norsk Medisinaldepot AS (Oslo, Norge), and sterilised water from Fresenius Kabi (Oslo, Norway). [\(^3\)H]-ifenprodil was purchased from PerkinElmer (Waltham, MA, US), while verapamil, nifedipine and naloxone were obtained from Merck (Darmstadt, Germany). CGP 39551 was purchased from Novartis (Basel, Switzerland).

2.2. Animals and treatments

The animal experiments were approved by the Norwegian Animal Research Authority (Norwegian Food Safety Authority). All animals were handled in accordance with the Norwegian Animal Welfare Act and the EU Directive 2010/63/EU.

2.2.1. Rat offspring

Rat offspring (Sprague-Dawley; Taconic, Ebyj, Denmark) were prenatally exposed to methadone (10 mg/kg/day), buprenorphine (1 mg/kg/day), or vehicle (sterile water) by use of a 28-day osmotic minipump (delivering rate 2.5 μl/hour, 2ML4, Alzet Cupertino, CA, US) implanted in the dams 5 days prior to mating. Similar doses have proven to elicit developmental effects in rat offspring for methadone (Hutchings et al., 1992) and buprenorphine (Robinson and Wallace, 2001). The pump delivered the drug continuously throughout the gestation. The animals were housed in the animal facility at the Department of Comparative Medicine, University of Oslo, with a 12-h light/dark cycle, temperature of 21 ± 2 °C, humidity of 50 ± 10%, and food and water ad libitum. The day of birth was noted as postnatal day (PND) 0. After birth the pups were kept with their respective dam. On PND 1, 7, 14 and 21, one or two pups were randomly chosen from each litter and decapitated (n = 6). The brains were removed and the cerebella were dissected and snap-frozen by submerging them in liquid nitrogen. The cerebella were stored at -80 °C until further processing for western blot analysis.

2.2.2. Chicken embryo

Eggs (Gallus gallus) were obtained from Nortura Samvirkekylling (Våler, Norway) and incubated at 37.5 °C and 45% relative humidity in an OvaEasy 380 Advance EXII Incubator (Brinsea, Weston-super-Mare, UK). In accordance with the 3R principles, the mother is excluded from the experiments and the number of fertilized eggs are easy to control. On embryonic day (E) 13, eggs were trans-illuminated with a LED lamp (Brinsea) to ensure live embryos, characterised by spontaneous movements. Trans-illumination was also used to select an injection site, aiming to avoid blood vessels during the injection. On E13 and E14, a 20 mg/kg dose of morphine, methadone or an equal volume of 0.9% saline was injected into the chorioallantoic membrane with a 30-gauge needle. Naltrexone treatment has been shown to precipitate withdrawal symptoms after opioid exposure with similar concentrations to those used in this study (Newby-Schmidt and Norton, 1981). Earlier work in our lab has shown that injection of 10 mg/kg methadone gave a maximum peak of 9.8 ± 1.7 μM in the brain after two hours, and that morphadone could still be detected in the brain 48 h after injection (Hadera et al., 2017). Morphine was chosen as a full MOR agonist for egg injection, due to its water solubility (National Center for Biotechnology Information, 2019; Guo et al., 2007). On E17, the embryos were anesthetised by hypothermia by submerging the eggs in crushed ice for 7 min, hatched, and immediately decapitated. The skull was opened along the cranial sutures and the cranium was removed to expose the brain. The cerebellum was then isolated with a spatula and the meninges were removed with forceps. For western blot analysis, the cerebella were snap-frozen in liquid nitrogen and stored at -80 °C until further processing. The results of the present study represent average effects on male and female cerebella.
2.3. Western blot analysis

Cerebella were harvested for western blotting as described by Austdal et al. (Austdal et al., 2016). The membranes were probed with primary antibodies against GluN2B (1:1000), MOR (1:250) and β-actin (1:10000) followed by a HRP-conjugated secondary antibody (1:10000). For the rat (n = 6) and chicken samples (n = 12), values were normalised against control (n = 6), and for the chicken cerebella

Fig. 1. GluN2B protein expression in 1–21 days old rats prenatally exposed to opioids. GluN2B protein expression is shown as relative values to the internal control protein β-actin expression. Samples in both A and B originated from PND1, PND7, PND14 and PND21 old pups. Variation is shown as SD, n = 6 and * p < 0.05 was calculated with Kruskal-Wallis test, and Dunn’s test for multiple comparisons. Below graphs: Representative examples of western blots of GluN2B and β-actin. A: Time line of GluN2B protein expression in cerebella from 1 to 21 days old rats prenatally exposed to sterile water. The values are normalised to the expression level at PND1. The mean of 1–3 technical replicates were used as one individual value (n, and n = 6 per time point). B: GluN2B expression in cerebella from pups prenatally exposed to sterile water (control), methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day). The values are normalised to control. 1: PND1, 2: PND7, 3: PND14, 4: PND21.
time series, values were normalised to the level at E18 (n ≥ 3 per time point).

### 2.4. Cerebellar granule neurone culture

The chicken cerebella were harvested as described above, and the tissue was cut into pieces by a scalpel. The tissue was trypsinised before the cells were seeded on 35 mm dishes or 96-well plates pre-treated with poly-L lysine (Wilkin et al., 1976; Jacobs et al., 2006) at a density of 1.7 × 10^6 cells/ml (Cohen et al., 1979). The cultures were grown in BME supplemented with heat-inactivated chicken serum (7.5%), KCl (22 mM), L-glutamine (2 mM), insulin (100 nM) and PenStrep (1%) (Mathisen et al., 2013). Chicken cerebellar culture has been shown to consist of 80% neurons (Jacobs et al., 2006).

### 2.5. Calcium influx measurements

The procedure has been described previously (Fjellidal et al., 2019). Briefly, the relative intracellular Ca^{2+} level was measured using the ratiometric fluorescent Ca^{2+} indicator Fura-2. Fluorescence was measured using a CLARIOstar® plate reader (BMG Labtech, Germany). The effect of magnesium ions (Mg^{2+}, 50 μM) and MK-801 (10 μM) on the basal intracellular Ca^{2+} level was tested against treatment with buffer lacking Mg^{2+}. Inhibition curves for Ro 04–5595, methadone or morphine in twofold dilution ranging from 20 nM to 1 μM (for MK-801: 2 nM to 1 μM) were established (n = 4 per concentration), and each inhibition curve experiment was repeated at least three times. Verapamil and nifedipine were used at a concentration of 10 μM, and naltrrexone was used at 30 μM (n = 3, with replicates > 7 per treatment). Maximum inhibition by MK-801 (10 μM) and CGP 39551 (100 μM) were also tested (n = 3, with replicates > 16 per treatment). The baseline values in the presence of Mg^{2+} (50 μM) were subtracted. When comparing maximum NMDA/glycine evoked Ca^{2+} influx in cerebellar cultures pre-treated with morphine, methadone or saline in ovo, the experiment where the control and saline groups gave significantly different results was excluded from further analysis (n = 1).

### 2.6. Receptor binding

Cerebella from opioid exposed chicken were homogenised in ice-cold 0.32 M sucrose. Binding studies were performed as previously described (Kvello et al., 2019). In short, samples were pre-incubated in Tris–HCl buffer (pH 7.4, for 10 min in 25°C) before the addition of 4 nM [3H]-ifenprodil (60 minutes, 25°C). The reaction was terminated by washing the filters (Whatman™, GF/B grade; VWR, PA, US) 4 times with ice-cold Tris–HCl. Next, 4 mL of UltimaGold (#6013329, PerkinElmer, MA, US) was added to each filter. The radioactivity was counted in a liquid scintillation analyser (Trip-Carb 2810TR; PerkinElmer, MA, US). Unspecific binding was determined by adding 50 μM ifenprodil to parallel samples. The protein amount was measured as described previously (Lowry et al., 1951). Specific receptor binding was calculated as total binding minus nonspecific binding and converted to femtomoles [3H]-ifenprodil bound per milligram tissue protein.

### 2.7. Analysis and statistics

GraphPad Prism 8 (© 2018 GraphPad Software, San Diego, CA, USA) was utilised to generate inhibition curves and IC_{50}-values. Outlier values were tested for by the built-in feature in GraphPad (Robust regression and Outlier removal, Q = 1%) and normality was checked with the D’Agostino-Pearson omnibus normality test. Statistically significant differences were evaluated by Kruskal-Wallis’ test, with Dunn’s multiple comparison test included as post hoc-test when appropriate.

### 3. Results

#### 3.1. Prenatal exposure to buprenorphine reduces GluN2B protein expression in developing rat cerebellum

First, we established a timeline of GluN2B expression in cerebella from pups (PND1–21) prenatally exposed to sterile water (vehicle). The expression of GluN2B increased from PND 1 to PND 7, before it declined significantly at PND21 (Fig. 1A). In buprenorphine exposed pups, the GluN2B expression level was significantly reduced at PND14 compared to control. There was no significant difference between methadone and buprenorphine treated pups at PND14. Moreover, no significant alteration of GluN2B level was observed on PND1, PND7 or PND21 (Fig. 1B1–4).

#### 3.2. Methadone blocks NMDA-mediated calcium influx in chickengranule neurone cultures

We first examined the expression of the GluN2B subunit in the developing chicken embryo cerebellum from E12 to E20 in unexposed chickens, and found an increase in the expression towards E18, before it declined until E20 (Fig. 2A). We also confirmed the presence of MOR receptors in the cerebellum at E17 (Fig. 2B). To study NMDAR function, chicken granule neurone cultures were prepared on E17. We found that Mg^{2+} significantly lowered the intracellular Ca^{2+} level in non-stimulated cell cultures compared to the control group. There was no difference in intracellular Ca^{2+} level between cultures treated with the non-competitive, open channel NMDA receptor blocker MK-801 or Mg^{2+}. Stimulation with NMDA and glycine led to a significant increase in the GluN2B expression level at E18 (n ≥ 3) comparing all samples against each other using Kruskal-Wallis test, and Dunn’s test for multiple comparisons. Below graph: Representative examples of western blots of GluN2B and β-actin expression. B: Example of western blots of MOR protein expression and β-actin expression at E17 in chicken embryo cerebellum.

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Fig. 2. Chicken embryo cerebellar GluN2B expression at E12-E20 and MOR expression at E17. A: Timeline of GluN2B protein expression in homogenised chicken embryo cerebella, from E12 to E20. GluN2B protein expression is given relative to the internal control protein β-actin. The values are normalised to the expression level at E18 (n ≥ 3). * p < 0.05 comparing all samples against each other using Kruskal-Wallis test, and Dunn’s test for multiple comparisons. Below graph: Representative examples of western blots of GluN2B and β-actin expression. B: Example of western blots of MOR protein expression and β-actin expression at E17 in chicken embryo cerebellum.
in intracellular Ca\(^{2+}\) concentration (Fig. 3A).

The maximum inhibitive effect of MK-801 (10 μM) was compared to the effect of the competitive antagonist CGP 39551 (100 μM) and it was found that MK-801 blocked significantly more of the NMDA/glycine induced Ca\(^{2+}\) influx than CGP 39551 (Fig. 3B). The L-type calcium channel blocker verapamil (10 μM) decreased intracellular Ca\(^{2+}\) level significantly by 7% compared to NMDA/glycine stimulated samples (Fig. 3C).

MK-801 and the selective GluN2B non-competitive antagonist Ro 04–5595 dose-dependently inhibited NMDA-induced Ca\(^{2+}\) influx with IC\(_{50}\) values of 51 ± 28 nM and 257 ± 226 nM, respectively (Fig. 4A–B). The effect of acute opioid exposure on the NMDA/glycine evoked Ca\(^{2+}\) influx was then investigated. Methadone dose-dependently inhibited the influx, while morphine showed no effect (Fig. 4C–D). Morphine did not affect the inhibition curve of NMDA/glycine-induced Ca\(^{2+}\) influx by MK-801 or Ro 04–5595, while the inhibition of the NMDA/glycine-induced activation of Ca\(^{2+}\) influx by both antagonists was considerably augmented by the presence of methadone (Fig. S1).

3.3. In ovo opioid exposure increases chicken cerebellar NMDAR function with no effect on GluN2B expression

Cerebellar cultures were prepared at E17 from chicken embryos exposed to methadone or morphine at E13 and E14. Maximum NMDA/glycine evoked Ca\(^{2+}\) influx was measured at day \(1\) in vitro (DIV 1) and both methadone- and morphine-treated groups had significantly higher Ca\(^{2+}\) influx than the saline and control groups (Fig. 5A). The level of GluN2B subunit expression in homogenised cerebella from chicken embryos exposed to methadone or morphine at E13 and E14 and sacrificed at E17, was determined by western blotting and binding studies. Western blotting experiments showed no statistically significant change in GluN2B expression (Fig. 5B). Binding studies, using the GluN2B specific compound [\(^{3}H\)]-ifenprodil did not show any significant differences in GluN2B levels of opioid-exposed cerebella compared to the control group (Fig. 5C), supporting the western blot experiment result.

4. Discussion

In this study, we tested if continuous prenatal exposure to opioids during foetal brain development affects the expression of cerebellar GluN2B in rat pups the three first weeks after birth. We also investigated if short-term exposure of chicken embryos in ovo changes the cerebellar GluN2B expression levels or NMDAR function in cerebellar cultures.

In rodents, the cerebellum displays a characteristic pattern in NMDAR mRNA subunit expression and the GluN2B subunit is primarily expressed prenatally. During the first two weeks after birth it is gradually replaced by GluN2A and GluN2C, which continue to dominate tissue neurons (Akazawa et al., 1994). The transient GluN2B expression, confirmed in the present work, coincides temporally and functionally with the migration of cerebellar granule neurons (Hager et al., 1995). Earlier studies have shown that opioid exposure led to downregulation of GluN2A and GluN2B subunits in rat periaqueductal grey matter (PAG) tissue neurons (Posa et al., 2015). Chronic ethanol exposure of cerebellar granule neurons in vitro delayed the developmental switch from GluN2B to GluN2A (Snell et al., 2001), demonstrating that the NMDA receptor composition can be affected by different compounds.

The opioid receptor expression in rat cerebellum is temporarily upregulated during early postnatal maturation (Barg and Simantov,
1989), coinciding with the migration phase. Endogenous opioids have shown an inhibitive effect on proliferation and differentiation in the cerebellum (Hauser et al., 2000) and administration of external opioids has been noted to augment this effect (reviewed in (Hauser et al., 2003)). NMDARs and MORs have been shown to physically interact in different areas of the brain (Rodríguez-Muñoz et al., 2012), supporting the theory that they act in concert to regulate cerebellar growth and maturation.

Buprenorphine and methadone are used in OMT of heroin addiction (Bart, 2012) to maintain a stable opioid blood concentration and prevent abstinence symptoms. The rat embryos in this study were continuously exposed to OMT drugs, starting prior to conception and lasting until birth. Consequently, the cerebellum was not directly exposed to opioids during its highly proliferative and migratory phase (PND 0–14) (reviewed in (Hatten a and Heintz, 1995)). We observed a significant decrease in GluN2B expression in buprenorphine-treated rat pups at PND14 compared to control pups. Surprisingly, there was no significant difference between methadone or buprenorphine treated pups, indicating that the lowering of GluN2B expression was unrelated to NMDAR inhibition. Hence, our findings suggest that the effect on the GluN2B expression was opioid receptor-mediated.

The expression of GluN2B in the chicken cerebellum displayed two peaks, one at E15 (not significant) and one at E18. Interestingly, this expression pattern is closely correlated with chicken cerebellar Pax6 expression (Austdal et al., 2016), an essential regulator of granule neurone differentiation and migration (Yamasaki et al., 2001). The expression of GluN2B also matches the cerebellar growth rate observed from E13-E18 (Austdal et al., 2016). These features closely resemble rat cerebellar development (Zhong et al., 1995), but occurs in ovo in the chicken as opposed to postnatally in the rat.

Granule neurones were grown in vitro to investigate if in ovo exposure of opioids triggered long-lasting changes in NMDA receptor function, measurable with the Ca²⁺ influx assay. NMDARs are mechanosensitive (Paoletti and Ascher, 1994) and can be activated by the change of media or plate handling between measurements, which probably led to the increased Ca²⁺ level observed in the non-stimulated control cells. Hence, Mg²⁺ was added to the wash-buffer to generate the baseline values. Similar inhibition of Ca²⁺ influx was observed for Mg²⁺ and the open channel blocker MK-801, indicating that the use of Mg²⁺ to establish a stable Ca²⁺ baseline was appropriate. The effect of MK-801 on NMDA/glycine evoked Ca²⁺ influx resembled observations in other species (Nonaka et al., 1998) and the GluN2B-specific inhibitor Ro 04–5595 gave similar IC₅₀-values as we have previously observed in chicken forebrain culture (Fjellidal et al., 2019). Similarly, acute

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**Fig. 4. Inhibition curves of NMDAR antagonists and opioids.** IC₅₀ values were determined by Ca²⁺ influx measurements in E17 chicken cerebellum cultures at DIV 1. Ro 04–5595, methadone and morphine were tested with twofold dilution series from 20 nM to 10 μM, while MK-801 was tested with 2.0 nM to 1 μM. Ca²⁺ influx was induced with NMDA (200 μM) and glycine (100 μM) and the intracellular Ca²⁺ level was measured by the fluorescent ratiometric Fura-2 assay, as described in Material and Methods. Inhibition curves are normalised, and A–D are representative examples. A: NMDAR channel blocker MK-801. IC₅₀-value: 50 ± 27 nM (n = 12). B: GluN2B specific antagonist Ro 04–5595. IC₅₀-value: 257 ± 226 nM (n = 8). C: Methadone. IC₅₀-value: 5445 ± 3430 nM (n = 3). D: Morphine. IC₅₀-value: Not applicable (n = 3).
exposure to morphine or methadone produced expected outcome, showing that methadone inhibited NMDA-induced Ca\(^{2+}\) influx, while morphine did not (Laurel Gorman et al., 1997). The IC\(_{50}\)-value of methadone in chicken cerebellum culture was comparable to values found in rat brain (Matsui and Williams, 2010).

The maximum inhibition by the channel blocker MK-801 gave negative values in the inhibition curve experiments, suggesting that it inhibited more than NMDA/glycine induced Ca\(^{2+}\) influx. This effect may stem from MK-801 inhibition of nicotinic receptors (Ramoa et al., 1990), as nicotinic receptors that contain alpha 7–9 subunits are Ca\(^{2+}\) permeable (Fucile, 2004). The nicotinic receptors can be activated by extracellular Ca\(^{2+}\) (Vernino et al., 1992), and are found in human and rat cerebellum in the period corresponding to the time point our experiments in the chicken were performed (Hellström-Lindahl et al., 1998). Nicotinic receptor expression has also been reported in chicken cerebellum (Daubas et al., 1990). The fact that MK-801 inhibits significantly more of the NMDA/glycine induced Ca\(^{2+}\) influx than CGP 39551, supports this theory. The L-type voltage gated calcium channel blocker verapamil decreased NMDA/glycine induced Ca\(^{2+}\) influx slightly, but significantly, compared to control. This effect has been reported earlier (Qiu et al., 1998; Melena and Osborne, 2001) and indicates that L-type channels contribute to a small portion of NMDA induced Ca\(^{2+}\) influx, possibly caused by NMDA-mediated depolarisation.

The embryos were exposed to methadone or morphine before the first peak in GluN2B expression, as we expected the brain to be sensitive at that time point. In addition, a single or repeated dose of morphine between E12-14 in the chicken embryo has been shown to induce dependence, proved by precipitation of withdrawal symptoms by injection of naloxone (Bronson and Sparber, 1989). Yet, in contrast to the observed reduction in GluN2B expression in rat pups at PND14 after prenatal buprenorphine exposure, the chicken embryo cerebella exposed to methadone or morphine, at E13 and E14 showed no significant effect on GluN2B expression in western blotting or binding studies. However, a significantly elevated NMDA/glycine induced Ca\(^{2+}\) influx was observed in vitro at DIV1. This effect also appears to be opioid receptor-mediated, not NMDAR related, as both methadone- and morphine-treatment gave similar results. An earlier study suggested that long-lasting NMDAR potentiation was mediated by G-protein- activation of protein kinase C, initiated by MOR agonist (DAGO) binding.

Fig. 5. NMDAR function and GluN2B protein expression in opioid exposed chicken. Eggs were injected with 20 mg/kg of morphine or methadone or an equal volume of saline at E13 and E14, before sacrifice at E17. For further details, see Materials and Method. Variation is shown as SD. A: NMDA/glycine evoked Ca\(^{2+}\) influx was investigated in E17 chicken cerebella cell culture at DIV 1. Ca\(^{2+}\) influx was induced with NMDA (200 μM) and glycine (100 μM) and the intracellular Ca\(^{2+}\) level was measured by the fluorescent ratiometric Fura-2 assay as described in Material and Methods. **** p < 0.0001, using Kruskal-Wallis and Dunn’s multiple comparison test (n = 3). B: GluN2B protein expression in cerebella from prenatally opioid-exposed chicken embryos, sacrificed at E17. The values were normalised to the average of saline controls, and statistical significance was calculated using the Kruskal-Wallis test and Dunn’s multiple comparisons test (n = 10). C: [\(^{3}H\)]-ifenprodil binding (femtomoles/milligram protein) in chicken cerebellar cultures exposed to morphine or methadone in ovo. Statistical differences were tested with Kruskal-Wallis’ test and Dunn’s multiple comparisons test (n = 6).
Activated PKC reduced the physiological Mg\(^{2+}\) block of NMDA receptors (Chen and Huang Marine, 1991). MOR agonist (DAMGO) has also been shown to augment Ca\(^{2+}\) influx in both NMDARs and L-type Ca\(^{2+}\) channels in cultured hippocampal neurons (Przewlocki et al., 1999). Reciprocally, activation of NMDA receptors attenuates opioid receptor/G-protein coupling, possibly by PKC activation caused by elevated intracellular Ca\(^{2+}\) level. This may cause a vicious circle where PKC mediates both the activation of NMDARs and leads to opioid receptor/G-protein uncoupling, ultimately driving the development of opioid tolerance (Bailey et al., 2006), and references therein). In addition, the expression of PKC itself can also be affected by prenatal opioid exposure (Wang et al. (2017)).

The decrease in GluN2B expression observed in rat offspring, two weeks after the opioid exposure was terminated could indicate that withdrawal has an impact on GluN2B expression level. Previous studies have shown significant downregulation of all subunits three days after morphine injection in the nucleus accumbens, while their expression was upregulated compared to control after three weeks (Jacobs et al., 2005). If the potentiating effect of embryonal opioid exposure on intracellular Ca\(^{2+}\) concentration is similar and persistent in rat and chicken, chronically elevated Ca\(^{2+}\) influx during gestation might be associated with the observed downregulation of GluN2B in the rat cerebellum. Elevated intracellular calcium levels have been shown to increase GluN2B turnover (Araújo et al., 2005). Interestingly, Bergmann glial (BG) cells also express NMDA receptors (López et al., 1997; Müller et al., 1993). The function of glial NMDARs is debated, but they are proposed to modulate plasticity in neural synapses (Verkratovsky and Kirchhoff, 2007). BG cells guide neuronal migration in the cerebellum (Xu et al., 2013), and if their NMDAR composition and function is disrupted by opioids, they may contribute to affect cerebellar development.

In conclusion, we report that opioid exposure during development leads to long-term changes in NMDAR expression in postnatal rat and affects NMDA receptor function in chicken embryo cerebellum, suggesting NMDARs as an important neurodevelopmental target of opioid effects. These effects appear to be mediated by opioid receptors, rather than being caused by NMDAR antagonism.

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Declarations of interest: none.

Appendix A. Supplementary data

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References


