Neurodevelopmental toxicity of escitalopram and venlafaxine in PC12 and chicken cerebellum granule neurons, and kinetic studies of escitalopram in chicken embryo

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Acknowledgments

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Abstract

Depression during pregnancy can be a danger for both mother and fetus. Therefore, treatment is often considered necessary even though pregnant women are often excluded from clinical trials, and the knowledge of the potential risk of the drug for the fetus is often lacking. Therefore, an increased understanding of the effects of escitalopram and venlafaxine, the first treatment choice for pregnant women, on neurodevelopment is desired.

In vitro viability studies were performed in PC12 cells and primary cultures of chicken cerebellum granule neurons with MTT assay after exposure to escitalopram and venlafaxine. In addition, western blot analysis, real-time PCR, and live cell imaging with IncuCyte were done for chicken granule neurons. High-content imaging for chicken cerebellum granule neurons stained for synaptogenesis was also done. The chicken embryo can be used for preclinical neurotoxicological studies. However, there is no available data on the distribution of escitalopram and its effect in the chicken fetus. Therefore, a kinetic analysis of the drug was performed.

The studies in this thesis showed a decreased viability in both PC12 cells and chicken cerebellum granule neurons 72 hours after exposure to 100 µM escitalopram. However, no changes were found in the viability of chicken cerebellum granule neurons after exposure to venlafaxine.
A significant reduction in neurite length was found in chicken cerebellum granule neurons for both escitalopram (100 µM) and venlafaxine (200 µM) 68 hours after exposure.
The kinetic study of escitalopram injected onto CAM found that chickens can metabolize escitalopram into demethylescitalopram and that escitalopram reaches the brain in concentrations clinically relevant to human use.

In conclusion, valuable information about the potential neurodevelopmental impact of antidepressants may be found in developing neuron cultures. Chicken embryo shows promise as an alternative in vivo model to testing pharmacokinetics and pharmacodynamic effects in the embryo.

Abbreviations

BDNF	Brain-derived neurotrophic factor
CGNs	Cerebellum granule neurons
CNS	Central nervous system
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
MAP2	Microtubule-associated protein-2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NET	Noradrenaline transporter
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PBS +	Phosphate buffered saline added magnesium and calcium
PSD95	Post-synaptic density protein 95
SERT	Serotonin transporter
SNRI	Serotonin and noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SYP	Synaptophysin

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1. Introduction

1.1 Depression and treatment during pregnancy

All drugs used during pregnancy can potentially affect the fetus, but pregnant women are often excluded from clinical trials because of concerns for the safety of the fetus, ethics, and difficulty in designing appropriate trials (1). Because of this lack of research, one is generally conservative in treating pregnant women with drugs. However, for some diseases, the necessity to treat the mother outweighs the potential risk for the fetus. Depression can be one of these instances. With a prevalence of up to 10-12 % of all pregnant women (2, 3), depression is a major disability and cause of impairment for many. In the Nordic countries, the selective serotonin reuptake inhibitor (SSRI) escitalopram is the most used antidepressant during pregnancy, while venlafaxine is the most used serotonin and noradrenaline reuptake inhibitor (SNRI) (4, 5). Many studies have evaluated the safety of antidepressants during pregnancy, and escitalopram and venlafaxine are generally considered safe to use during pregnancy (6). Still, the knowledge of how the drugs may affect developing neurons is more limited. Neurons act differently during development and in their mature state (7). Therefore, the effect of antidepressants might be different in the developing fetus than in an adult. Investigating the effects of antidepressants on the developing nervous system is because of this crucial for understanding the potential risks of antidepressant drug treatment during pregnancy. This knowledge may guide future recommendations for the choice and dosage of antidepressants during pregnancy.

1.2 Escitalopram, venlafaxine, and mechanisms of depression

1.2.1 Escitalopram

Escitalopram is a selective serotonin reuptake inhibitor (SSRI) (8). It is the S-enantiomer of citalopram and exerts its effect on the postsynaptic reuptake of serotonin at the terminals and cell bodies of serotonergic neurons through the serotonin transporter (SERT), as shown in Figure 1.1 (9). This increases the serotonin levels in serotonergic synapses in the brain, enhancing the serotonergic neurotransmission (9). Escitalopram is metabolized by CYP2C19, CYP2D6, and CYP3A4 to the primary metabolite S-demethylcitalopram (10). The metabolite has a weak affinity to SERT and does not contribute appreciably to the therapeutic activity of escitalopram (10).

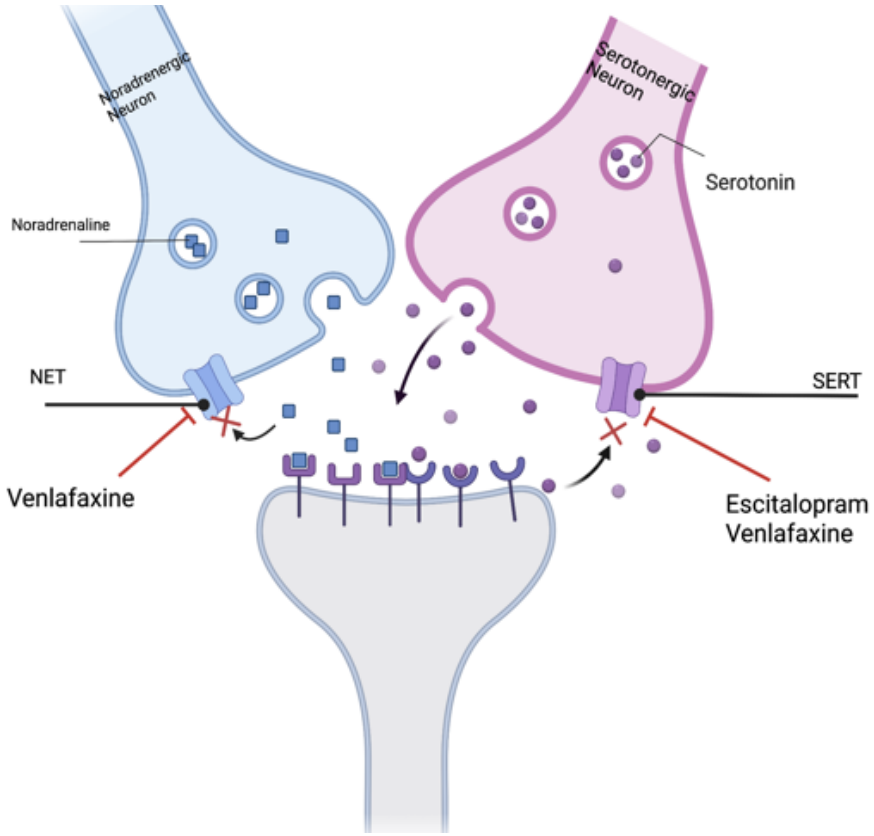

Figure 1.1. SSRI and SNRI mechanism of action. *NET = noradrenaline transporter, SERT = serotonin transporter. Venlafaxine inhibits reuptake of serotonin and noradrenalin through SERT and NET. Escitalopram inhibits reuptake of serotonin from the synaptic cleft through SERT. Created with BioRender.com*

1.2.2 Venlafaxine

Venlafaxine is a serotonin and noradrenaline reuptake inhibitor with a lower potency for serotonin reuptake than SSRIs and noradrenaline reuptake than tricyclic antidepressants, respectively (11). It exerts its effect on the SERT and noradrenaline transporter (NET) (figure 1.1) (11).

1.2.3 Mechanism of depression

Although the pathophysiology behind depression is not fully understood, the increase in synaptic serotonin forms the basis of the monoamine hypothesis of depression treatment, long thought to be the cause of the antidepressant effect of the drugs (9, 12, 13). This is, however, disputed in the literature (12, 14). A newer hypothesis suggests the pathology might also be related to proteins regulating neuroplasticity and neurogenesis regulated by neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (12, 13). Concentrations of BDNF in patients with depression are found to be reduced but normalized again with the treatment of

antidepressants, thus supporting this hypothesis (15). BDNF mainly interacts with the TrkB receptor, as shown in Figure 1.2, but its pro-form (pro-BDNF) has a high affinity for the P75 receptor (16). While activation of the Trk receptors by mature neurotrophins will promote cell survival and neurite outgrowth, activating the P75 receptor by the proneurotrophins will mediate apoptosis (17). A better understanding of the mechanisms behind changes in protein expression and neurite outgrowth is necessary. Different inhibitors can give a clue as to what causes any changes and through what pathway the drugs affect the cells. Inhibitors of TrkA, TrkB, and P75 are commercially available and can be used to research these pathways. The Trk receptor proteins are essential in both the signaling and development of neurons. Therefore, inhibiting these receptors might provide information on the mechanism of action for any changes in the development of the neurons or cell cultures. TrkA is the first receptor discovered and interacts with NGF. TrkB has a high affinity for brain-derived neurotrophic factor (BDNF)(18). BDNF is broadly expressed in the adult and developing brain, and its effect through stimulating the TrkB signaling pathway is critical for neuronal survival, morphogenesis, and plasticity (19). BDNF affects neurons positively or negatively through various intracellular signaling pathways triggered by activating TrkB or P75 (19).

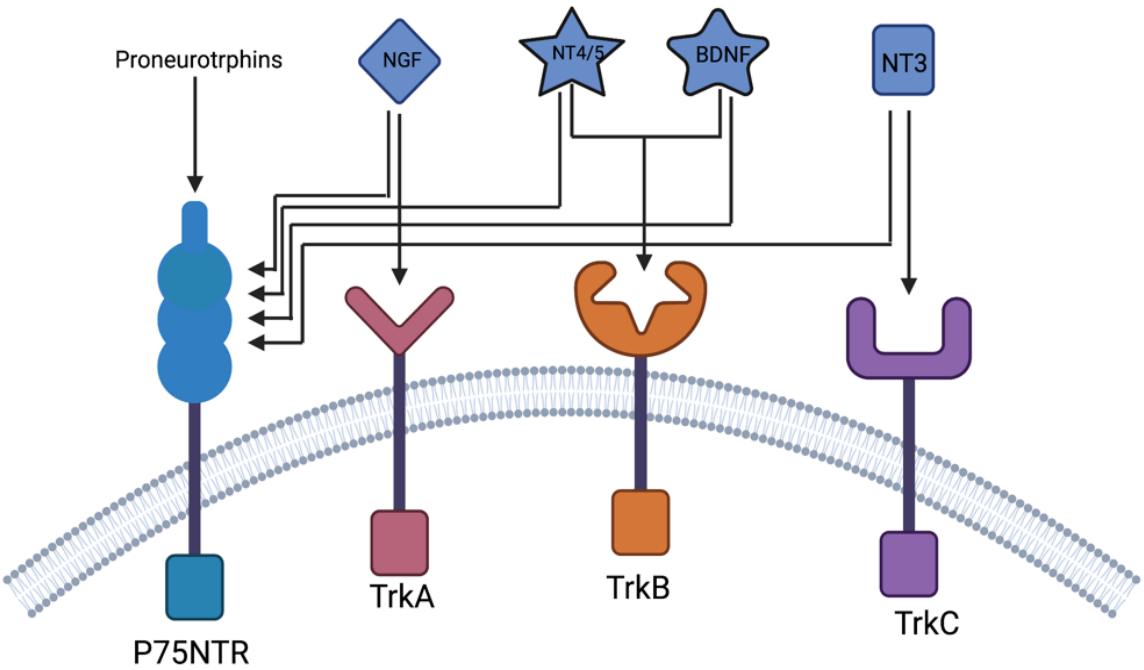

Figure 1.2. Neurotrophin receptors. *The neurotrophins NGF, NT3, NT4, NT5 and BDNF bind to their respective Trk-receptor. The P75NTR receptor can bind all neurotrophins with low affinity and proneurotrophins with high affinity. Created with Biorender.com with inspiration from "Basic Neurochemistry – Principles of Molecular, Cellular, and Medical Neurobiology" (2012).*

1.3 Development of the nervous system

Neurodevelopment takes a long time in humans, spanning from the third gestation week all the way to adolescence, and includes many complex cellular processes such as proliferation, migration, apoptosis, and differentiation, as shown in figure 1.3 (20). The main stages of brain development are neurulation, cellular proliferation and differentiation, neuronal migration, axonal guidance and pathfinding, organization, and myelination (21). In embryonic development, neural progenitor cells can differentiate into all the cells that comprise the brain and CNS except immune cells that migrate in at a later stage. Formation of the neural tube begins around embryonic day 19 (21). The neural progenitor cells at the most rostral part of the neural tube will give rise to the brain, while cells in a more caudal position will become hindbrain and spinal column (21). At embryonic day 42, the neurogenesis starts, and the neural stem cells will differentiate into neuroblasts and glioblasts. As they develop, neuroblasts migrate to different brain areas, differentiating into neurons and building up neural networks (20). By the end of the embryonic stage (week 8), most of the rudimentary structures of the brain and the CNS are established (20). A network of neurites will create synapses used to transfer signals (22). This synaptogenesis can be investigated in cell cultures by staining synaptophysin (SYP) and postsynaptic density protein (PSD-95) (23, 24). DAPI can stain cell nuclei, and MAP2 can stain microtubules (25, 26).

Both proliferation and apoptosis of neuronal cells are important for the development of the brain and CNS (20). During early development, apoptosis is active and necessary, while mature neurons have long time survival capabilities and no longer proliferate (7). Apoptosis of as many as 50 % of all neurons is necessary to establish the complex networks of the developing brain (20). During this development, external factors can also affect the fetus and cause disruptions in normal physical or cognitive behavior later in life (27). Critical time points are sensitive time windows during which the system is most subject to change, and the plasticity of specific physiological or behavioral phenotypes is heightened relative to other developmental stages (28). Figure 1.3 shows some of these critical time points, such as proliferation, migration, and apoptosis.

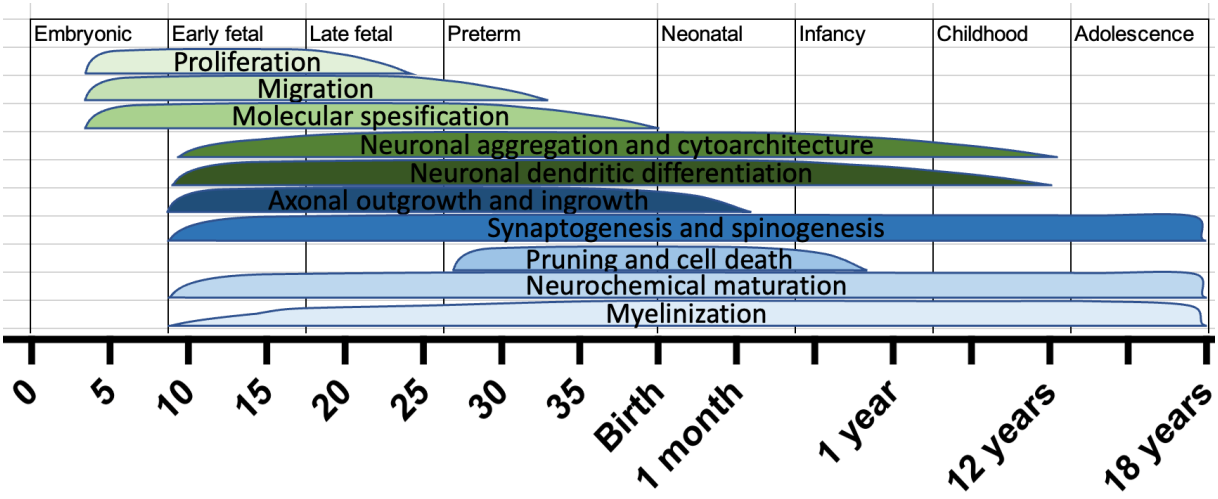

Figure 1.3. Developmental periods in human cortical histogenesis. *The figure is created with inspiration from (29).*

1.4 Serotonin and noradrenalin in the developing CNS

An intact serotonergic system is critical for normal CNS function, and during embryogenesis, serotonin is important as a growth factor (30). Furthermore, serotonin receptor activity forms an integral part of the cascade leading to changes in brain structure, and the serotonergic system interacts with BDNF and other chemical messengers, including dopaminergic transmitter systems (30). Disruptions of these processes could contribute to CNS disorders that have been associated with impaired development (30). There are several different serotonin receptors in the CNS, with the 5-HT_{1A} receptor (5-HTR1A) being the most extensively distributed (31).

Noradrenaline is also essential for normal CNS development as it regulates Cajal-Retzius cells, which are proposed to be instrumental in neuronal migration and laminar formation (32). Therefore, as SSRIs and SNRIs directly affect these systems by altering the serotonergic activities, they could affect the normal development of the CNS (30).

1.5 Cerebellum

The cerebellum is widely conserved between species and can be used as a model to reflect general CNS development (33). It was long believed only to contribute to the planning and execution of movements but is now found also to be involved in cognition, mood, and behavior (34, 35). In patients with mood disorders, an altered expression of TrkB signaling in the cerebellum is found, possibly causing neuroplastic rearrangement leading to a progressive functional and structural modification (35). Evidence of the importance of the cerebellum in

fine-tuning both emotional and cognitive functions in addition to motor function strengthens the relevance of this part of the brain in studies of depression and antidepressants. The numerous cerebellar granule neurons in the cerebellum constitute the most homogenous neuron population in the brain, and they can be grown in vitro (33). As mentioned above, apoptosis, migration, proliferation, and differentiation are essential aspects of CNS development. As cerebellar granule neurons undergo all these processes, the cells can be used to model these mechanisms (33). An important marker for migration and differentiation is Pax6 (36).

1.6 Model systems

In researching the potential effects of drugs on the brain, humans are excluded from experiments as the brain must be harvested for analysis. Therefore, alternative models like animal models or in vitro models are necessary. With all cell models, one must consider factors such as differences in gene expression, signaling pathways, and drug metabolism when mimicking in vivo actions. Since the goal is to attain knowledge about use in humans, the models chosen must be relevant to the drugs researched and the information gathered with them transferable to humans.

1.6.1 PC12-cells

The PC12 cell line has proven useful for neurobiological and neurochemical studies (37). Exposure to nerve growth factor (NGF) causes the cells to resemble noradrenergic neurons with reversible neurite growth (37). This makes it a relevant and valuable model in studies of the effects of escitalopram and venlafaxine on neuronal development. It's a clonal cell line from a pheochromocytoma of the rat adrenal medulla (38). The ability to start differentiation with NGF enables the use of the cell line to observe and compare differences in responses in differentiated and undifferentiated cells after exposure to drugs (38). This ability also makes it a useful cell line to study processes associated with neuronal differentiation.

1.6.2 Chicken and chicken cerebellum granule neurons

Rat CGNs are often used for neurotoxicology studies but are expensive to maintain because of the husbandry and facilities needed, and the cells need to be grown in depolarized culture conditions that don't mimic physiological conditions (39). Because chicken cerebellum granule neurons (cCGNs) can be grown in conditions closer to physiological conditions and

only need limited facilities and husbandry, the model is a cheaper and simpler option. The chicken cultures respond to glutamate excitotoxicity similar to rat cultures and can be an alternative model to rats (39). Chickens also enable testing of the effects of drugs in the major organ systems such as the cardiovascular, respiratory, and central nervous system (33). This is a requirement in drug development and is commonly done in mammals like e.g., pigs, monkeys, or zebrafish (33). Chicken embryos are physiologically closer to humans than zebrafish, another alternative species widely used, with a convenient size and a short incubation time of 21 days. With the use of this model, the three R´s (replace, reduce, and refine) in animal research experiments are improved compared to more classical animal studies in mammals because the chick is never hatched and therefore doesn't live under suboptimal conditions for the species.

Furthermore, no animal has to be injected or put under major stressing factors except euthanasia. One drawback of the model is that, in general, the drug tested should be administered in the same way as intended for therapeutic use in humans. As this is orally for escitalopram and venlafaxine, it is impossible to achieve this before after hatching. In addition, some physiological differences between chickens and mammals cannot be mimicked in ovo, for example, the ability of the mother to metabolize drugs before they reach the fetus or the placental barrier in mammals (33). The fetus, however, is exposed to the drugs through the bloodstream. This can be replicated in chickens. The chorioallantoic membrane (CAM) is the highly vascularized outermost extra-embryonic membrane, shown in figure 1.4 (40). By injecting drugs onto CAM, absorption of the drug through the bloodstream is enabled, and the

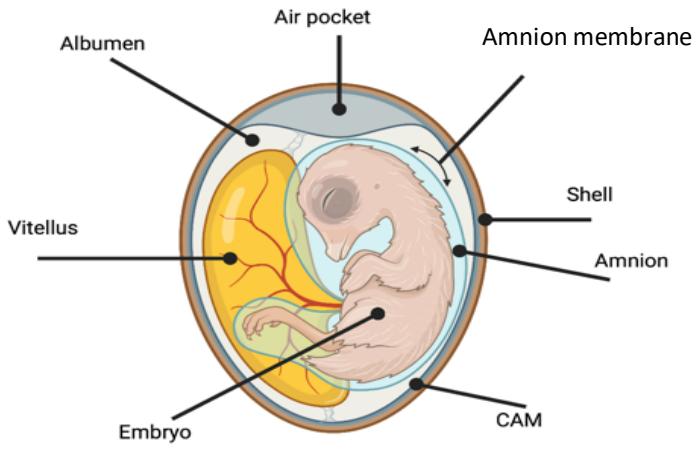

Figure 1.4. Diagram of a chicken egg at embryonic day 14. *Created with Biorender.com*

chicken fetus is exposed with minimally invasive methods. Thus kinetics, metabolism, distribution, and effects of drugs on the developing fetus can be studied.

1.7 Aim of the study

The need for medical treatment of depression during pregnancy often outweighs the potential risk of the drug for the fetus, as the disease itself can be damaging. There is a substantial knowledge gap on the potential effect of these drugs on the neurodevelopmental processes. Research on this will give practitioners and patients a broader foundation for their risk analysis when considering medical treatment. This thesis aims to investigate the effect of escitalopram and venlafaxine on developing neurons using PC12 cells and the chicken cerebellum as models. Furthermore, the developing chicken fetus is used to investigate the kinetics and metabolism of escitalopram.

Secondary objectives have been:

1. Investigate changes in viability of developing neuronal cells with exposure to escitalopram and venlafaxine.
2. Investigate changes in neurite length after exposure to escitalopram and venlafaxine.
3. Run a pilot experiment of gene expression.
4. Investigate if escitalopram injected onto CAM reaches the brain in concentrations relevant to humans and if it is metabolized to demetylescitalopram.

2. Materials and methods

2.1 Materials and equipment

For the full materials and equipment list, see Appendix A1 and A2.

2.2 Cell cultivation and in vitro exposure

2.2.1 PC12 cells

The PC12 cell line was used for viability assays. The PC12 cells were split twice weekly (Monday and Thursday) and incubated at 37 °C and 5 % CO_2. When seeding the cells, a density of $7x10^4$ cells/ml was used. One day after plating, differentiation was induced by reducing the serum concentration (10 % medium and 90 % serum-free medium) and adding NGF (50 µg/ml). When changing the medium, the cells were exposed to escitalopram (100, 10, and 1 µM), and venlafaxine (200, 20, and 2 µM). The cells were then incubated under the same conditions for 72 hours. For the full protocol, see appendix B.1-B.2.

2.2.2 Chicken cerebellum granule neurons

Fertilized eggs of the breed Ross 308 delivered by Nortura Samvirkekylling were incubated in an OvaEasy 380 Advance EXII incubator at 37 °C and a humidity of 45 %. The incubator provides automatic turning of the eggs and lowers and raises the temperature to simulate natural brooding. At day 17 of incubation, the chicken cerebellum granule neurons were harvested and plated on desired plates precoated with poly-L-lysine, depending on the later use. For full protocol, see Appendix B3-B5. The cells were exposed to escitalopram (100, 10 and 1 µM) or venlafaxine (200, 20 and 2 µM) one day after plating. Serotonin (5 µM) and the inhibitors of P75 (100 nM), TrkA (5 µM, GW) and TrkB (Ana12, 10 µM) were also used. All procedures were done in accordance with Norwegian regulations on the use of animals in experiments (FOTS-id: 28992).

2.3 MTT-assays

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a quick and easy way to measure the viability of cells after exposure to drugs and can be combined with many exposures at the same time (41). Live and proliferating cells with an active metabolism will reduce the MTT dye to purple-colored formazan crystals, which can be dissolved in DMSO (42). Through spectroscopic measurements, increases or decreases in cell

survival or proliferation can be indirectly measured. There will be less MTT converted to formazan crystals in wells where the cells are exposed to substances that negatively impact their proliferation or cause increased apoptosis. In this thesis, MTT analysis was done on PC12 cells and chicken cerebellum granule neurons. The cells were seeded on blank 96-well plates and incubated for 24 hours at 37 °C and 5 % CO_2, and exposed as described in 2.2. After 72 hours, the medium was aspirated, and MTT was added before further incubation under the same conditions for 3 hours. The medium was removed again, and DMSO was added to dissolve the crystals before scanning the plate in a ClarioStar plate reader at 570 nm. See the full protocol in Appendix B6.

2.4 Live Cell Imaging with IncuCyte

In order to find changes in neurite length during exposure to venlafaxine and escitalopram, the IncuCyte live cell imaging system was used. Chicken cerebellum cells were harvested on day 17 of incubation and seeded on 96-well plates. Because of evaporation effects on the outermost wells on the plate, only 60 wells in the middle of each plate were used for cells. The rest of the wells on the edges was added PBS. The plates were then incubated for 24 hours before exposure to escitalopram, venlafaxine, and inhibitors of P75, TrkA, and TrkB. As soon as possible after exposure, the plates were put in the IncuCyte, and four pictures were taken of each well with 10x magnification every four hours for up to 72 hours. The IncuCyte incubator had 5 % CO_2 and a temperature of 37 °C. The IncyCyte Zoom live-cell analysis software (Essen BioScience) was then used to analyze the changes in neurite length. The settings for analysis were trained on representative pictures from all technical replicates and set as follows: segmentation adjustment 0,5; hole fill 0; Adjust Size 0; Min cell with 7 µm. Neurite parameters: Filtering: best; neurite sensitivity: 0,65; neurite width: 1 µm.

2.5 Western blotting

In short Western blotting is a semi-quantitative method to measure the amount of proteins in a sample (43). The negative charge of SDS-treated proteins allows us to use electrophoreses to force the proteins through a gel. Larger proteins will take longer to travel this gel than smaller proteins, and thus they can be separated.

2.5.1 Harvesting of cells for western blot analysis

The cell lysate needs to be collected. This was done in a radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 5 mM EDTA (pH 8), 50 mM Tris (pH 8), 1 % Tritin-X100, 0,5 % sodium deoxycholate, 0,1 % sodium dodecyl sulfate, and dH_2O. The protease inhibitors leupeptin, pepstatin A, PMSF, and the phosphate inhibitor Na_3VO_4 were added to the RIPA buffer right before use. After 72 hours of exposure, chicken granule neurons were harvested and frozen for later protein analysis. All the medium was removed from the plates, and the cells were washed twice with ice-cold PBS. 200 µl of RIPA buffer with leupeptin, pepsatin A, PMSF, and Na_3VO_4. A cell scrape was used to harvest all the cells in the well, and the suspension was transferred to Eppendorf tubes before being sonicated three times for 2 seconds with a minute on ice in between each time and then kept at - 20 °C until further analysis. All the work was done on ice to prevent the degradation of proteins. For the full protocol, see Appendix B.7.

2.5.2 Protein measurement

It is necessary to know the protein content in the samples to calculate how much to load into the gel wells to ensure that samples are compared on an equal basis (43). The amount of protein was 25 µg per well. Measurements were done using the Pierce™ BCA Protein Assay Kit. Peptide bonds between amino acids reduce Cu^{2+} to Cu^{1+} (44). Cu^{1+} can then form chelation bonds with two BCA molecules, forming a complex with a strong purple color and a peak absorbance at 562 nm (44). A standard curve is made with increasing concentrations of bovine serum albumin (BSA), and after absorbance measurement, the amount of protein in the samples can be approximated. See full protocol in appendix B.8.

2.5.3 SDS polyacrylamide gel electrophoresis

The lysate is added to a laemmli buffer, often called the loading buffer. This buffer contains SDS, glycerol, 2-mercaptoethanol, bromophenol blue, and Tris-HCl. The glycerol makes the solution heavier to ensure that it sinks into the well on the gel, while the bromophenol blue colors the solution so that it is possible to see how far the separation has progressed (43). The SDS will stretch out the proteins, and heating will denature them, giving them a negative charge (43). The samples were added 4x Laemmli buffer and 2-mercaptoethanol (30 + 6 µl per 100 µl sample) before 5 minutes of boiling at 95 °C and then loaded into the well of the gel. For the full protocol, see appendix B.9.

2.5.4 Western blotting

The nitrocellulose membrane and filters were wetted with a 1x trans-blot buffer added EtOH. A sandwich was then made by putting a stack of filters on the bottom, the nitrocellulose membrane, the gel, and a stack of filters on the top. The cassette was closed, and the transfer was made for 30 minutes and 26 v. When the blotting was complete, the nitrocellulose membrane was removed, submerged into a Ponceau solution to stain the proteins, and then rinsed in distilled water. The membrane was then cut to analyze proteins of different sizes simultaneously.

2.5.5 Blocking

Blocking was done to prevent unspecific binding of antibodies, reduce background noise, and prevent false positives. The Licor Intercept® blocking buffer was used. The membrane is put in this solution and placed on a roller for one hour.

2.5.6 Antibodies

The primary antibodies are added after blocking. The membrane is put into a falcon tube containing blocking buffer, TBS with tween (TBS-T), and the primary antibodies. The concentration in these experiments is listed in table 2.1.

Table 2.1 Antibodies used in Western blot

Antigens	Species	Concentration
BDNF 14 kDA	Rabbit	1:1000
GluN2B 178 kDa	Rabbit	1:1000
Pax6 46-47 kDa	Rabbit	1:5000
β-Actin 42 kDa	Mouse	1:1000
Secondary antibodies:		
Goat, anti-Rabbit 800 and 680	Rabbit	1:10000
Goat, anti-Mouse 680	Mouse	1:10000

The tubes are then incubated on a roller mixer overnight at 4 °C. The next day the blocking solution is discarded, and the tube is washed with 5 ml of 1x TBS-T 3 times for 10 minutes. After this, the secondary antibodies are added and incubated for one hour. The membrane was then washed three times with TBS-T for 10 minutes. After this, the membrane is ready for detection.

2.5.7 Detection

The fluorescent secondary antibodies will emit light that can be detected by the scanner, and the bands can be further analyzed with EmpiraStudio. The detection of the bands was done with the Odessey-CLx Imaging system.

2.6 Real-time qPCR

Quantitative polymerase chain reaction (qPCR) is a widely used method to quantify differences in gene expression levels (RNA) between samples (45). This is useful to support findings in western blot analyses or other phenotypic observations (45). When done in real-time, the amount of PCR product produced in each cycle can be quantified, giving a quantified measurement of the PCR products accumulated over the course of the reaction (46). SYBR Green is an intercalating dye that fluoresces when bound to double-stranded DNA (46). Primer-mediated replication of the target sequence during PCR enables multiple molecules of SYBR Green to bind to the product and emit a strong fluorescent signal that is easily detected (46).

2.6.1. Harvesting of cells for qPCR

The RNeasy® Plus Mini kit was used for the qPCR procedures. Ice-cold PBS was used to wash the cells once before 350 µl of the RLT lysis buffer was added. Then a cell scraper was used to scrape the well briefly before the lysate was transferred to an Eppendorf tube and stored at -80 °C until further analysis. For full protocol, see appendix B.10.

2.6.2 RNA isolation

The RNeasy® Plus Mini kit was used to isolate the RNA from the samples. For the full protocol, see appendix B.11. After isolation 3 µl were aliquoted for RNA measurement, and the rest of the samples were stored at -80 °C.

2.6.3 RNA quantification

The concentration of RNA in the samples was measured with the nanodrop system. The spectrophotometer was calibrated with RNA-free water, and one µl of sample was placed on the spectrophotometer to measure the content.

2.6.4 RNA conversion

To convert the RNA to cDNA, calculations were made based on the quantification to ensure that 100 ng of converted RNA would be made from every sample. Up to 9 µl of the samples were diluted with RNA-ase-free water to 9 µl, and 10 µl of 2XRT buffer and 1 µl of 20X enzyme mix were added. The samples were then incubated at 37 °C for 60 minutes and heated to 95 °C for 5 minutes to stop the reaction. RNase-free water (180 µl) was added, and the samples were kept at -20 °C until further analysis.

2.6.5 cDNA measurement

The cDNA was measured with primers for different genes of interest. GADPH was used as the housekeeping gene. 3 µl of cDNA solution was added to a white 96-well plate with 1 µl of reverse and forward primer for the gene of interest (table 2.3) and 5 µl of SYBR Green for a total volume of 10 µl in each well. A negative control without cDNA was also added to ensure there was nothing in the water that would bind primer. Detection was done at the BIORAD CFX96 Touch Real-Time PCR detection system with the protocol described in table 2.2.

Table 2.2. Protocol used for cDNA measurement.

1.	95 °C for 30 minutes
2.	95 °C for 10 seconds
3.	60 °C for 30 seconds
	+ Plate read
4.	72 °C for 10 seconds
5.	Go to step 2, repeated 45 times
6.	Melt curve 65 to 90 °C, increment 0.5 °C for 5 seconds + plate read

Table 2.3. Primers used for PCR.

Primer	Species	Sequence	Producer
GADPH Forward	Gallus Gallus	GAT GGG TGT CAA CCA TGA GAA A	Thermo Fisher
GADPH Reverse	Gallus Gallus	TGG TGC ACG ATG CAT TGC	Thermo Fisher
BDNF Forward	Gallus Gallus	GAA AAG TCT GCA CAT GAG GGC	Thermo Fisher
BDNF Reverse	Gallus Gallus	GTG TGG CAT TGC TG AAG GG	Thermo Fisher
Pax6 Forward	Gallus Gallus	CCA CAT CCC CAT CAG CAG TA	Thermo Fisher
Pax6 Reverse	Gallus Gallus	TGA AAG AGG AAA CGG GGG TG	Thermo Fisher
HTR1A5 Forward	Gallus Gallus	GTT GCC GTT CTT CAT CGT GG	Thermo Fisher
HTR1A5 Reverse	Gallus Gallus	GAG TAG CCC AGC CAG TTG AT	Thermo Fisher

2.7 High-content imaging of chicken granule neurons for synaptogenesis

The cells were harvested on day 17 in the same way as previously described and seeded out on blank Corning Biocoat 96-well plates (PLL precoated). For a full overview, see section 2.2. The cells were exposed to 10 and 1 µM escitalopram or 20 and 2 µM venlafaxine the day after seeding them. After 72 hours of incubation in 5 % CO_2 and 37 °C, the medium was removed. The cells were washed with PBS twice before adding 4 % formaldehyde in PBS and incubating the plate at room temperature for ten minutes. After removing the formaldehyde, the wells were added 200 µl PBS, and the plates were stored in the fridge until the staining of the cells and adding antibodies (table 2.4). The PBS was removed from the cells manually and carefully so as not to dislodge them. Since some of the antigens are intracellular, the cells must be permeabilized. This was done with 0,1 % Triton X100 in 1x PBS added magnesium

and calcium (PBS+) for 10 minutes at room temperature. This allows for the antibodies to enter the cell and bind to intercellular proteins. Blocking buffer with 3,5 % BSA in PBS+ was added for 30 minutes at room temperature. This is to prevent unspecific and unwanted binding of the antibodies and false positives. DAPI (1:1000) and primary antibodies for MAP2, PSD95, and SYP (table 2.4) were added to the cells in blocking buffer and incubated at 4 °C overnight. The cells were then washed twice with BPS+ before the secondary antibodies were added, and the plate was incubated at room temperature for one hour. For the full protocol, see Appendix B.12. The cells were then photographed with the Terumo Fisher CellInsight CX7 Laser at 10 x magnification.

Table 2.4 Antibodies for High Content Imaging

Protein	Marker Function	Dilution Factor	Supplier	Catalog number	Species	Poly/Monoclonal
MAP2	Neurite outgrowth	1:5000	Abcam	Ab5392	Chicken	Polyclonal
PSD95	Post-synaptic marker	1:300	Abcam	Ab13552	Mouse	Monoclonal
SYP	Pre-Synaptic marker	1:200	Abcam	Ab14692	Rabbit	Polyclonal

2.8 Kinetic studies of escitalopram in chickens

2.8.1 Injection and harvesting

To better understand the absorption, distribution, and metabolism of escitalopram in the chicken fetus, a kinetic study was done. First, the eggs were weighed. The eggs were then injected with 0.3 mM escitalopram in sterile physiological NaCl onto CAM (shown in figure 1.4). This was done by first illuminating the eggs to find a suitable area for injection where there were no blood vessels and avoiding the embryo and yolk. The appropriate spot was then marked, and a hole was carefully made with a needle, ensuring not to push further than 3-4 mm into the egg. The drug solution was then injected based on the weight of the eggs (1 µl per g egg). Because injecting the eggs with such an accurate dose was difficult in practice, the

weight of the eggs was rounded up or down to the closest 10 g, then injected with 50, 60, or 70 µl, depending on their weight. Then at different time points after injection, the whole brain, both lungs and egg yolk, were harvested and frozen in liquid nitrogen before the tissue was kept at – 80 °C until further processing.
A pilot experiment was done first, with harvesting at 0,5, 1, 2, 4, 6, 8, and 24 hours after injection. Later the experiment was expanded with harvesting at 3, 5, 7, 12, and 18 hours after injection in addition to the original time points. Three eggs were injected and harvested for every time point, so there were three parallels of every tissue from every time point in both experiments.

2.8.2 Homogenization of the tissue

The tissue was weighed, and MQ-water was added in a 1:1 ratio for the brain and lungs and 1:2 for egg yolk, as this tissue has a higher viscosity. The homogenization was done with the tissue on ice and with a Micro-Pellet homogenizer for about 1 minute until there were no remains of whole visible tissue or lumps. 100 µl of the homogenized tissue was then transferred to kinetics tubes, refrozen in liquid nitrogen, and stored at – 80 °C until further analysis.

2.8.3 Analysis of escitalopram and its metabolite demethylescitalopram

The analysis of escitalopram and its metabolite demethylescitalopram was performed at the Department of Forensic Sciences at Oslo university hospital by LC-MS/MS. The samples were analyzed and compared to an internal standard for escitalopram and demethylescitalopram. Six concentrations of the drug and its metabolite were prepared beforehand to create a standard curve for the concentrations. A volume of 50 µl of the internal standard was added to all the samples except for the blind samples. 75 µl of borate buffer (pH 11) was added before the samples were shaken on a whirl mixer. Then 1,2 ml of ethyl acetate and heptane (80:20) were added, and the tubes were lightly shaken again before being put on a roller mixer for 10 minutes. This allows the drug and its metabolite to leave the matrix and enter the supernatant. The tubes were then centrifuged for 10 minutes at 4500 rpm. A volume of 900 µl of the supernatant was then transferred to a Nunc plate and evaporated at 50 °C and 5 psi. The samples were then resolved in 200 µl 5 mM ammonium acetate buffer (pH5) and acetone nitrile (75:25) and shaken for 1 minute.

The quantification was done on a Waters Aqcuity UPLC machine with a 2,1 x 50 mm column (C18, 1,7 µm).

2.9 Statistics

The results are presented as mean, ± standard deviation where the data had a Gaussian distribution. If not Gaussian distributed, the data is presented as a boxplot. Statistical analysis was performed with GraphPad Prism 9. Data with Gaussian distribution were tested with One-Way ANOVA with Dunnets post hoc test. $P < 0.05$. Outliers were removed with ROUT (Q=1) or Grubbs (α=0.05). Data that did not exhibit normal distribution were analyzed with Kruskal-Wallis and Dunn's hoc test.

3. Results

3.1 Cytotoxicity of antidepressants in PC12 and chicken cerebellum granule neurons

The viability of PC12 cells was measured with MTT-assay 72 hours after exposure to 1-100 µM escitalopram. Both differentiated and undifferentiated cells were analyzed. Chicken cerebellum granule neurons were exposed to both escitalopram (1-100 µM) and venlafaxine (2-200 µM) for 72 hours. A significant reduction in viability was seen for 100 µM of escitalopram in the PC12 cells, both in differentiated and undifferentiated cells, compared with the control (figure 3.1). A reduction in viability was not shown for PC12 cells with full serum medium after exposure to escitalopram. For the lower concentrations, no difference in viability was found. Escitalopram significantly reduced viability in chicken cerebellum granule neurons, but only in combination with the inhibitors of P75 and TrkB (Ana12) (figure 3.1). Hydrogen peroxide was used as positive control and showed no viability. Exposure to venlafaxine did not change the viability of the chicken granule neurons, as shown in figure 3.1.

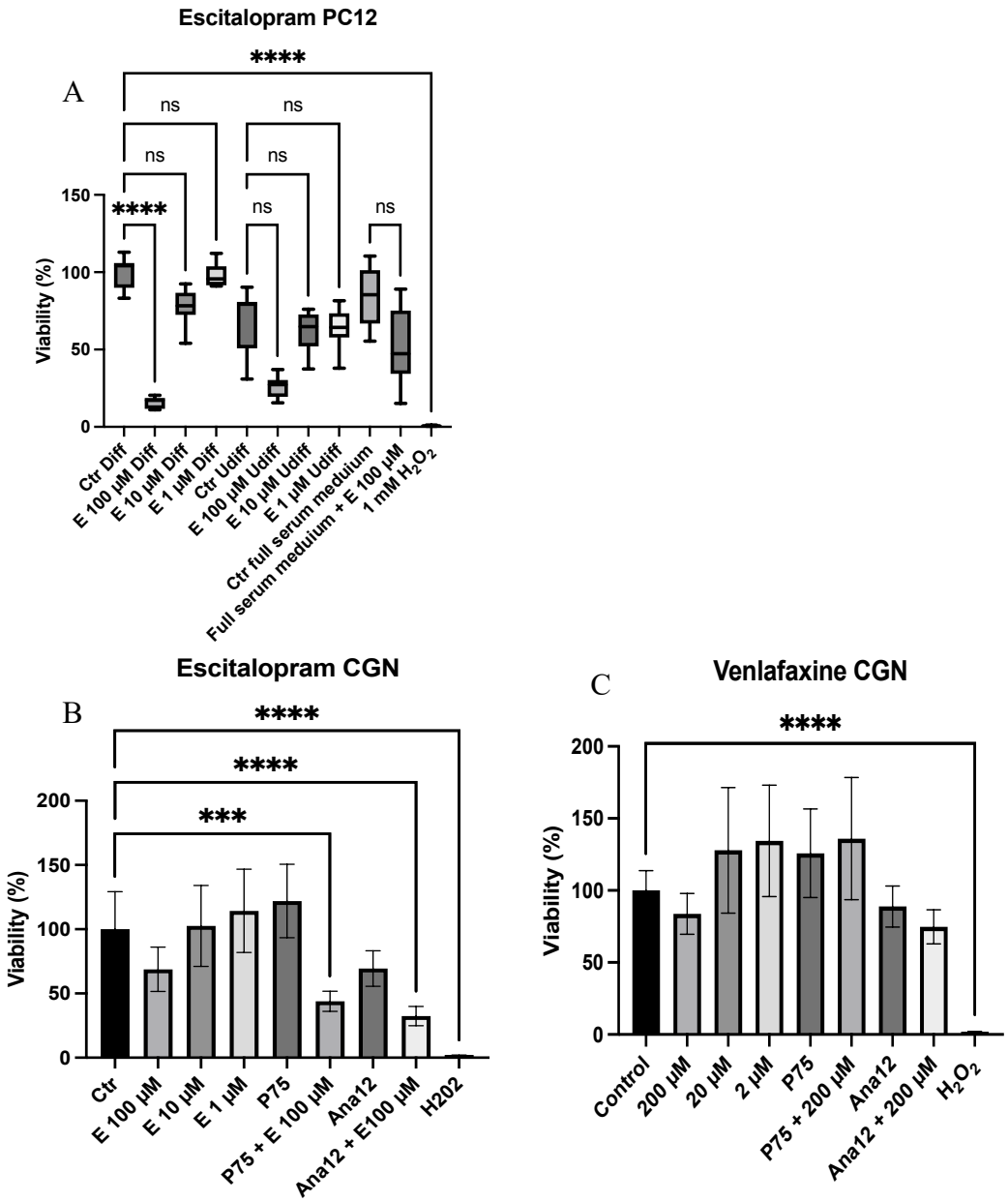

Figure 3.1. Viability of differentiated, and undifferentiated PC12 cells, and chicken cerebellum granule neurons 72 hours after exposure to escitalopram. *PC12 cells were exposed to escitalopram (1-100 µM) one day after plating. Chicken cerebellum granule neurons were exposed to escitalopram (1-100 µM) and venlafaxine (2-200 µM) one day after plating. Viability was analyzed with MTT assay. (A) The data is presented as a box plot with 25 and 75 % quartiles of three biological replicates (n=8) normalized against the control. Statistical analysis was done by Kruskal-Wallis with Dunn's hoc test (α=0.05). One outlier was removed (Grubbs, α=0,05). * Shows a significant change compared with the control (* p=0.013, **** p < 0.0001). (B and C) Data is presented as means with standard deviation of two biological replicates (n=6) normalized against the control. Statistical analysis was done by one-way ANOVA (α=0.05). * Shows significant change compared to control (*** p<0.002, **** p<0.0001) No outliers were removed (ROUT Q=1). One-way ANOVA was also used to compare the inhibitors in combination with escitalopram or venlafaxine, with E100 or V200 µM as control. No significant difference were found.*

3.2 Neurite Length with LiveCell Imaging

To identify possible neurotoxic effects of escitalopram and venlafaxine in chicken granule neurons, the neurite length was quantified using EssenBioScience IncuCyte Zoom NeuroTrack software. Tracking the development of neurite length over 68-72 hours using live cell imaging with IncuCyte revealed a trend of reduced neurite length in the chicken granule neurons after exposure to venlafaxine. Representative phase contrast images used for the analysis are shown in figure 3.2 and 3.7. For the highest concentration of venlafaxine (200 µM), there was a 22,6 ± 5.6 % reduction in neurite length 68 hours after exposure compared to the control, as shown in figure 3.3. For the lower concentrations of venlafaxine, no significant changes were observed at this time point.

Two controls were added to the plate, one on the left and one on the right. The left control was used for the drug exposures, and the right was used for the inhibitors. There was a difference between the controls, and the plate was therefore split in two for analysis.

No difference in neurite length was found 68 hours after exposure to either of the inhibitors or the combination of inhibitors and venlafaxine (figure 3.6). With the highest concentration of escitalopram (100 µM) a reduction in neurite length of 44.6 ± 15.4 % 68 hours after exposure was observed (figure 3.4). For the lower two concentrations of escitalopram, no changes were observed. The chicken granule neurons exposed to both escitalopram and inhibitors of P75, Ana12 and trkA (GW) did not significantly change the neurite length as shown in figure (3.5). There is a trend of reduced neurite length with the 100 µM of escitalopram here as well but it is not significant.

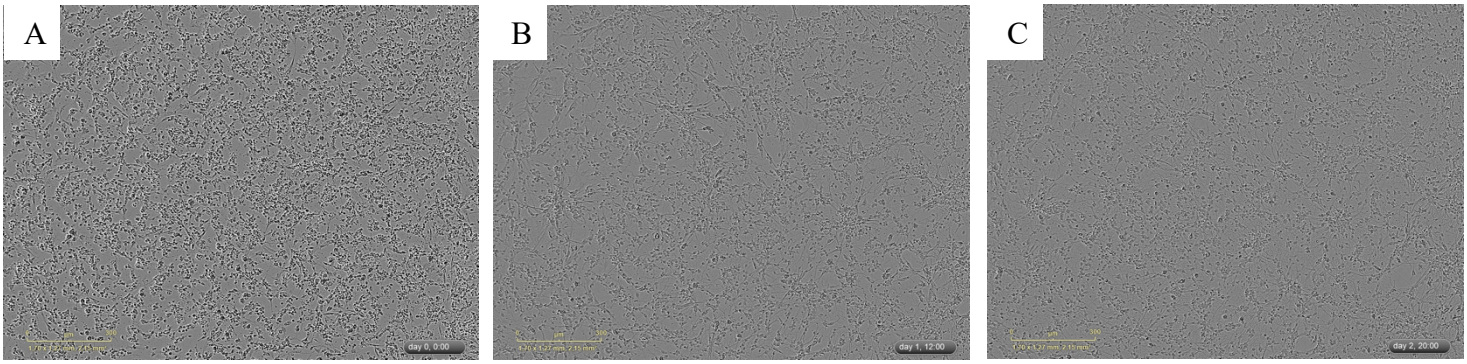

Figure 3.2. Representative phase contrast images used for analysis taken at 0, 36 and 68 hours after exposure*. The pictures show the same area of the same control well with 10x magnification. (A) 0 hours, (B) 36 hours and (C) 68 hours.*

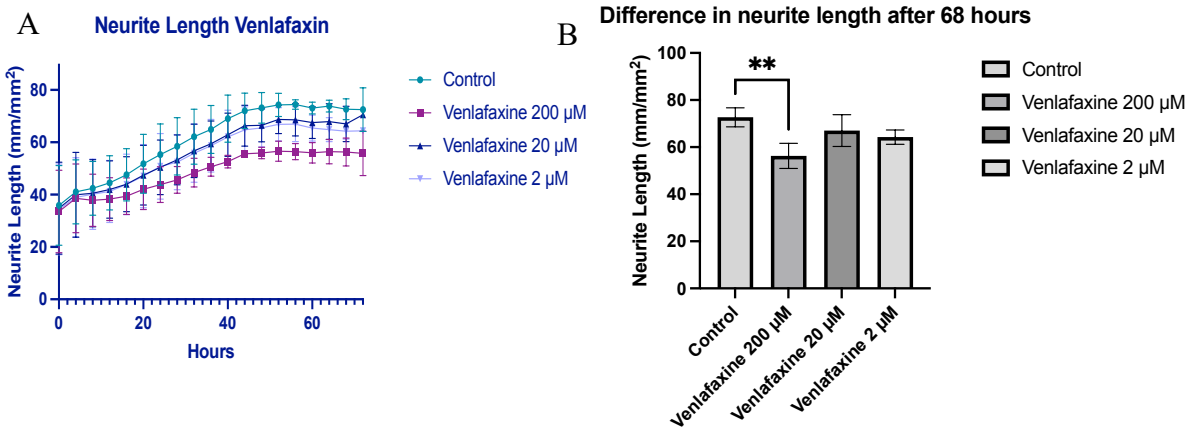

Figure 3.3. Differences in neurite length in chicken granule neurons*. The cells were exposed to 200, 20 and 2 µM of venlafaxine one day after harvesting and photographed every four hours for 68-72 hours with the IncuCyte LiveCell imaging. The resultant phase contrast images were used to quantify neurite length. (A) The data presented are the means with standard deviation of three biological replicates (n=6), except for the 72-hour values where there are two biological replicates. Analysis is based on four images per well. No outliers were removed ROUT (Q=1). (B) A reduction of 22,6 % ± 5.6 % in neurite length at 68 hours for the group exposed to 200 µM venlafaxine using one way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (α = 0.05, P=0,0098). For the other two exposures no significant changes were found at 68 hours.*

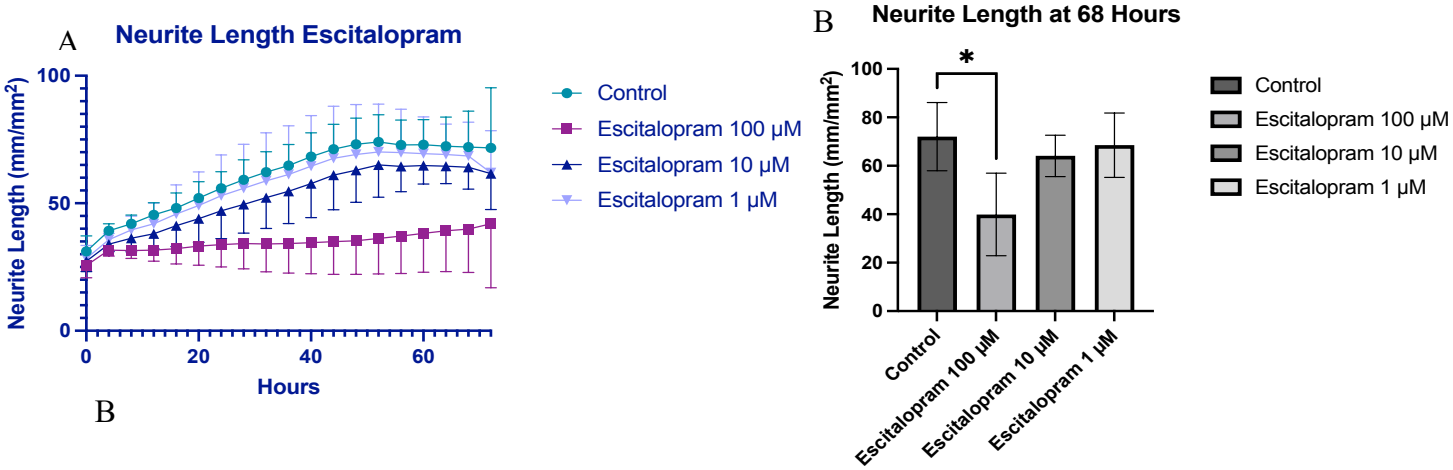

Figure 3.4. Differences in neurite length in chicken granule neurons. *The cells were exposed to 100, 10 and 1 µM of escitalopram one day after harvesting and photographed every four hours for 68-72 hours with the IncuCyte LiveCell imaging system. The resultant phase contrast images were used to quantify neurite length. (A) The data presented are the means with standard deviation of three technical replicates (n=3), except for the 72-hour values where n=2. Analysis is based on four images per well. No outliers were removed ROUT (Q=1). (B) A reduction of 44.6 ± 15.4 % in neurite length at 68 hours for the group exposed to 100 µM venlafaxine using one way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (α = 0.05, P=0,0484). For the other two exposures no significant changes were found at 68 hours.*

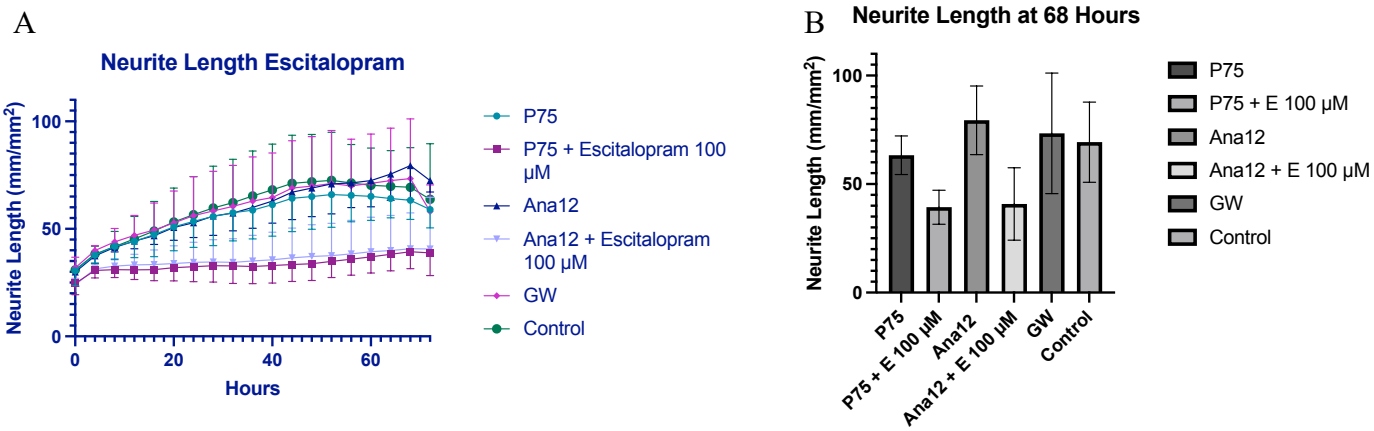

Figure 3.5. Effects on neurite length for chicken granule neurons 0-72 hours after exposure to inhibitors of P75, TrkB (Ana12) with and without escitalopram, and TrkA inhibitor (GW). *The data in (A) are presented as means with standard deviation based on three biological replicates. (B) presents the differences in neurite length at 68 hours as means of three biological replicates (n=6). No changes were observed at this timepoint with one-way ANOVA using Dunnett's multiple comparisons test, $\alpha = 0,05$.*

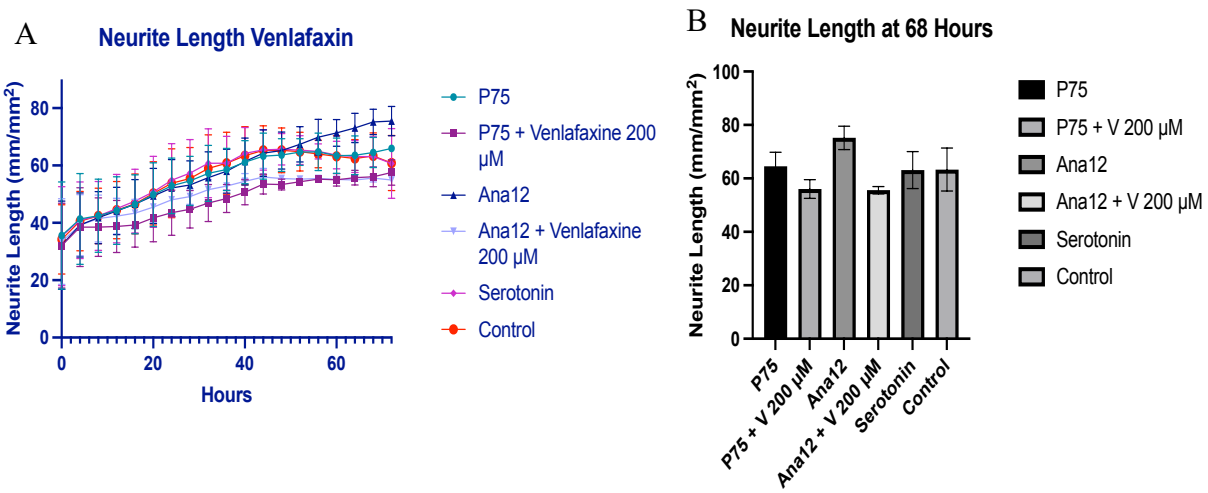

Figure 3.6. Differences in neurite length in chicken granule neurons. *The cells were exposed to 200 µM of venlafaxine, P75, Ana12 and serotonin one day after harvesting and photographed every four hours for 68-72 hours with the IncuCyte LiveCell imaging. The resultant phase contrast images were used to quantify neurite length. (A) The data presented are the means with standard deviation of three biological replicates (n=6), except for the 72-hour values where there are two biological replicates. Analysis is based on four images per well. No outliers were removed ROUT (Q=1). (B) No difference in neurite length was found at 68 hours using one way analysis of variance (ANOVA) with Dunnett's multiple comparisons test ($\alpha = 0.05$).*

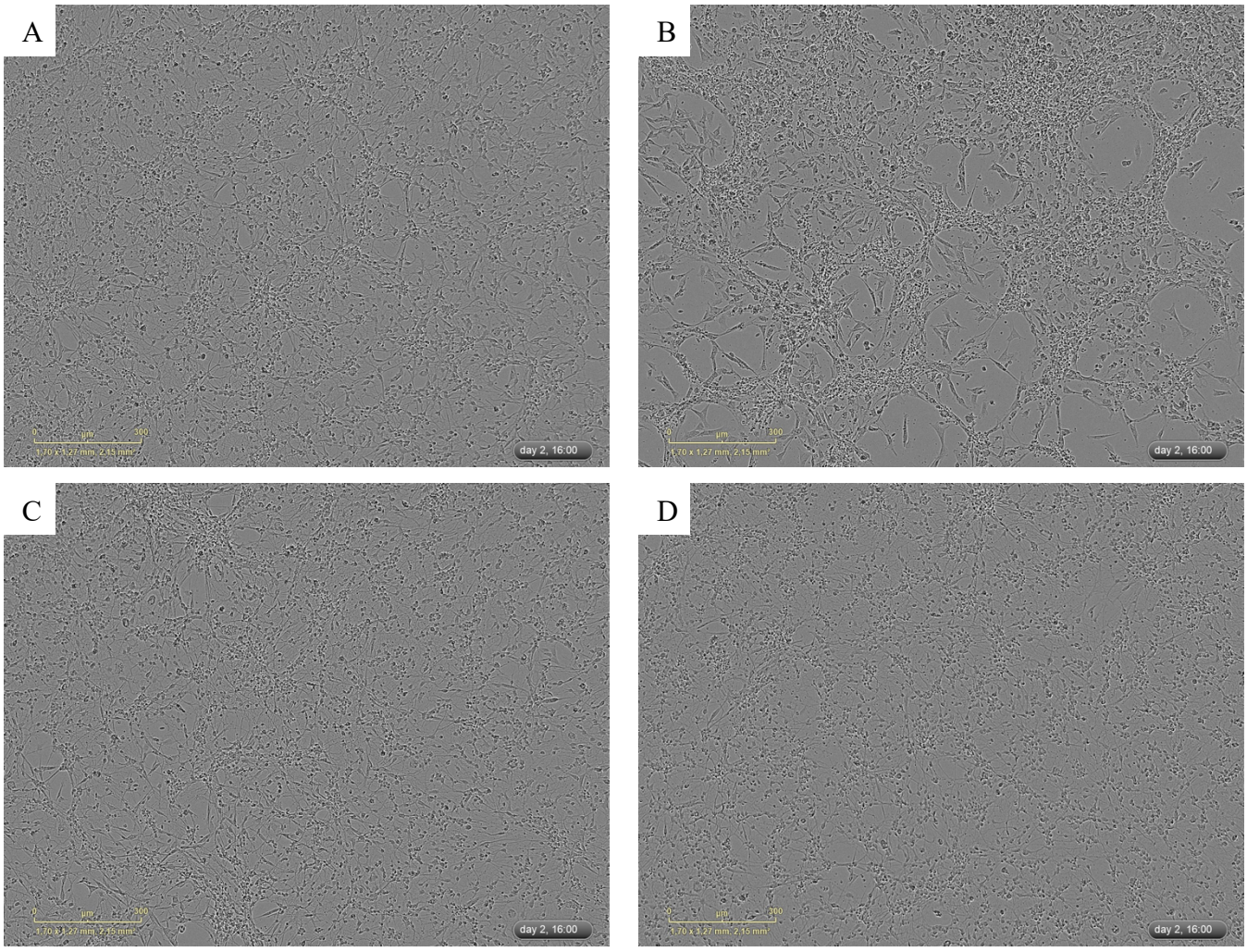

Figure 3.7. Representative phase contrast images 68 hours after exposure to escitalopram. *(A) control, (B) 100 µM escitalopram, (C) 10 µM escitalopram and (D) 1 µM escitalopram.*

3.3 Realtime qPCR and Western blot analysis

Changes in gene expression were investigated with real-time qPCR. Only one biological replicate was performed, so no statistics could be run. The results are shown in Figure 3.8. There is a trend of dose-dependent decrease in BDNF expression in cells exposed to venlafaxine and an increase in the cells exposed to 1 and 10 µM of escitalopram. The inhibitors of TrkB (Ana12) and P75 gave a reduction in BDNF expression both alone and for the drugs in combination with the inhibitors (figure 3.8).

Escitalopram gave a reduction in 5HTR1A expression, while venlafaxine 200 µM showed an increase. Inhibition of TrkB also gave an increase in 5HTR1A expression. No change was observed for P75.

For Pax6, no changes were observed except for the toxic dose of escitalopram (100 µM).

For the western blot analysis, background noise and unspecific binding of the antibodies made analysis difficult. For BDNF and HTR1A5, there were no bands on the membrane.

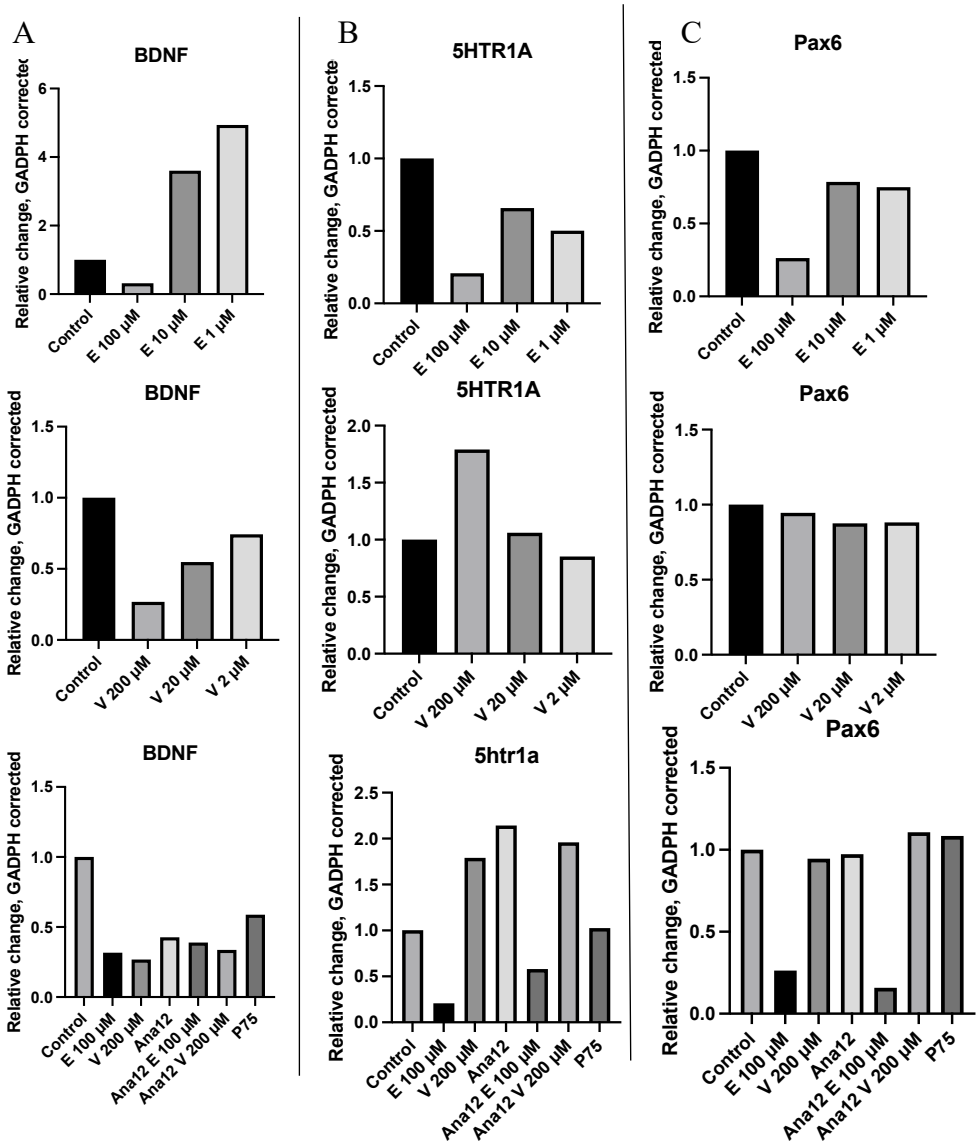

Figure 3.8. Fold change of BDNF, Pax6 and 5HTR1A in chicken cerebellum granule neurons 72 hours after exposure to escitalopram (100, 10 and 1 µM) or venlafaxine (200, 20 and 2 µM), and the inhibitor of TrkB (Ana12) or P75. *GADPH was used as housekeeping gene and the values presented are normalized to control. One biological replicate, with two technical replicates were performed. Values above one indicates increase, values below one indicates a decrease. (A) BDNF expression, (B) 5HTR1A expression, and (C) Pax6 expression.*

3.4 High Content Imaging

High Content Imaging of chicken cerebellum granule neurons was performed after exposure to escitalopram (10 and 1 µM) and venlafaxine (20 and 2 µM) for 72 hours. The cells were fixated with 4 % formaldehyde and incubated with DAPI and antibodies for MAP2, PSD95, and SYP to investigate any effects on synaptogenesis. The protocol followed was optimized for human stem cells, and part of the experiment was to test and optimize the procedure for chicken granule neurons. The resultant high-content images revealed that the fixation method worked for chicken cerebellum granule neurons with very little loss of cells. The pictures show that DAPI worked and reveal a large degree of clustering for all exposures (figure 3.9). MAP2 is primarily expressed in the cell cluster bodies and, to a lesser degree, in the neurites (figure 3.9). PSD95 and SYP were expressed but at a relatively small degree. The composite images in figure 3.9 E, show all channels at once and reveal some background and unspecific binding. The composite images reveal some overlap of SYP and PSD95 expression.

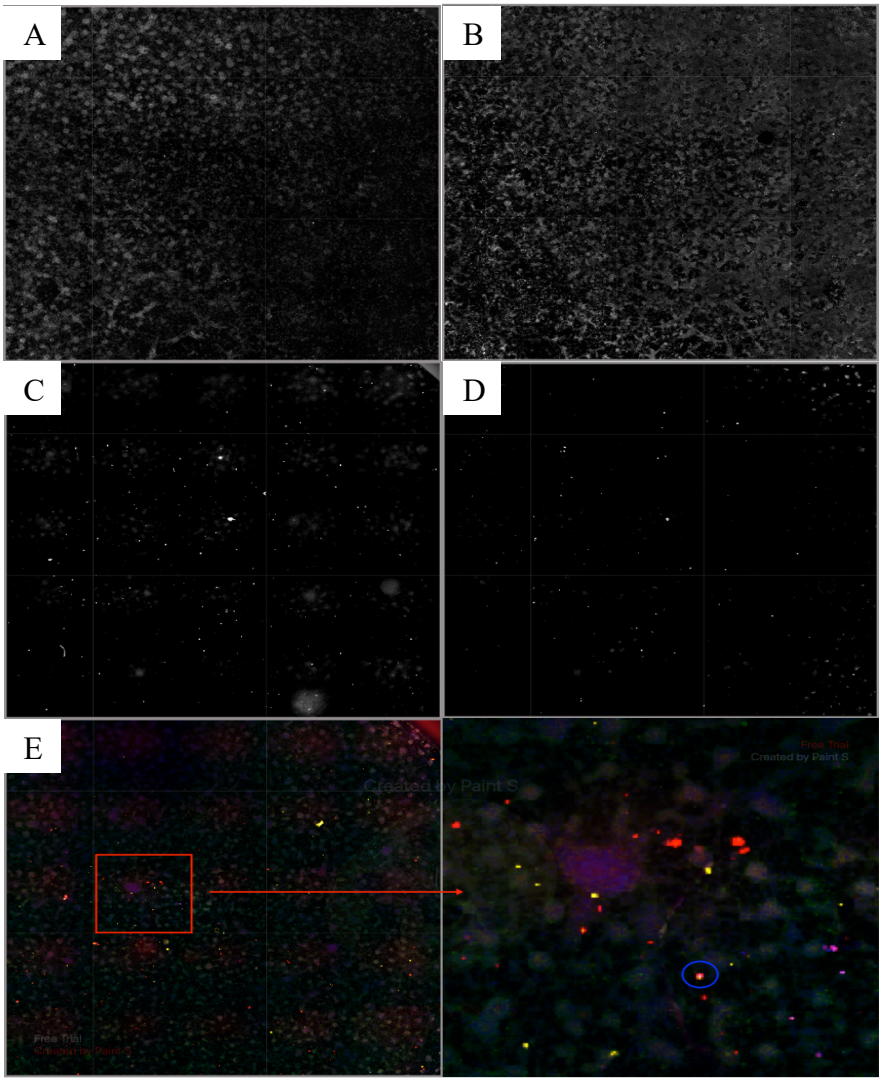

Figure 3.9. Representative high content images of chicken cerebellum granule neurons incubated with antibodies for DAPI, MAP2, SYP and PSD95. *Exposure: (A) DAPI, (B) MAP2, (C) SYP, (D) PSD95, (E) Composite of all channels. Blue ring shows SYP/PSD95 overlap.*

3.5 Kinetic studies of escitalopram and demethylescitalopram

The metabolism and kinetics of escitalopram were studied in eggs injected with 0,3 mM escitalopram (97 µg/kg) in physiological saline water on day 13 of incubation. The fetuses were then harvested at different time points. The tissue analysis in Figure (3.10) shows that the drug injected into the egg reaches the brain within 30 minutes and C_{Max} between 30 minutes and 2 hours. The highest concentration was 700,2 ± 95,0 nM in the brain and 499,7 ± 124,2 nM in the lungs after one hour. The graph of the concentration of escitalopram over time shows distinct absorption and elimination phases. With the intervals chosen for this study, the maximum concentration in the brain comes in somewhere around one hour. Assuming even distribution in the egg, the concentration in the brain was at C_{max} 0.23 % of

the injected drug concentration. It reaches above clinical human serum concentrations in the lungs, brain, and yolk. After the peak value at two hours after injection, the concentration of escitalopram in the brain rapidly decreased and reached clinically relevant concentrations after 5 hours. The distribution characteristics of escitalopram are described in table 3.1. The elimination constant (K_e) describes the fraction of the drug eliminated per hour.

Table 3.1. Drug distribution characteristics for escitalopram in the brain, lungs, and yolk*. Estimated with exponential one-phase decay model and area under the curve analysis in GraphPad Prism. No curve was found to be appropriate for the yolk data, and these values are therefore omitted.*

	Dose	Cmax, nM	Tmax, hours	$AUC_{0.5\text{-}24\ h}$, nM/h	Cmax/D	K_e, h^{-1}	Half-life Hours
Brain	0.3 mM (97 µg/kg)	700.2	1	3162	0.0023	0.3119	2.22
Lung	0.3 mM (97 µg/kg)	499.7	1	2497	0.0016	0.3569	1.94
Yolk	0.3 mM (97 µg/kg)	-	-	33668	-	-	-

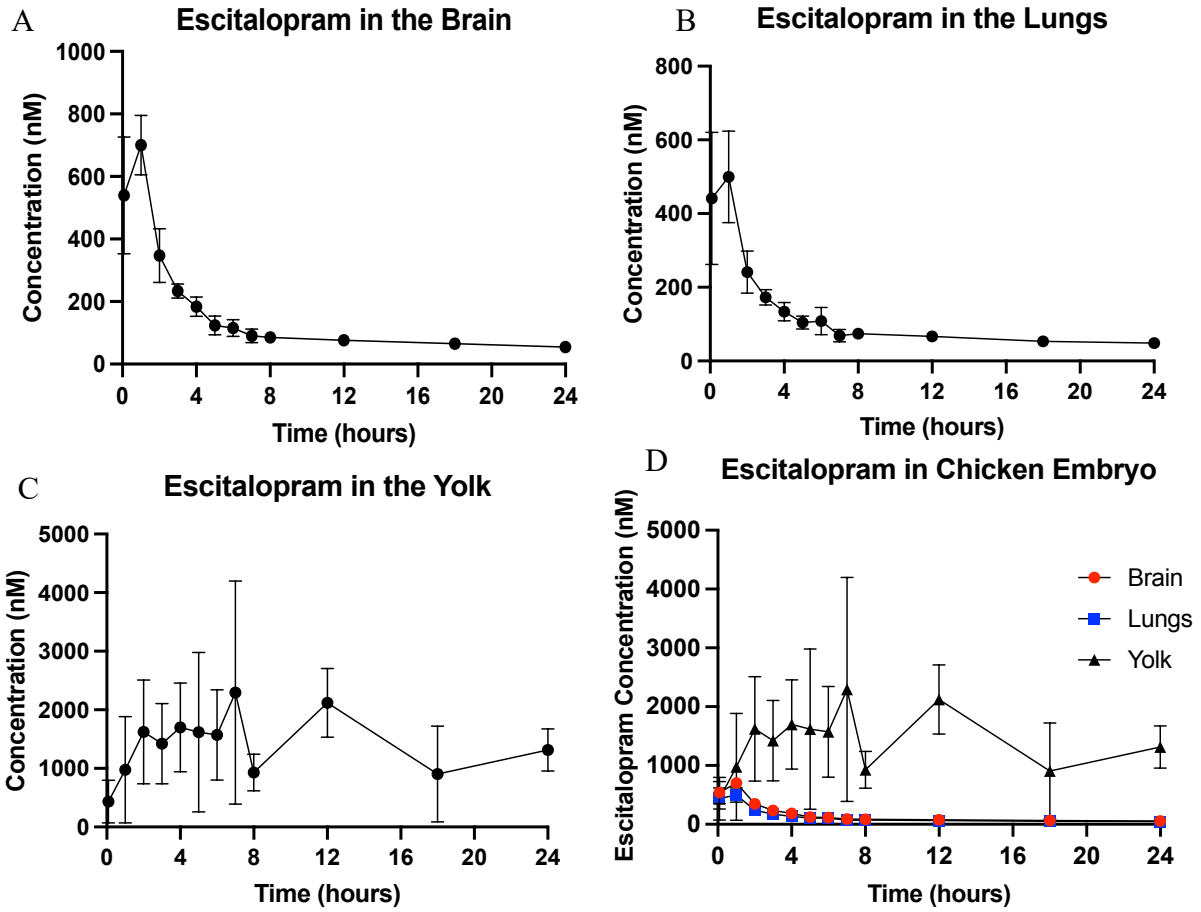

Figure 3.10. Measurements of escitalopram in chicken fetus 0.5-24 hours after injection*. Eggs were injected with 0.3 mM escitalopram (97 µg/kg) in saline water on day 13 of incubation. Brain, lungs and yolk were thereafter harvested at different timepoints, and the tissue concentration of escitalopram was analyzed using LC-MS/MS. Data is presented as means with standard deviation of 6 (0.5, 1, 2, 4, 6, 8 and 24 hours) or 3 (3, 5, 7, 12 and 18 hours) individual chickens. (A) Concentration in the brain, (B) concentration of escitalopram in the lungs, (C) in the yolk and (D) in all three tissues. No outliers were removed with ROUT (Q=1).*

The analysis of the escitalopram metabolite demetylescitalopram showed that chickens can metabolize the drug to this metabolite. The concentration was found to be increasing in the egg yolk over time while plateauing in the lungs and slowly decreasing in the brain (figure 3.11). The highest concentration in the brain was 5 nM after four hours. The highest concentration in the lungs was found after 5 hours (10.5 nM), and in the yolk at 24 hours (37.2 nM).

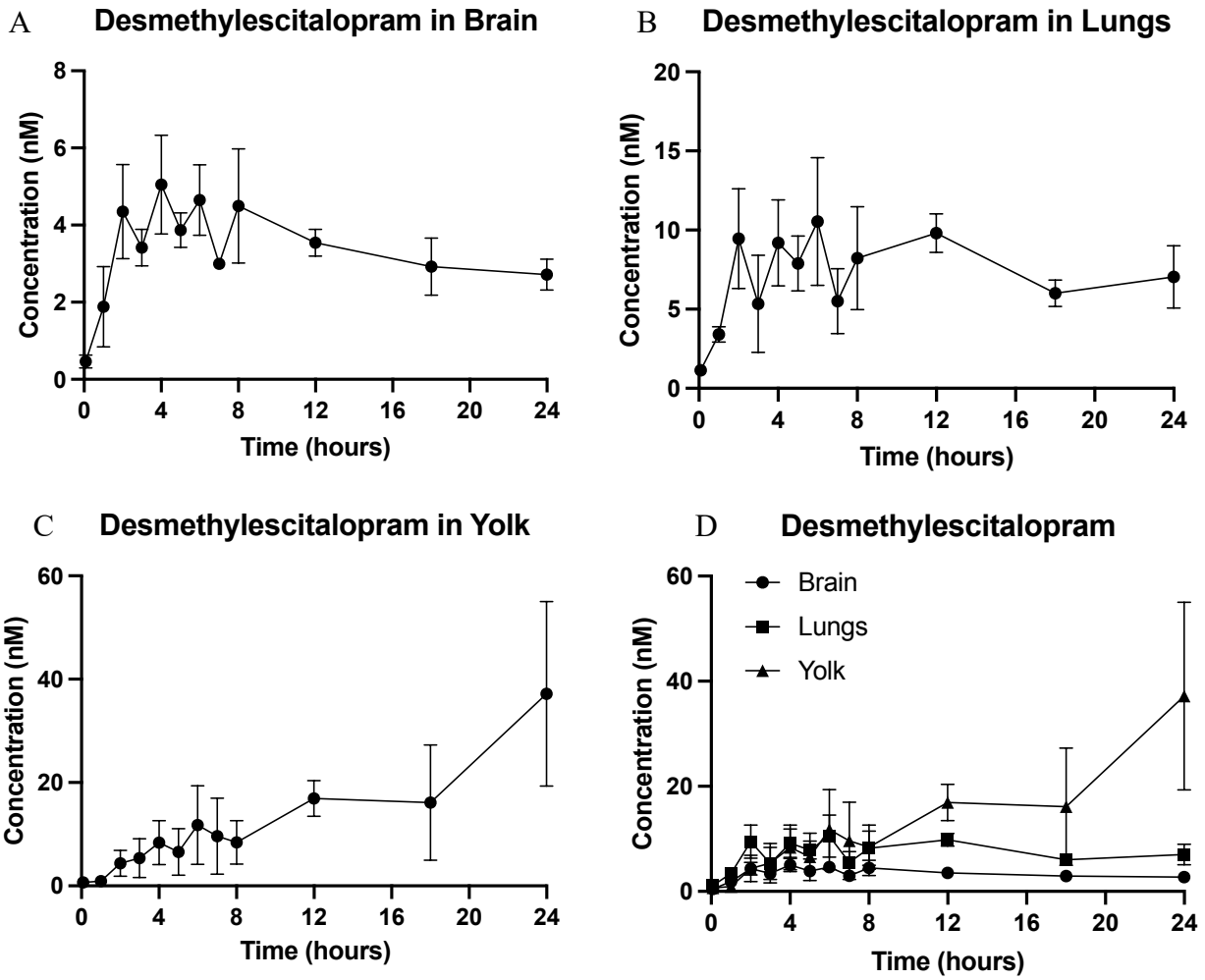

Figure 3.11. Concentration of the metabolite demethylescitalopram in chicken fetus in brain, lungs and yolk*. The eggs were injected with escitalopram at day 13 of incubation and the tissue was then harvested at different timepoints between 0.5-24 hours and analyzed for the metabolite. The data presented in the graphs are the mean with standard deviation of 6 (0.5, 1, 2, 4, 6, 8 and 24 hours) or 3 (3, 5, 7, 12 and 18 hours) individuals. Three outliers were removed by Grubbs ($\alpha = 0.05$). (A) Brian, (B) Lungs, (C) Yolk and (D) all tissues together.*

4. Discussion

This work was done to increase our understanding of the effect of antidepressants escitalopram and venlafaxine on developing neurons. The use of antidepressants during pregnancy has been shown to potentially cause perturbations to the neurobiology, behavior, and risk of neurodevelopmental disorders in exposed offspring (47). On the other hand, the risk of maternal suicide or other negative effects can come from leaving the mother untreated for depression. Therefore, it is important to investigate further the effects of these drugs on neurons and the developing brain to find the safest alternatives and dose regimens.

4.1 Chicken as a preclinical model

The goal of researching the effect of antidepressants on neurons is to gather knowledge relevant to human use. It is, therefore, important to choose models whose results can be transferable to humans (48). A minireview from 2015 concluded that the chicken embryo model could supplement other preclinical drug safety studies, including studies investigating effects on the CNS (33). Zebrafish are widely used for preclinical safety and toxicology studies on CNS. Chickens are phylogenetically closer to humans than fish (33), and chickens have a large degree of genetic sequence homology from our last common ancestor (49).

As used in this thesis, the cerebellum in chicken undergoes important neurodevelopmental processes such as proliferation, migration, differentiating, and apoptosis (33). The chicken CGNs will, if grown under the correct conditions, maintain many of their characteristics. In contrast to primary granule neurons, cell lines may carry properties as malignant cells.

The chicken embryo is with its yolk sac and chorioallantoic membrane (CAM) similar to the mammalian placenta (50). They both develop in a similar way and share important roles in the exchange of gasses and nutrients (50).
The chicken model was used for this research to further validate the model as an alternative animal model more in line with the guideline for ethical animal research's three R´s (reduce, replace, and refine). Reducing the number of animals needed for the model is a big benefit. Since fertilized eggs can easily be obtained from commercial actors, the need for housing and breeding of adult animals is eliminated. This also lowers the cost of the model as it is expensive to provide care and husbandry. The eggs are self-sufficient, have a short incubation time of 21 days, and only require simple facilities with an incubator to provide a humidity of

45-55 % and a temperature of 37-39 °C to develop. As the fetuses develop separately from their mother, there is also no need to inject or euthanize the mother to study the effects on her offspring, thus again reducing the number of animals used compared to studies in mammals like rats or mice. This also means that each individual is independent of the other and introduces higher genetic variability than, for example, using a litter of mammalian pups.

Optimally, the administration of drugs in animal studies should replicate that of intended human use. The antidepressants studied in this thesis would be taken orally by the mother, but the fetus would be exposed over the placenta. It is, therefore, a drawback with this model that the drugs must be injected into the egg if the goal is to research the effect in adults. However, since the fetus is of interest here, the injection onto CAM replicates the human exposure over the placenta. The model does, however, not replicate the mother's metabolism of the drugs studied in the way they would be in a pregnant human. This means that the method could be less transferable to human use since the human fetus will also be exposed to metabolites of the drugs the mother has taken. If desired, this can be alleviated by injecting a mix of the drug and its metabolites. The egg is also a closed compartment, and waste substances cannot be excreted. This can increase the time and amount of drug exposure depending on the distribution and metabolism of the drug in the egg. This can be advantageous as it might reduce the number of injections necessary and prolong exposure. The kinetics of escitalopram is discussed in 4.9.

4.2 PC12 cells as model system

Although a clonal cell line derived from a pheochromocytoma of the rat adrenal medulla, PC12 cells respond to exposure to NGF by differentiating to resemble sympathetic neurons (38). Different cell types can have cell type-specific reactions to exposure (51), and including different cell types in researching the hypothesis will therefore strengthen any biological findings. It is commonly used as a model for neuroscientific research (52). The cell line is also relevant for the research of antidepressants because the PC12 cells express both SERT and NET transporters that escitalopram and venlafaxine inhibit (53, 54). Previous studies have found that the model system can show differences in cells exposed to antidepressants like SSRI and SNRI (55). Changes in endpoints like neurite outgrowth are sometimes cell type-specific, and effects should therefore be shown in other cell types as well (51). With all cell

models, one must consider factors such as differences in gene expression, signaling pathways, and drug metabolism when using cell lines to mimic actions in vivo.

4.3 Choice of exposures

The therapeutic serum concentration for escitalopram is 20-120 nmol/l, and for venlafaxine, it is 400-2000 nmol/l (56, 57). Studies in umbilical cord blood from fetuses exposed to escitalopram and venlafaxine show an escitalopram concentration of 4.5-94 ng/ml in maternal plasma and 4.4-54 ng/ml in the umbilical cord (58). This corresponds to 0.011-0.23 µM and 0.011-0.013 µM. For venlafaxine, the study found maternal plasma concentrations of 7.8-2283 ng/ml and umbilical cord concentrations of 1.4-1694 ng/ml, corresponding to 0.025-7.3 µM and 0.004-5.4 µM (58). I chose to use some concentrations in the therapeutic range and some above. In in vitro experiments in this thesis, I used concentrations 1, 10, and 100 µM of escitalopram. For venlafaxine, I used 2, 20, and 200 µM. By including three increasing concentrations, it is possible to investigate any dose-response effects, and it is also relevant in case of overdose situations and to discover neurotoxic effects. An upconcentration of citalopram (racemate of escitalopram) in cerebrospinal fluids compared to serum concentrations has been shown (59). Because of this, a higher exposure than that found in serum and the umbilical cord was chosen. For escitalopram, the dose of 100 µM might be in the toxic range (60).

4.4 Viability assays in PC12 and chicken granule neurons

The MTT assay in PC12 cells showed decreased viability in cells exposed to 100 µM of escitalopram. This implies that the drug is toxic for the cells in this dose and confirms data from previous toxicology studies of escitalopram (60). By adding more concentrations to the assays, a EC_{50} concentration can be found for the toxicity of escitalopram. Both differentiated and undifferentiated cells had reduced viability at this concentration. There is also a trend of reduced viability for lower doses of escitalopram, but this change was not significant. Hydrogen peroxide was used as the positive control as this would kill the cells, and the reduced viability for this exposure shows that the MTT assay works as intended.

For the chicken cerebellum granule neurons, escitalopram gave a trend of reduced viability in the highest concentration (100 µM) and increased viability in the lowest (1 µM). As activation of the P75 pathway is important for mediating neural apoptosis during development (61),

inhibition of this receptor in CGNs may have increased viability, although not significant, it supports other results for P75 in this cell type from mice and rats (62). In a study using MTT assay to analyze the viability of primary hippocampal neurons from rats, concentrations of up to 80 μM did not change the viability (63). These cells were exposed for 24 hours, and it is possible that the reduced viability found in this thesis, therefore, comes from the longer exposure with 100 μM or differences in the cell type researched.

Inhibition of the TrkB receptor led to reduced viability, but the change was not significant. It is expected to see reduced viability when inhibiting this pathway as activation of the TrkB receptor by BDNF is important for cell survival (64). A further reduction of viability was found for the cells exposed to both the inhibitor of TrkB and escitalopram compared to the control.

The chicken cerebellum granule neurons exposed to venlafaxine did not show any significant changes in viability with the MTT assay. There is, however, a trend of reduced viability for the cells exposed to 200 μM, and increased viability for cells exposed to 20 and 2 μM. Further studies must be done to confirm this in chicken granule neurons. Still, if later proven significant, it would supplement other studies that have found a decreased apoptosis in, for example, PC12 cells exposed to venlafaxine (65). As for the escitalopram group, an increase in viability was found when inhibiting the P75 receptor and a decrease when inhibiting the TrkB receptor.

Replicating the procedure with more accuracy and refinement of the technique and protocol could help reduce the variability. The MTT assay cannot differentiate between cytotoxic and cytostatic effects, so it is not possible to say from this experiment whether the reduction is caused by apoptosis or reduced mitochondrial activity. Trypan blue and propidium iodide will stain cells with lost membrane integrity, i.e., dead cells, and could be used to distinguish cell death from reduced cell proliferation or reduced mitochondrial activity. Other viability assays could also have been considered. The Resazurin viability assay, for example, does not, unlike the MTT assay, kill the cells and therefore has the advantage that other experiments can be done on the cells too (41).

4.5 Neurite length

Neurite outgrowth is a critical cellular process in CNS development and can be quantified using image analysis (51). The endpoint for these experiments was neurite length. To measure this, the IncyCyte Zoom analysis software tracked neurite length based on phase contrast images taken every four hours after drug exposure. Previous studies have shown increased dendrite outgrowth in hippocampal neurons from rats after exposure to escitalopram and other antidepressants, possibly because of action through CaMKII, protein kinase A or PI3K signaling pathways (66). Increased neurite length has also been found in PC12 cells for escitalopram (55) and venlafaxine (67), but for venlafaxine only in a concentration up to 6 µM. In this thesis a reduction in neurite length was observed for both escitalopram and venlafaxine with signs of a dose-effect response. The higher concentration of both drugs reduces neurite length at 68 hours after exposure. As previously discussed, the highest dose of escitalopram might be in the toxic range. It could therefore show a reduction in neurite length because of increased apoptosis instead reduced neurite growth. The IncuCyte Zoom analysis software cannot differentiate between dead and living cells. As previously mentioned, SSRI and SNRI have showed an increase in neurite outgrowth. However, a reduction in neurite length in SH-SY5Y cells exposed to escitalopram in non-toxic concentrations has been seen (Zosen et al., manuscript under revision). Differences may be explained by cell-specific effects and their stage of development, highlighting the importance of experiments in different models.

The inhibitors of TrkB and P75 were added to investigate the possibility of escitalopram having effects through the brain-derived neurotrophic factor (BDNF) or its pro from proBDNF. The reduction in neurite length for both venlafaxine and escitalopram in the developing chicken cerebellum granule neurons could point towards upregulation of pro-BDNF with a higher affinity to p75. However, inhibition of P75 alone showed more or less no difference to the controls. There might be different pathways that are more important for neurite length in chicken cerebellum granule neurons. Inhibition of the TrkB receptor showed a trend of increased neurite length. These results do not overlap with MTT, suggesting different growth factor pathways to be involved in cell viability and neurite outgrowth. While not significant, it is unexpected as activation of TrkB is essential in differentiation morphogenesis (19). No significant changes in neurite length were found at 68 hours compared to the control for escitalopram in combination with inhibitors of TrkB and P75. There seems to be a trend of reduced neurite length for 100 µM escitalopram, but again this could be because of the toxic concentration causing increased apoptosis. For venlafaxine,

none of the concentrations were in the toxic range, and a significant reduction in neurite length is seen with the highest concentration of 200 µM. There is also a trend of decreased neurite length with increasing concentrations.

4.6 Real-time qPCR and Western blot analysis

Measuring the amount of gene expression alone is not proof of physiological changes, as an increase in RNA expression does not necessarily mean the amount of protein it codes for is increased. Nonetheless, changes in RNA expression could help explain the changes seen in neurite length and viability seen in this thesis. Among the genes tested, BDNF showed some intriguing trends, while 5HTR1A was difficult to interpret. Pax6 showed no change. When measuring BDNF, there is a trend of a dose-dependent reduction in BDNF expression for venlafaxine. As only one parallel was done, this cannot be confirmed in this thesis. However, as the BDNF cascade pathway through the TrkB receptor is crucial for cell survival, plasticity, and morphogenesis, a reduction in expressed BDNF should be investigated further in future studies, also in other cell types and in vivo. Furthermore, if an increase in proBDNF can be shown, the negative effect of the antidepressant on developing neurons might come from an increased P75 receptor activity. For escitalopram, an increase in BDNF expression is seen with the nontoxic concentrations. This preliminary data shows interesting potential for further RNA and protein experiments for escitalopram and venlafaxine in chicken as BDNF might be a target for antidepressants (68). Furthermore, the antidepressants have increased BDNF in other adult animal models (69, 70). Pax6 did not change with exposure to escitalopram, venlafaxine, or the inhibitors of P75 and TrkB. A reduction was only seen for the toxic dose of escitalopram.

For 5HTR1A, an increase in expression was found with exposure to venlafaxine and a decrease with exposure to escitalopram. The inhibitor of TrkB also showed an increase in 5HTR1A expression. As this experiment was only done once, the trends cannot be confirmed and only gives an indication of possible changes. In the literature, the effect of escitalopram and venlafaxine on these proteins and their gene expression seemed lacking, and no prior reports were found.

Investigation of the BDNF pro-BDNF, GluN2B and Pax6 was attempted through western blot analysis in chicken granule neurons to confirm and extend qPCR data. Finding antibodies that worked in chicken proved difficult, and a lot of unspecific binding and background noise

made analysis impossible for the experiments in this thesis. As chickens are not used as models to the same degree as, for example, mice and rats, there is a lack of commercially available antigens.

4.7 High Content Imaging

High-content image analysis can be used to assess synapse formation in primary cultures with comparable results to lower throughput methods (71). This method could findings from the IncuCyte (4.5). This thesis investigated high-content imaging as a possible method to research synaptogenesis in chicken cerebellum granule neurons after exposure to escitalopram and venlafaxine by staining DAPI, MAP2, PSD95, and SYP. As this had not been done before with this method, the aim was to test and optimize it on the chicken neurons. The cells were stained with DAPI as this will identify nuclei (25). The DAPI staining worked well for the chicken neurons, but the seeding density could advantageously have been reduced. The images from channel one (DAPI) showed a large degree of clustering, and the high density reduced the antibodies' ability to penetrate the cell walls. Therefore, some parts of the wells, especially around the edges, were not stained optimally, and the fluorescence was reduced in these areas. MAP2 was four days after seeding, mainly expressed in the cell bodies and the cell clusters, not in the neurites. As MAP2 is a protein mainly found in dendrites upon maturation (26), the high overlap of signal from DAPI and MAP2 could suggest the cells were fixated too early in development. Seeding the cells with a lower density and giving them more time to develop might alleviate this in future studies. SYP is a presynaptic marker, while PSD95 is a postsynaptic marker, and their overlap can be used to identify synapse formation (25). In this experiment, I found relatively few overlapping signals from the two markers and some highly fluorescent areas that stem from unspecific binding. The antibodies do appear to work on the chicken granule neurons, as signals are found. However, the low number of signals could mean that they don’t work optimally on chicken granule neurons, that the seeding density was too high, or that the cells were fixated too early in development. In previous research, SYP and PSD95 are found mainly in the cell body at a early stage of neuronal maturation (24). No visible changes were observed for the different exposures, but the experiment should be repeated after optimizing the method.

4.8 Kinetic studies of escitalopram

To get a better understanding of the in vivo distribution and metabolism of escitalopram when injected into the egg, a kinetic study was done. Before the kinetics study in this thesis was done, it was unknown if chickens could metabolize escitalopram to the same metabolites as humans. The analysis was performed at the Department of Forensic Sciences at Oslo university hospital. The standard that was used for the metabolite of escitalopram was a couple of years old, and the data for the metabolite should therefore be regarded as semiquantitative as the accuracy could not be guaranteed by the department.

Escitalopram reaches the lungs, brain, and yolk within 30 minutes of injection. This shows that absorption and distribution happen quickly with this administration method, and the drug is probably absorbed through the peripheral bloodstream and not distributed entirely by diffusion. Other studies have shown a similar uptake of drugs in the brain. For example, Zosen et al. for lamotrigine and valproate (72), Yadav et al. for persistent organic pollutants (73), Khoder for opioids (74), and Mozaffari for venlafaxine (75).
There is a clear absorption and elimination phase in the brain and lungs. The elimination phase can be attributed to both metabolism of escitalopram and re-distribution within the egg. The concentration of escitalopram in the yolk showed a trend of continued increase one hour after injection, while the concentrations in the brain and lungs are decreasing. This could mean that the egg yolk works as a reservoir for the drug because the embryo will absorb nutrients from the yolk and, over time, also the drug/metabolites found here. Escitalopram in humans has a quite large distribution volume of 12-26 l/kg, thought to be because of its high lipophilicity (76). This means that it is not found only in serum but distributed to other tissue as well, which could help explain why it accumulates in the yolk despite being a closed system.

The eggs were injected on day 13 of incubation, and this is the start of critical cerebellar development that happens from day 13 to 17 of incubation (77). This is also at a time when the blood-brain barrier starts to be able to reduce and block drugs from entering the brain, which has been shown to start around day 14 (78). The concentration of escitalopram that reached the brain was almost six times higher than the highest serum concentration in the therapeutic window in humans. In further studies injecting escitalopram at a lower concentration should therefore be considered. Since the concentration decreased to clinically relevant concentrations after just five hours, injecting a lower dose on day 13 might

necessitate repeated injections to ensure the brain is appropriately exposed until harvesting. In the viability and neurite outgrowth experiments undergone in this thesis, a concentration of about eight times the clinically relevant doses did not show significantly reduced viability or neurite outgrowth on chicken cerebellum neurons. Therefore, it is not necessarily problematic for such a short amount of time but should be taken into consideration.

The pharmacokinetic model for escitalopram in the chicken embryo seems to be best described by a multicompartment model, as the elimination phase of the drug is not linear. The elimination of escitalopram seems to follow first-order elimination with a constant half-life. The half life of escitalopram in the brain was found to be 2.22 hours. In rats similar half-live values of 1.6 -1.9 hours have been found (79). Using rats as model system, however, requires the use of an infusion pump for the animals to be appropriately exposed over time, making it a more invasive procedure than the chicken model. In humans the half-life of escitalopram is 27-33 hours (10).

The concentration of the metabolite was higher in lung tissue than in the brain. As the lungs have a high blood flow, the concentrations here are most likely the closest to the serum concentrations. The lower concentrations in the brain, therefore, suggest a developed blood-brain barrier and peripheral metabolism of escitalopram at embryonic day 13. CYP2D6 and CYP3A4, two of the enzymes metabolizing escitalopram in humans, are also found in chicken (80)

Because it is impossible to measure the blood concentration from the same animal over time with the chicken model, all data are based on different individuals that are euthanized. Despite this, the variation in the brain and lungs is relatively low. This consistency shows that the model is reliable. The concentrations in the yolk samples, however, did have a lot of variation. This could be because optimal pipettes were not used in the process of homogenizing the tissue for further analysis. Some tissue, especially that of the yolk, was very viscous and, therefore, difficult to pipette out accurately in the correct volume. This is potentially one of the reasons the variation is bigger in the yolk tissue than in the other tissue. Using a Gilson pipette instead could have alleviated this and reduced the variation in these samples. Furthermore, the harvesting of yolk samples was not executed optimally. Some of the yolk samples were contaminated with highly vascularized tissue surrounding the yolk, which can have affected the final concentrations measured.

With this experiment, I have shown that escitalopram reaches the brain in concentrations relevant to humans when injected onto the CAM. Therefore, it can be used as a suitable model for researching the neurotoxic effects of the drugs. For future studies with the injection of escitalopram in chicken eggs to research the effects in the cerebellum, like western blot analysis or qPCR, a reduced concentration could be appropriate.

4.9 Future perspectives

Further investigation of BDNF/proBDNF should be performed through western blot and PCR in chicken cerebellum granule neurons and other cell lines. This might give new insight into the pathway of reduced cell viability and neurite length through the TrkB/p75 receptors.
As it is now shown that escitalopram reaches the brain in clinically relevant concentrations when injected onto CAM, in ovo experiments with the drug can be carried out in the future. This will also enable research on other parts of the brain with a higher density of adrenergic neurons than the cerebellum.

5. Conclusion

In PC12 cells and chicken cerebellum granule neurons, a reduction in viability for 100 µM escitalopram was shown. Venlafaxine did not cause any changes in viability for chicken cerebellum granule neurons.
Both escitalopram and venlafaxine caused reduced neurite length in chicken cerebellum granule neurons 68 hours after exposure to the highest concentrations tested.
Changes in gene expression for BDNF in cCGNs showed an interesting trend with exposure to escitalopram and venlafaxine and should be further investigated.
It was also shown that escitalopram injected onto CAM reaches the brain in concentrations relevant to human use and that the chicken fetus can metabolize escitalopram demethylescitalopram.

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Appendix

A. Materials

A.1 Chemicals and biological products

Chemicals and biological products	
10x Tris/Glycine/SDS Buffer	Bio-Rad Laboratories, USA
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, USA
2-Mercaptoethanol 50 mM	Life Technologies Ltd., Paisley, UK
Albumin Standard	Thermo Scientific, Rockford, USA
Anti-Adherence Rinsing Solution	Stemcell Technologies, Vancouver, Canada
AraC	Sigma-Aldrich, St. Louis, USA
Basal Medium Eagle (BME), Gibco	Thermo Fisher Scientific, Rockford, USA
BioWhittaker's DMEM (Dulbecco's Modified Eagle's Medium)	Lonza Bioscience, Basel, Switzerland
Bromophenol blue	Merck KGaA Darmstadt, Germany
BSA 35% in DPBS (for immunostaining)	Sigma-Aldrich, St. Louis, USA
BSA, fraction V	Cytiva HyClone Laboratories, Utah, USA
Calcium chloride dihydrate	Sigma-Aldrich, St. Louis, USA
Chicken serum, Gibco	Thermo Fischer Scientific, Rockford, USA
Citric acid (AnalaR)	BDH Chemicals l.td., Poole, UK
Copper sulfate pentahydrate (CuSO4 x 5H2O)	Merck KgaA, Darmstadt, Germany
Cytosine β-D-arabinofuranoside	Sigma-Aldrich, St. Louis, USA
DAPI	Termo Fisher Scientific, Rockford, USA
Deocyribonuclease I from bovine pancreas Type IV, lyophilized powder	Sigma-Aldrich, St. Louis, USA
Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x $2H_2O$)	Sigma-Aldrich, St. Louis, USA
DMSO (dimethyl sulfoxide)	Sigma-Aldrich, St. Louis, USA
Escitalopram oxalate	Sigma-Aldrich, St. Louis, USA
Ethanol	Antibac, Oslo, Norway
Ethylenediaminetetraacetic acid (EDTA)	VWR Life Science, Radnor, USA
Fetal calf serum, Gibco	Thermo Fischer Scientific, Rockford, USA

Formaldehyde, 37 wt. % solution in water	Sigma-Aldrich, St. Louis, USA
Glucose	Merck KGaA, Darmstadt, Germany
Glutamine	Sigma-Aldrich, St. Louis, USA
H-transferrin	Sigma-Aldrich, St. Louis, USA
HCl	VWR Life Science, Radnor, USA
Horse serum, Gibco	Thermo Fischer Scientific, Rockford, USA
Insulin	Sigma-Aldrich, St. Louis, USA
Blocking Buffer Intercept®	Li-COR Biosciences, Lincoln, USA
KCl	Sigma-Aldrich, St. Louis, USA
L-Glutamine	Sigma-Aldrich, St. Louis, USA
Magnesium sulfate	Sigma-Aldrich, St. Louis, USA
Na_3VO_4	Sigma-Aldrich, St. Louis, USA
NaCl	VWR Life Science, Radnor, USA
NaOH	Merck KGaA, Darmstadt, Germany
Nerve growth factor (NGF)	Sigma-Aldrich, St. Louis, USA
Penicillin-Streptomycin	Invitrogen, Carlsbad, USA
Pepstatin A	Sigma-Aldrich, St. Louis, USA
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich, St. Louis, USA
Poly-L-Lysine hydrobromide	Sigma-Aldrich, St. Louis, USA
Ponceau S	Sigma-Aldrich, St. Louis, USA
Precision Plus Protein All Blue Standard	Bio-Rad Labratories Inc., Hercules, USA
Propidium iodide	Sigma-Aldrich, St. Louis, USA
Putrecine dihydrochloride	Sigma-Aldrich, St. Louis, USA
Retinoic acid (RA)	Sigma-Aldrich, St. Louis, USA
Sodium deoxycholate monohydrate	Sigma-Aldrich, St. Louis, USA
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4H_2O$)	VWR Life Science, Radnor, USA
Sodium dodecyl sulfate (SDS)	Shelton Scientific (Sigma-Aldrich, USA)
Sodium pyruvate	Thermo Fischer Scientific, Rockford, USA
Sodium selenite (Na_2SeO_3)	Sigma-Aldrich, St. Louis, USA
Stripping Buffer (NewBlot Nitro)	Li-COR Biosciences, Lincoln, USA
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich, St. Louis, USA
Trans-Blot Turbo 5x Transfer Buffer	Bio-Rad Laboratories Inc., Hercules, USA

Tri-iodide-L-thyronine sodium salt (T3)	Sigma-Aldrich, St. Louis, USA
Tris base	Sigma-Aldrich, St. Louis, USA
Tris-HCl	Sigma-Aldrich, St. Louis, USA
Triton™ X-100	Sigma Aldrich, St. Louis, USA
TrkA inhibitor (GW 441756)	Tocris Bioscience, Bristol, UK
Trypsin from bovine pancreas	Sigma Aldrich, St. Louis, USA
Trypsin inhibitor	Sigma-Aldrich, St. Louis, USA
Trypsin-EDTA (0,05 %)	Thermo Fischer Scientific, Rockford, USA
Tween® 20	Sigma-Aldrich, St. Louis, USA
Venlafaxine hydrochloride	Sigma-Aldrich, St. Louis, USA

A.2 Equipment

Equipment	
Alcohol Pads	B. Braun Medical, Melsungen, Germany
Blotting machine (Trans-Blot Turbo Transfer System)	Bio-Rad Labratories Inc., USA
Cell culture dishes	Thermo Scientific, Waltham, MA, USA
Cell scraper (Costar® Cell Lifter 3008)	Corning Inc. New York, USA
Cooling centrifuge (Heraeus Fresco 21)	Thermo Fischer Scientific, Rockford, USA
Disposable scalpels	Swann-Morton®, Sheffield, England
Disposable syringes	B. Braun Medical, Melsungen, Germany
Electrophoresis apparatus (BioRad Power Pac 300)	Bio-Rad Laboratories Inc., Hercules, USA
Electrophoresis vessel	Bio-Rad Laboratories Inc., Hercules, USA
Eppendorf tubes (SafeSeal 1,5 ml)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Fine weight (CPA225D)	Sartorius Lab Instruments GmbH & Co, Goettingen, Germany
Hemocytometer (Neubauer 0.100 mm Tiefe Depth Profondeur)	Marienfeld Superior, Lauda-Königshofen, Germany
Incubator (OvaEasy 380 Advance EXII)	Brinsea, Weston-super-Mare, UK
IncuCyte	

Insulin needles with syringe (Omnican®50)	B. Braun Australia Pty Ltd, Bella Vista, Australia
LAF bench (Holten LaminAir, modell 1.2)	Eco Holten AS, Denmark
Magnetic stirrer (RCT basic)	IKA®, Staufen, Germany
Microscope (Leica DM LS)	Leica, Wetzlar, Germany
Microscope (Nikon Inverted Microscope Eclipse TE300)	Nikon Corporation, Tokyo, Japan
Mini-PROTEAN TGX Gels	Bio-Rad Laboratories Inc., USA
Needles (sterile) BD Microlance™ 3 Nr.18	Becton, Dickinson and Company Limited, Co. Louth, Ireland
Odyssey-CLx Imaging System	Li-COR Biosciences, Lincoln, USA
Pasteur pipette	Thermo Fischer Scientific, Rockford, USA
Petri dishes	Sigma-Aldrich, St. Louis, USA
Pierce BCA Protein Kit	Thermo Scientific, Rockford, USA
Pipette tips	Sartorius Blohit Liquid Handling Oy, Helsinki, Finland
Pipettes (sterile)	Corning Inc., Corning, USA
Plate Reader (CLARIOstar®)	BMG Labtech GmbH, Ortenberg, Germany
Plates for IncuCyte, 96-well (TPP®)	Sigma-Aldrich, St. Louis, USA
Plates, 6-well (TC Plate 6 well, Standard, F)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Plates, 96-well, clear bottom	Thermo Scientific, Waltham, MA, USA
Roller (Stuart Rollermixer SRT9)	VWR Life Science, Radnor, USA
Sterile filter 0,2 µm (Whatman™)	GE Healthcare UK limited, Buckinghamshire, UK
Water bath (Sub Aqua 12)	Grant Instruments, Royston, UK
Weight (Valor 3000 XtremeW)	Ohaus, Switzerland
Whirl mixer	Terumo Lab AS, Leuven, Belgium

B. Protocols

B.1 Splitting PC12-cells

Medium used:

500 ml DMEM + 500 ml Penisilium/Streptomycin + 5 ml pyruvate + 50 calf serum + 25 ml horse serum.

1. Old medium is discarded
2. 10 ml 37 °C new medium is added
3. The cells are dislodged from the bottom by hitting the bottle repeatedly with the hand
4. Mix well by pipetting up and down repeatedly
5. All the medium is pipetted out of the bottle
6. 1,5 ml is transferred to a new bottle or back to the original if splitting into the same bottle
7. 20 ml of 37 °C new medium is added
8. Mix well by pipetting up and down
9. Incubate in 5% CO_2 at 37 °C

B.2 Plating PC12-cells on a 96-well plate

Medium used:

500 ml DMEM + 500 ml Penisilium/Streptomycin + 5 ml pyruvate + 50 calf serum + 25 ml horse serum

1. Old medium is discarded
2. 10 ml 37 °C new medium is added
3. The cells are dislodged from the bottom by hitting the bottle repeatedly with the hand
4. The cells are counted on a hemocytometer
5. The amount of cell suspension needed is calculated, the medium is added, and the suspension is mixed well by pipetting up and down repeatedly

Formula used for calculation:

$$ml\ cell\ suspension = \frac{98\ wells * 0{,}2\ ml * 7 * 10^4}{Amount\ of\ cells\ (mean) *\ 10^4}$$

6. 200 µl of cell suspension is added to every well
7. Incubate at 37 °C and 5 % CO_2

B.3 Coating with poly-L-lysine

1. Mix 10 ml of sterile water in a sterile bottle of poly-L-lysine (sigma 5 mg)
2. Transfer to 500 ml of sterile water

3. 200 µl per well is used for 96-well plates, 2 ml for 35 mm wells or 5 ml for 100 mm dishes
4. Add to all wells and incubate on for 1 hour
5. Aspirate the solution from all wells and leave them in the LAF bench so the dish dries, preferably overnight

B.4 Preparation of Chicken cerebellum granule cells primary culture and seeding

Equipment autoclaved before use:
Scissors and tweezers for dissection of the brain
2 sharp tweezers for extraction of the cerebellum
Pasteur pipettes with a long tip, trypsinization bottle and four glass flasks with cap
1 spatula to remove the cerebellum

Cell culture medium used:
500 ml basal medium eagle (BME)
37.5 ml heat inactivated chicken serum
825 mg KCl
5 ml Penicillin/Streptomycin
146 mg glutamine
5.7335 mg Insulin

Solution 1:	
Bovine serum albumin (BSA)	1,5 g
Krebs-ringer solution 10x	50 ml
$MgSO_4$ (3,82 g / 100 ml)	4 ml
Distilled water	to 500 ml
Solution 2:	
Trypsin	12,5 mg
Solution 1	50 ml

Solution 3:	
DNase	3,1 mg
Trypsin inhibitor	13,0 mg

$MgSO_4$ (3,82 g / 100 ml)	250 µl
Solution 1	to 25 ml

Solution 4:

Solution 3	8 ml
Solution 1	50 ml

Solution 5:

$MgSO_4$ (3,82 g / 100 ml)	320 µl
$CaCl_2$ (1,20 g / 100 ml)	320 µl
Solution 1	40 ml

All solutions are sterile filtered before use

1. 17 days old chicken eggs are removed from the incubator
2. 6 and 6 eggs at a time are put in ice for 7 minutes for anesthesia
3. The eggs are washed in 70 % ethanol before they are cracked (in 14 cm petri dishes). A sterile scalpel is used to decapitate the chick before the cerebellum is removed with scissors, tweezers, and a spatula under the LAF hood.
4. The cerebellum is put in a dish with solution 1
5. The meninges are removed with tweezers and the cerebellum is put in a new dish with solution 1
6. Solution 1 is removed, the cerebellums are centered in the dish and cut into small pieces with a sterile scalpel. Cut in two directions perpendicular to each other. 10 ml of solution 1 is added to the dish
7. The pieces are transferred to a sterile 50 ml falcon tube with 10 ml of solution 1 and the tube is centrifuged at 1000 rpm for 1 minute
8. The supernatant is removed
9. 8 ml of solution 2 is added. Pipette carefully up and down to dissolve the pellet
10. The cell suspension is transferred to a sterile trypsinization bottle. The bottle is put on a water bath at 37 °C for 15 minutes. Shake the bottle periodically. The caps on the bottle must not be tightened completely.

11. The pieces from the trypsinization bottle are sucked up and transferred to a sterile 50 ml falcon tube with 15 ml of solution 4. Fill the tube with solution 4
12. The tube is centrifuged at 1000 rpm for 2 minutes. If the supernatant is not clear, a small amount of solution 3 can be added and the centrifugation is repeated
13. The supernatant is removed
14. The tube is added 3 ml of solution 3. The pellet is carefully dissolved by pipetting up and down 15-20 times with a sterile Pasteur pipette with a rounded tip. When the cell lumps have sunk to the bottom, the top, lump free part of the solution is transferred to a sterile 50 ml falcon tube with 50 ml of solution 5
15. 2 ml of solution 3 is added to the tube with the lumps, and the step above is repeated until there are no more visible cell lumps present. Transfer the rest of the solution to the tube with solution 5
16. Centrifuge at 900 rpm for 7 minutes
17. The supernatant is removed
18. 10 ml cell culture medium with chicken serum is added. The pellet is dissolved and transferred to a sterile bottle with the same medium. A small sample is taken out for counting on a hemocytometer after the solution is mixed well
19. The plates have on beforehand been coated with poly-L-lysine (B3)
20. The plates are incubated at 37 °C and with 5 % CO_2

B.5 Exposure of chicken cerebellum granule neurons

Medium used:

BME	500 ml
KCl	825 mg
Glutamine	146 mg
Penecillin/Streptomycin	5 ml
Holotrasferine	50 mg
Putrescine	4.8 mg
Insulin	12.5 mg

1. New medium is mixed with drugs/inhibitors to desired concentrations
2. Old medium is discarded
3. New medium with the desired treatment is added
4. The plate is incubated at 37 °C and 5 % CO_2

B.6 MTT-analysis in a 96-well plate

1. Aspirate all medium from every well except from column 1
2. 1 ml 5 mg/ml MTT dissolved in PBS is added to 10 ml of medium
3. Incubate the plate in 5 % CO_2 and 37 °C for 3-4 hours
4. Aspirate the medium from all wells
5. 100 µl technical DMSO is added to all wells
6. The plate is incubated for 1 hour with 5 % CO_2 and 37 °C
7. The absorbance is measured on the ClarioStar plate reader at 570 nm

B.7 Harvesting for western blotting

1. Remove all medium from the wells
2. Wash twice with 3 ml of cold PBS, aspirate well
3. Add 200 µl RIPA buffer with:

Leupeptin 5 mg/ml, 1 µl to 1 ml

Pepstatin A 1 mg/ml, 5 µl to 1 ml

PMSF 100 mM, 3 µl to 1 ml

Na_3VO_4 10 mM, 10 µl to 1 ml

4. Scrape with cell scraper in two directions perpendicular to each other
5. Transfer the solution to an Eppendorf tube and put on ice
6. Sonicate the samples for 2 seconds three times, with one minute on ice in between
6. Store samples at – 20 °C

B.8 Protein measurement

1. Working reagentd prepared from Pierce™ BCA Protein Assay Kit (50 part reagent A: 1 Part reagent B)
2. Increasing volume of the standard solution (0, 1.25, 2.5, 5, 10, 20, 40 µL of 0.5 mg/) in duplicate are added to a blank 96-well plate (the first two are blank). 0,5 mg/ml standard is prepared by diluting 2 mg/ml standard with PBS.

3. 5 µl (more or less depending on the amount of protein) of the 1:20 dilution preparation is added to the well, starting row number 3
4. 200 µl of the working reagent is added to all the wells (1 Reagent B:50 Reagent B, BCA)
5. The 96-well plate is then put in an incubator at 37 °C for an hour (1-1,25 hrs)
6. Absorbance is measured at 570 nm in the Clariostar plate reader.
7. Launch Clariostar program
8. Export report in Excel format.
9. Use the standard curve and the absorbance of samples to calculate the amount of protein in the samples

B.9 SDS page

1. Calculate the volume of each sample needed to load the desired amount of protein based on the protein measurement.
2. Calculate the amount of 4x Lamelli buffer (25 %), mercaptoethanol (5 %) and MQ water to make a solution containing 25 µg of protein per 15 µl
3. Find desired gel, remove the bottom tape and the well protection.
2. Insert the gels in the rig
3. Add electrophoresis buffer in between the gels and fill it up high enough for the current to move
4. Add 3 µl of the standard to the first well
5. Add the 15 µl to be loaded into each well
6. Put on the lid
7. Run at 150V for 45-60 min

B.10 Harvesting cells for qPCR

1. Aspirate medium and wash twice with ice-cold PBS (1-2 ml)
2. Aspirate PBS (remove as much as possible) and add 350 µl of RLT lysis buffer
3. Scrape the plate briefly, then remove the RLT buffer with a pipette and transfer the cell lysate to an Eppendorf tube
4. Store at -80 °C

B.11 RNA isolation:

RNeasy® Plus Mini Kit (cat. nos. 74134 and 74136)

1. Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.
2. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied)
3. Centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) in the Heraeus Fresco 21 sentrifuge. Discard the column and save the flowthrough. Add 1 volume (350 µl) of 70% ethanol to the flow-through and mix well by pipetting. Do not centrifuge. Proceed immediately to step 4
4. Transfer up to 700 µl of the sample, including any precipitate, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.
5. Add 700 µl Buffer RW1 to the RNeasy Mini spin column (in a 2 ml collection tube). Close the lid, and centrifuge for 15 s at 8000 x g. Discard the flow-through.
6. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through
7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm). Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to further dry the membrane
8. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x g to elute the RNA

B.12 Immunocytochemistry protocol

(SOP for Neurol Stem Cell cultivation practices, Anna J, Lauvås, 25/10-22)

5.1 Solutions and Reagents

- 1x PBS with Ca^{2+} and Mg^{2+} (PBS+)
- Permeabilization buffer: 0,1 % Triton-X100 in 1x PBS+. Can be stored at 4 °C
- Blocking buffer: 3,5 % BSA in 1x PBS+. Prepare fresh
- 4 % Formaldehyde. Prepare fresh

5.2 Procedures:

When working with formaldehyde, work in LAF-hood to remove risk of exposure

5.2.1 Fixation

1. Remove the culture medium by manual pipetting and add 4 % formaldehyde for 10 minutes at room temperature. 96-well plate: 50 µl/well
2. Aspirate formaldehyde completely by manual pipetting
3. Gently wash twice with 1x PBS+. Always aspirate by manual pipetting
4. Cells can be stored at 4 °C in 1x PBS+ prior to analysis. Use parafilm to seal the lid to avoid evaporation. 96-well plate: 200 µl/well

5.2.2 Immunostaining:

1. Remove 1x PBS+ by manual pipetting
2. If the antigen is intracellular, permeabilize cells with 0,1 % Triton X-100 in 1x PBS+ for 10 minutes at room temperature. If not intercellular, proceed to the next step. 96-well plate: 50 µl/well
3. Aspirate permeabilizer by manual pipetting
4. Incubate with blocking buffer (3,5% BSA in 1x PBS+) for 30 minutes at room temperature. 96-well plate: 50 µl/well
5. Aspirate blocking buffer by manual pipetting
6. Add primary antibodies: Incubate with primary antibodies at the suggested dilution in blocking buffer for either 1 hour in an incubator, 2 hours at room temperature, or overnight at 4 °C
7. Aspirate primary antibody by manual pipetting
8. Gently wash twice with 1x PBS+, always aspirating by manual pipetting. 96-well plate: 100 µl/well

   Work in darkness from this point on

9. Incubate the cells with secondary antibodies in blocking buffer (premade from the day before) for 1 hour, protected from light at room temperature. Work in darkness and wrap in aluminum foil. 96-well plate: 50 µl/well
10. Gently wash twice with PBS+. Always aspirate by manual pipetting
11. Store cells in PBS+ at 4 °C until analysis. Seal the lid with parafilm and wrap the plate in aluminum foil to protect it from light. 96-well plate: 200 µl/well
12. Take images with the Thermo Scientific CellInsight CX7 Laser