MEASURING DOPAMINE AND MELATONIN CONCENTRATIONS FROM THE MOUSE RETINA

Descriptive Literature Review

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Bachelor's Thesis
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TIIVISTELMÄ

Tampereen ammattikorkeakoulu
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ANTTONEN SANNA & KOKKILA JOANNA:
Dopamiini- ja melatoniinipitoisuuksien määritys hiirten verkkokalvopreparaateista
Kuvaileva kirjallisuuskatsaus

Opinnäytetyö 47 sivua, joista liitteitä 1 sivu
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Tämän opinnäytetyön aiheena oli selvittää dopamiinin ja melatoniinin pitoisuuksien määritysmenetelmät hiirten verkkokalvoista. Tavoitteenamme oli selvittää kuvailevan kirjallisuuskatsauksen avulla mitä erilaisia pitoisuuksien määritysmenetelmiä tieteellisissä tutkimuksissa on käytetty. Tarkoituksenamme oli tuottaa työn toimeksiantajalle, professori Petri Ala-Laurilan neurotieteiden laboratoriolle, ajankohtaista ja koottua tietoa tutkimusmenetelmistä. Henkilökohtaisena tavoitteenamme oli perehtyä paremmin dopamiinin, melatoniinin, sirkadiaanisen rytmin sekä hormonien mittausmenetelmien teoreettiseen taustaan ja syventää aiemmin oppimaamme tietoa. Tavoitteena oli myös parantaa englannin kielen kirjoittamista ja ymmärtämistä. Opinnäytetyön tehtävänä oli selvittää eri mittausmenetelmät sekö niiden perusperiaatteet ja käytettävyys.

Opinnäytetyömme aineisto koostui eri tietokannoista kootusta materiaalista, joka sisälsi pääasiassa tutkimusartikkeleita ja kokoomateoksia. Määrittämillämme hakusanoilla ja hakukriteereillä haimme aineistoa seuraavista tietokannoista: Pubmed, ScienceDirect, EBSCO, Web of Science, Wiley Online ja Springer Link. Karsimme hakutulokset ensin otsikon perusteella työn aihealueeseen liittyviin tutkimuksiin, sitten kävimme artikkelien tiivistelmät läpi ja valitsimme yhteensä 35 artikkelia, joista vain kolme artikkelia oli mielestämme sopivia kuvailevaan kirjallisuuskatsaukseemme. Valitsimme mukaan myös yhden artikkelin hakukriteerien ulkopuolelta, sillä käytimme sitä pohjana katsauksessa ja tiedonhaussa.

Kuvailevan kirjallisuuskatsauksen tuloksena havaitsimme, että dopamiinin mittausmenetelmistä liittyen verkkokalvonäytteisiin löytyi tietoa hyvin. Melatoniinin mittausmenetelmistä emme onnistuneet löytämään yhtä paljon tietoa, joka olisi koskenut tutkimaamme aihetta. Osa tutkimuksista keskittyi esimerkiksi melatoniinin reseptoreihin verkkokalvolla, emmekä kokeneet sen vastaavan aihettamme. Ainoastaan yhdessä valitsemassamme tieteellisessä artikkelissa käsiteltiin myös melatoniinin mittausta. Dopamiinin yleisimpänä mittausmenetelmänä käytettiin korkean erotuskyvyn kromatografiaa ja sen erilaisia sovelluksia.

Johtopäätöksenä voidaan pitää ajatusta, että verkkokalvotutkimuksissa hallitsevana tutkimuskohteena ovat dopamiinin ja sen johdannaiset sekä niiden vuorokausivaihtelut. Myös melatoniini on osa tutkimuksia, mutta sen mittaamista suoritetaan harvoin verkkokalvonäytteiltä tai tutkimus kohdistuu melatoniinin johdannaisiin tai reseptoreihin. Työn suppean laajuuden takia emme voi varmasti sanoa kuinka paljon melatoniinia mitataan verkkokalvoilta. Tulevaisuudessa silmän sirkadiaanista rytmiä sekä dopamiinin ja melatoniinin osuutta siihen tullaan tutkimaan varmasti vielä enemmän.

Asiasanat: dopamiini, melatoniini, hiiri, verkkokalvo, sirkadiaaninen rytmi, korkean erotuskyvyn kromatografia, HPLC

ABSTRACT

Tampereen ammattikorkeakoulu
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ANTTONEN SANNA & KOKKILA JOANNA:
Measuring Dopamine and Melatonin Concentrations from the Mouse Retina
Descriptive Literature Review

Bachelor's thesis 47 pages, appendices 1 page
October 2015

The objective of this study was to discover assay methods used to measure dopamine and melatonin concentrations in the mouse retina. The purpose of this study was to provide current and compact information about the assay methods for our client, Professor Petri Ala-Laurila's neuroscience research laboratory. Other purposes was to increase our knowledge and improve our skills in writing and understanding English.

The method of this study was a descriptive literature review. The theoretical background of the study consisted of dopamine, melatonin and the circadian rhythm and their connection to the mouse retina. We also covered the basis of the assay methods concerning hormone measurements.

The main finding is that there are more research articles about measuring dopamine from the mouse retina than there are about measuring melatonin. Only one research article from our gathered data mentioned melatonin measurement. We found that the high performance liquid chromatography (HPLC) is most commonly used in measuring dopamine levels from retina samples.

The conclusion is that dopamine and its metabolites are dominating the research field in the studies of retina. Melatonin itself is usually a part of the research objective but its concentration is rarely or never measured from the retina. It usually concentrates on melatonin metabolites or its receptors. We suspect that future researches will concentrate more on the connection of dopamine and melatonin to the circadian rhythm.

Key words: dopamine, melatonin, mouse, retina, circadian rhythm, high performance liquid chromatography, HPLC

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1 INTRODUCTION

The subject of our study is to do a descriptive literature review about measuring methods of dopamine and melatonin concentration from the mouse retina. We chose this subject because it has connection to both neurophysiology and research work, which interest us. The idea for this study came from Professor Petri Ala-Laurila's neuroscience research laboratory that is located in the University of Helsinki. Their research is concentrated for example on the novel signal processing mechanisms and their adaptive dynamics at the synaptic and cellular-element level across the entire neural circuit of the retina. (Ala-Laurila Lab 2015.)

The objective of this study is to discover assay methods used to measure melatonin and dopamine concentrations in the mouse retina. The purpose of this study is to provide current and useful information about the assay methods for our client. In this study the theoretical background is based on the objective. Its core consists of dopamine, melatonin, the circadian rhythm, and the eye and the retina of the mouse. We also included theoretical information about the assays usually used to measure hormones, and about laboratory mice and their use in research.

Melatonin is a neurohormone produced in the pineal gland. The job of melatonin is to control the biological rhythm of different organs, which is why it is produced cyclically. (Leppäluoto, Kettunen, Rintamäki, Vakkuri, Vierimaa & Lätti 2012, 314.) The retinal cells of the eye also produce melatonin, but considerably less than the pineal gland. The effect of melatonin in the retina is not known very well, but it is hypothesized that it works as some sort of a neuromodulator. (Tosini, Baba, Hwang & Iuvone. 2012, 82.)

Dopamine works in the system as a transmitter and as a hormone (Tirri, Lehtonen, Lemmetyinen, Pihakaski & Portin 2001, 117). It is produced by retinal cells in the inner nuclear layer. The role of dopamine is to stimulate several light adaptive responses including the contraction of cones and distribution of pigments. Dopamine is released during the day, and it affects the circadian rhythms of both melatonin and dopamine. (Doyle, Grace, McIvor & Menaker 2002, 593–594.)

Every living thing has a daily rhythm, which originates within the organism. For example in humans the suprachiasmatic nuclei (SCN) of the hypothalamus controls the 24-hour rhythm. Many functions such as sleep, food intake, heart rate, and hormone release are connected to the circadian rhythm. Studies have shown, for example, that the central clock of the human brain is synchronized with external day by the circadian system. (Tosini, McMahon & Iuvone et al. 2014, 1–2.)

The retinal circadian clock was the first extra-SCN circadian system to be found in mammals. It has been found out in several studies that the many physiological, cellular and molecular rhythms of the retina are in fact controlled by the retinal circadian clock, or more likely a network of hierarchically organized circadian clocks. (Tosini et al. 2014, 1–2.)

The assignments of this study are outlined by the objective and the purpose. In this study we try to answer these questions: what different assay methods are there to measure dopamine and melatonin concentrations in mouse retina, the function principles of the assay methods, and the usability of them. We want to deliver a summary about the articles we discover. We are accustomed to using different databases and finding reliable information from the internet and literature based on our previous school assignments.

Based on our method we describe the used databases and the research words in our study. We also report the search result numbers from each database and the way we eliminated the articles not suited for our study. The chosen articles for this descriptive literature review are catalogued in the chart in the appendix of this study. We hope that Professor Petri Ala-Laurila's research laboratory finds this review as a useful information for their research.

2 OBJECTIVE AND PURPOSE

The objective of this study is to discover assay methods used to measure melatonin and dopamine concentrations in the mouse retina. The purpose of this study is to provide current and useful information about the assay methods for our client. Another purpose is to increase our own knowledge about melatonin, dopamine and circadian rhythm, and to improve our skills in writing and understanding English. We are using descriptive literature review as our study method.

Assignments of this study:

- Find out what different assay methods there are to measure dopamine and melatonin concentrations in the mouse retina.
- Work out the function principles of these assay methods.
- Find out the usability of the assay methods.

3 BASIS OF METHOD

We used descriptive literature review as the method in our thesis because it was the most efficient way to process the subject. The literature review was very easily selected as our method because of the assignment of our study. We followed the principles of the integrative descriptive literature review. The literature review can achieve many goals. It can serve in building a bigger picture of a specific area, and that is the description that best suited with our objective. The literature review is generally divided into three categories: the descriptive review, the systematic review and the meta-analysis. There are also other ways to divide literature review types. (Salminen 2011, 6–8.)

The descriptive literature review is one of the most commonly used literature review methods. It does not have strict and exact rules to follow. It can be called as a general review. The amount of material used in a descriptive literature review is huge. Methodological rules do not delimit the used material. The descriptive review can widely illustrate the research subject and classify its properties, if necessary. The difference between the systematic review and the descriptive review is that the descriptive review does have broader research questions compared to the systematic review. (Salminen 2011, 6–8.)

There are two types of the descriptive literature review: a narrative review and an integrative review. Especially the integrative review has some things in common with the systematic review. The narrative review is the lightest review type from the methodological point of view. Its purpose is to organize inconsistent data into a continuous process. The integrative review is used to describe the subject as diversely as possible. Compared to the systematic review, the integrative review gives out a wider picture from the literature material concerning the subject. The integrative review is also more critical than the narrative review. With the help of a critical evaluation the most important research material will be able to be densified as the base of the review. (Salminen 2011, 6–8.)

Even though the systematic review is more strict compared to the integrative descriptive literature review, we followed some of the stages represented at Fink's (2005) model. This model is to clarify the systematic literature review method, but for us it was helpful in organizing our work. The first step is to set the research question, and after this the literature and the databases are chosen (figure 1). The search terms are selected in the third stage. The search results are delimited to answer the research questions when the search terms are chosen carefully (figure 1). (Fink 2005, 4; Salminen 2011, 6–8.)

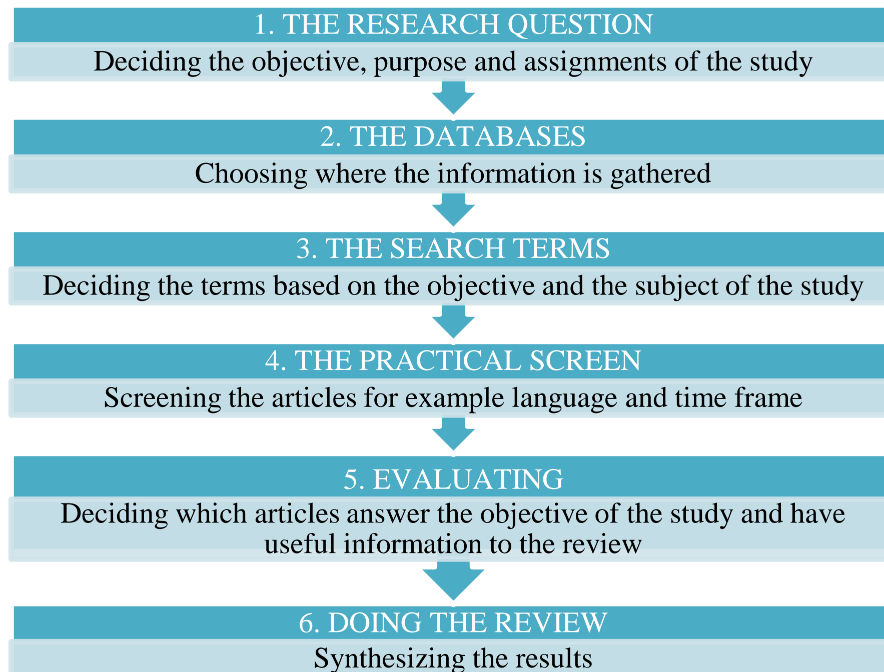


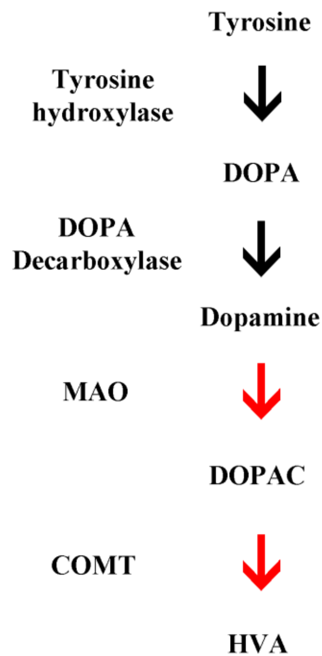
FIGURE 1. Process of literature review based on Fink's (2005) model and simplified for descriptive literature review. (Fink 2005, 4).

The next two phases are about screening (figure 1). At the fourth phase a practical screen frame is set to determinate which languages and publishing years are accepted as a delimited criteria. The next step is a methodological screen whose aim is to evaluate the scientific quality of the articles and the researches. Doing the review itself is the sixth phase (figure 1). In the systematic review a reliable and valid re-view calls for a standardized form of collecting information about the articles, but for the integrative review we were not so strict about which articles we could choose to include into the re-view. The last stage is the synthesizing of the results (figure 1). (Salminen 2011, 6–8.)

4 THEORETICAL BACKGROUND

4.1 Dopamine

Dopamine is the primary catecholamine of the retina. It is synthesized in a population of the cells of the inner nuclear layer that are either amacrine or interplexiform neurons. The neurons synthesize dopamine throughout their entire cell body. The synthesis (picture 1) starts with tyrosine that is conversed to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) which is a limiting enzyme for a DA synthesis. Then the L-DOPA is decarboxylated by an aromatic L-amino acid decarboxylase to form dopamine. (Tosini & Iuvone, 2014, 54.)



PICTURE 1. Dopamine synthesis (Tosini & Iuvone 2014, 51).

There are about 500 dopaminergic neurons in the mouse retina, which is a relatively small amount. Even though the percentage of the dopaminergic neurons is small compared to other cells, the axons of the neurons are so long they can spread the effect throughout the whole retina. Dopamine also affects the retina via diffusion from the blood circulation. (Tosini & Iuvone 2014, 54.)

The release of dopamine and the changes in its synthesis depend on the interaction between the dopaminergic neurons and the photoreceptors - dopamine is released when the neurons are stimulated by light. Because of that the dopamine release is higher during the day, and it is believed that it promotes light adaptation. A small amount of dopamine is also released during the night, as found when the test subjects were kept in darkness. This implies that the dopamine cycle exists even without light stimulation. (Tosini & Iuvone 2014, 54–55.)

The dopamine receptors belong to the family of G protein–coupled receptors (GPRCs), and there are five different subtypes (D_1-D_5) that are organized in to two different families (D_1- and D_2-like). The D_1 and the D_5 belong to the D_1 family and are positively coupled with adenylyl cyclase, meaning that their activation increases the intracellular levels of cAMP. The D_5 receptor has a much higher affinity to dopamine than the D_1 receptor, even though they have similar pharmacological properties with respect to selective agonists and antagonists. The D_2 family includes the subtypes D_2, D_3 and D_4 which are negatively coupled with adenylyl cyclase. (Tosini & Iuvone 2014, 55.)

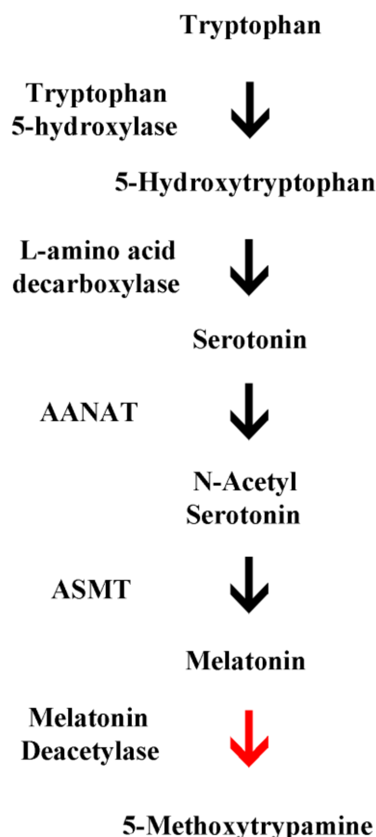
Because most of the cells in the retina express these receptors, dopamine has extensive effects in the regulation of many retinal functions. The D_1 receptors have been found in the inner and outer plexiform layers as well as within the cell bodies of horizontal cells, cones, amacrine cells and even ganglion cells. The D_5 are expressed by the retinal pigment epithelium (RPE). The D_2 receptors act as postsynaptic receptors and add autoreceptors that inhibit dopamine release. They are widely expressed in the retina by amacrine, bipolar, and ganglion cells. The D_4 receptors are expressed by the photoreceptors and can also be found in the interplexiform layer and the ganglion cells. There are no D_3 receptors in the retina. (Tosini & Iuvone 2014, 55–56.)

4.2 Melatonin

Melatonin (5-methoxy-N-acetyltryptamine) is a neurohormone that is involved in a variety of physiological processes. In the eye, melatonin modulates the activities of retinal neurons. In the outer retina, it regulates the activity of photoreceptors and also reduces the light responsiveness of cone-driven horizontal cells. (Huang, Wang, Weng, Sun & Yang 2012, 64–65.)

In addition, it potentiates inputs from glycinergic amacrine cells to ganglion cells in the inner retina. Melatonin is also a regulator in other processes including sleep, reproduction, and immune and vascular responses. It may also modulate the function of various types of neurons in the central nervous system by adjusting the activity of ligand- and voltage-gated ion channels. (Huang et al. 2012, 64–65.)

Melatonin is mostly synthesized in the pineal gland via the biosynthetic pathway (picture 2). The synthesis begins with the uptake of tryptophan, which is an amino acid, from the blood. Then the tryptophan in sequentially converted by a series of enzymatic reactions to 5-hydroxytryptophan, serotonin, N-acetylserotonin and lastly melatonin. The key step in the melatonin synthesis is catalyzed by arylakylamine N-acetyltransferase (AANAT). It converts serotonin to N-acetylserotonin and is also a subject to transcriptional and post-translational regulation. (Tosini & Iuvone 2014, 50.)



PICTURE 2. Melatonin synthesis (Tosini & Iuvone 2014, 51).

In the retina, the melatonin synthesis occurs in the night and it is controlled by the retinal circadian rhythm. The synthesis takes place primarily in the photoreceptor cells, but sometimes in other cells in the inner nuclear layer, or by the ganglion cells. (Tosini & Iuvone 2014, 50.) The melatonin synthesis in the retina occurs even if the pineal gland is removed. The retinal melatonin does not work as an endocrine hormone like the pineal melatonin does. The pineal melatonin is mostly cleaned out from the body via the liver, but the degradation of the retinal melatonin occurs in the retina. (Huang et al. 2012, 66.)

The role of melatonin in the eye is to produce dark-adaptive effects, such as modulating the sensitivity of photoreceptors and second-order neurons when there is little light. The role of melatonin differs a bit in different species, but in mice it increases the amplitudes of a- and b-waves and lowers the visual sensitivity. (Tosini & Iuvone 2014, 56.)

Melatonin applies its influence by binding to G-protein-coupled receptors (GPCRs) called melatonin receptor type 1 (MT_1) and type 2 (MT_2). They are both present in the vertebrate retina. The MT_1 receptors can also be expressed by dopaminergic neurons, which suggests that melatonin can directly modulate the activity of these cells and inhibit dopamine release. In mice the MT_1 receptors are located in the photoreceptors, inner retinal neurons and retinal ganglion cells (RGCs). Because the melatonin receptors are located in the same cells melatonin is synthesized, it is possible that it can regulate its own levels. The melatonin receptors are also expressed in the ciliary body, which suggests that it is involved in the regulation of the intraocular pressure (IOP). In mice there is also evidence of melatonin modulating the daily rhythm of the IOP. (Tosini & Iuvone 2014, 53–54; Huang et al. 2012, 66–68.)

4.3 Circadian rhythm

Every living thing has a daily rhythm, which originates within the organism. In humans suprachiasmatic nuclei (SCN) of the hypothalamus are controlling the 24-hour rhythms. The circadian clock controls many functions such as sleep, food intake, heart rate and hormone release. (Tosini et al. 2014, 1–2.)

The evidence of several accumulating studies has shown that the dysfunction on the circadian rhythm may affect the development of many pathologies for example jet lag or shift work that can contribute to a sleeping disorder. The retinal circadian clock was the first extra-SCN circadian system to be found in mammals. (Tosini et al. 2014, 1–2.)

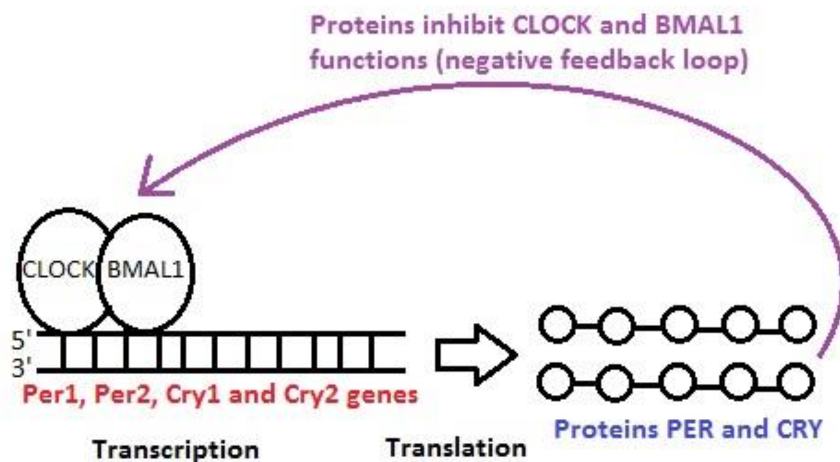
The vertebrate retina is a sensory organ as well as a 24-h biological clock (McMahon 2014, 69). It has an important role in the human circadian system because it synchronizes the central clock of the brain with external day (Tosini et al. 2014, 1). The circadian clock of the retina is endogenous and self-sustained and it controls many physiological and the metabolism rhythms of the retina. Thus the rhythms of the visual function are not just simple responses to the daily light-dark cycle. The circadian rhythms are generated by auto regulatory gene networks in eukaryotic cells. These networks spontaneously fabricate near-24-h cycles in gene and protein manufacturing with transcription-translation feedback loops. (McMahon 2014, 69–71, 87.)

The retinal circadian clock controls melatonin release, dopamine synthesis and circadian gene expression. It and its outputs influence also cell survival and growth processes in the eye for example the susceptibility of photoreceptors to degeneration from light damage. The endogenous circadian clock of vertebrate retina was first discovered from isolated Xenopus frog retinas. Tosini and colleagues later extended the research into mammalian retina and demonstrated the circadian rhythms of melatonin synthesis in isolated hamster, mouse and rat retinas. Other studies have shown that the endogenous retinal clock is widespread throughout the vertebrates. (McMahon 2014, 69–71, 87.)

The circadian rhythms of the retina generate from multiple sites. The photoreceptor layers and inner retinal layers are each capable of independently expressing circadian rhythms. The strength of circadian gene expression and the emphasis of rhythms expression vary across vertebrate retinas. In mammals clock activity is strong in the inner nuclear layer and in non-mammalian vertebrate the primary focus is in the photoreceptor layer. (McMahon 2014, 69–71, 87.)

4.3.1 The clock genes, melatonin and dopamine

The clock genes are identified as the *Period* genes Per1 and Per2, and the *Cryptochrome* genes Cry1 and Cry2. The transcription factors CLOCK and BMAL1 form a complex that transcripts the *Period* and the *Cryptochrome* genes (picture 3). This transcription results in PER and CRY proteins that form complex that suppresses their own transcription by stopping CLOCK and BMAL1 in the nucleus (picture 3). Thus the negative feedback is at the core of the molecular structure of the circadian clock. There are plenty of core circadian clock genes in the retina. In the brain the circadian clock can compensate for individual missing clock genes, but in the peripheral tissue clocks and in the retina, BMAL1, CLOCK, Per1 and Cry1 are each required for the producing of the rhythms. The circadian clock genes are expressed at some level in all the major retinal cell classes. (McMahon 2014, 69–71, 87.)



PICTURE 3. Core circadian clock genes and simplified core circadian molecular feedback loop.

Neurohormones melatonin and dopamine are involved in regulation of retinal physiology. They act as the drivers of retinal circadian rhythms. They have opposite roles in regulation and they are connected to each other. It is believed that critical factor of the regulation of circadian rhythms is the two-way feedback loop of melatonin and dopamine. The both neurohormones are synthesized and released under circadian control in many species but a clear evidence of the role of melatonin and dopamine systems in humans is still lacking. (Tosini & Iuvone 2014, 57–60.)

4.4 Laboratory mouse

For many years animal models have been used in scientific researches. At first the use of animals and their housing was not controlled very closely. These days there are many guidelines for taking care of laboratory animals, and people's awareness for animal welfare has been increased. The laboratory animal science concerns scientifical and technical information, and the skills and techniques involving laboratory animal care and use. Husbandry, nutrition, behaviour, health, production and management of laboratory animals is included into this. Because biotechnology and genetical engineering rodent models have been a growing field for many years, there is a steady demand on laboratory animals. (Hrapkiewich et al. 2013, 1.)

Harvard undergraduate Clarence Cook Little was the first to create an inbred strain of mice, which is named DBA, after the three recessive genes. In 1920 Leonell C. Strong made a series of crosses between the Bagg albino mice and the strain DBA. From the hybrids became several inbred lines called C3H, CBA, C, CHI and C12I. The C3H is widely used and it has been split into several different sublines. Most of the inbred strains were developed for use in cancer research. Biological material that can be designed by researchers was found necessary in scientific research. (Staats 1966.)

The mice are sorted into four general types that are common in use: inbred, hybrid, mutant-bearing and selected. When mice are used in breeding experiments, the parents, remote ancestors, all offspring and later descendants must be able to be identified. All laboratories must keep precise record of the individual mice and their traits. (Green E. 1966.)

Usually there are two objectives to gene modification. The first is to find out the development of pathological circumstances and the prevention of that. The second one is to figure out how much genes control the development process, and how do mutant genes affect this process. Researchers have found many loci from mice, which define the change of protein. (Green M., 1966.) In many retinal studies researchers use mice that have a melatonin production defiance in their genes, for example C57BL06J mice that are genetically incapable of melatonin synthesis (Doyle et al. 2002, 593).

Using animals in laboratory tests has been a method of natural sciences and psychology for a long time. Animal experimentation is justified with gained benefit and knowledge about life's phenomena. Understanding these phenomena has increased because of experimentation. Opponents against animal experimentation say that some animal testing is completely unnecessary, and some think that all animal testing should be forbidden. The opponents appeal that in animal experimentations the animal is only living out of humans' purpose, and it suffers unjustified pain and fear. The dialogue between the defenders and the opponents of animal experimentation is endless. Questions concerning animal testings vary from different fields. Laboratory animal ethics belong to both science ethics and prevention of cruelty to animals' ethics. The science ethics is a matter of the used principles concerning openness, fairness and responsibility of experimentation. The ethics of the prevention of cruelty to animals takes into consideration the good handling of animals and their potential rights. (Siitonen 2005, 13–17.)

People's attitude to animal testing has been studied a lot during the last 25 years. The attitudes vary depending on why the animal testing is done, which animals are used in the tests, and how much pain and suffering the testing produces. In general, the medical experiments are seen more positively than the psychology studies. Rats are seen as more positive test animals than the species commonly known as pets, such as dogs. Experimentations that cause less pain are more acceptable than pain inflicting experiments. (Pivetti 2005, 39–41.)

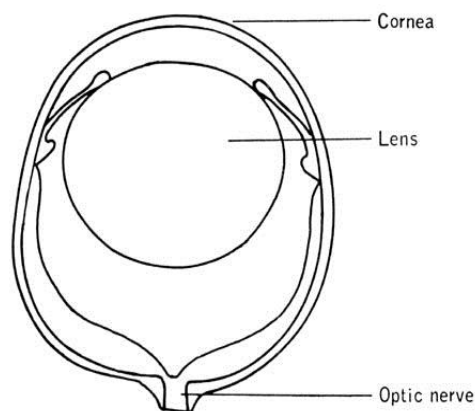
In 1985 Finland's law about prevention of cruelty to animals was included with a clause in relation to animal experimentation. In the law the scientific animal experimentation was determined as a procedure in which it is necessary to use animals when concerning human or animal healthcare, or when it is useful for scientific research or required for teaching purposes. (Hirsjärvi & Rydman 2005, 79–89.)

The test animals were also defined in the law to be vertebrate animals that are acquired or bred for animal testing. The law does not concern invertebrate animals. Previously it was possible to use pet animals that were given away, but the law changed this. The law about animal experimentation was finished in 2006. The essential change in relation to the previous clause is about animal experimentation permissions admission. Now there is a national board of test animals which is appointed by the Council of State. (Hirsjärvi & Rydman 2005, 79–89.)

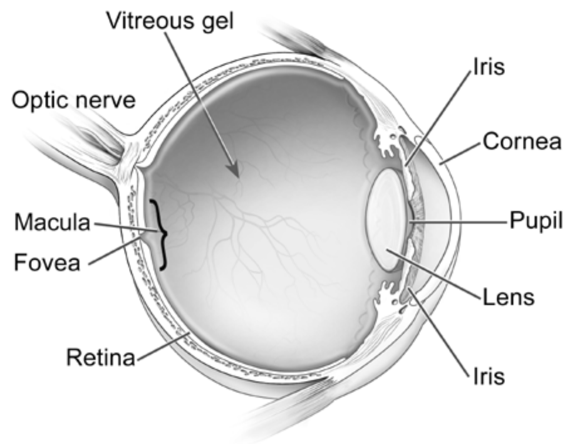
Research workers doing animal experimentation are required a university degree and remittance of a test animal course. In practice everyone under supervision of a qualified researcher is able to perform animal experiments. It is the responsibility of the supervisor to guide and supervise a non-qualified employee. Every animal experimentation requires a research plan which states the meaning of the experimentation, an approximate animal count, experimental procedures and an estimate of how much pain and agony the animals must endure during the experimentation. (Hirsjärvi & Rydman 2005, 79–89.)

4.4.1 The eye and the retina

The eye of the mouse is somewhat different from the human eye (picture 4, picture 5). Almost the whole of a mouse's eye is filled with the spherical lens. The function of the lens is to reflect a small and undetailed but bright image upon the retina. The cornea covers almost half of the surface of the eyeball, and the optic nerve is very small. (Fuller & Wimer 1966.) In the human eye the lens is much smaller and the cornea covers a smaller area. The different parts of the human eye are also better known than of the eye of the mouse. (Leppäluoto et al. 2012, 469-471.)



PICTURE 4. The eye of the mouse (Fuller & Wimer 1966).

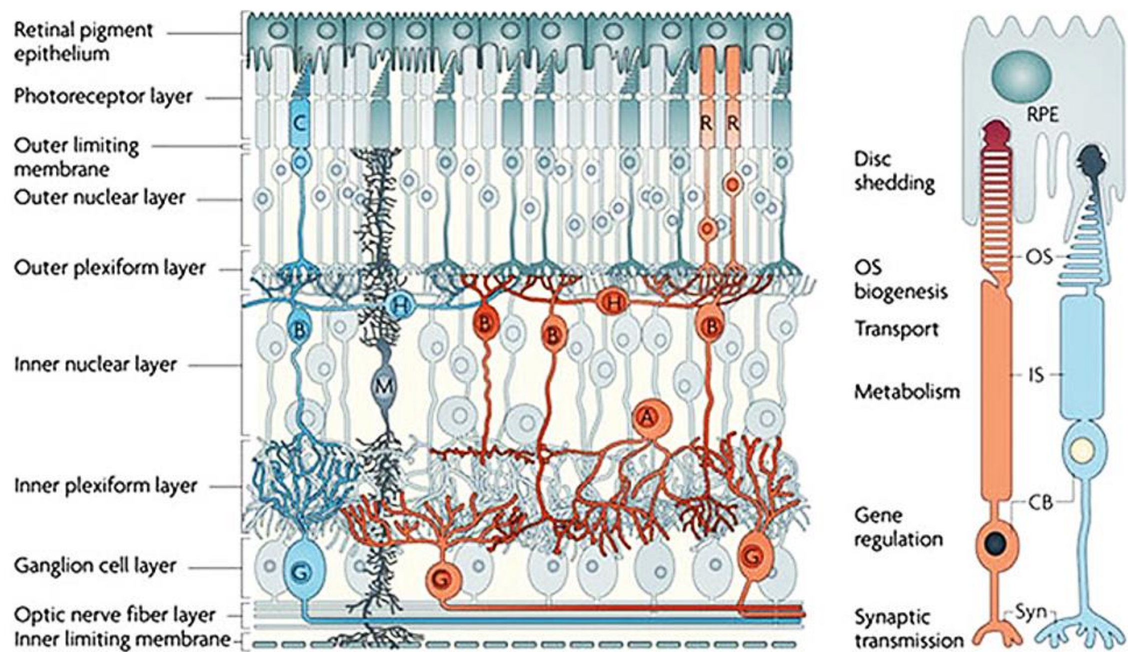


PICTURE 5. The human eye (The National Eye Institute).

At birth the mouse's retina consists of a ganglion cell layer, an inner fibre layer and a thick layer of undifferentiated cells. After 7 to 10 days the rods have developed, and the structure of an adult retina is established by the 12th day. The eyes typically open by the 14th day. Although the eyes are open, they are not fully mature. It takes three weeks to the hyaloid artery to stop supplying the embryotic lens, and it also takes four weeks for the rods to be organized. (Fuller & Wimer 1966.)

The retina is basically a piece of the brain that is located in the back of the eye. The role of the retina in the eye is to transform light energy into electric signals which the optic nerve then forwards to the brain. The retina has approximately 60 different types of neurons belonging to five classes. The different neurons extract different information, for example position in space, contrast, intensity, chromatic content and movement. The information is then conveyed to the visual centre of the brain by the optic nerve. The information also passes to the central clock located in the brain so that it gets information about whether it is night or day. (Strettoi & Parisi 2014, 3.)

The mammalian retina is about 200 µm thick. That is because the light has to pass through it completely and with minimal distortions in order to get to the photosensitive elements on the other side of it. In the retina, neurons are placed in functional units that are repeated in order to cover the surface of the retina. It is called the retinal pathway (picture 6). The neurons are placed in a way that the first ones the light needs to get to are facing the back of the eye. (Strettoi & Parisi 2014, 4.)



PICTURE 6. The retinal pathway (Strettoi & Parisi 2014, 5).

The five classes of the neurons are photoreceptors (rods and cones), horizontal cells (two types), bipolar cells, amacrine cells (30 types) and ganglion cells (about 20 types). The different classes have different functions according to their arrangement in the retina. When the light first absorbs into the eye, it activates the photoreceptor cells. Then it goes through the bipolar cells and the horizontal cells and then through the amacrine cells. After that it reaches the ganglion cells that form the optic nerve with their axons. (Strettoi & Parisi 2014, 4–5.)

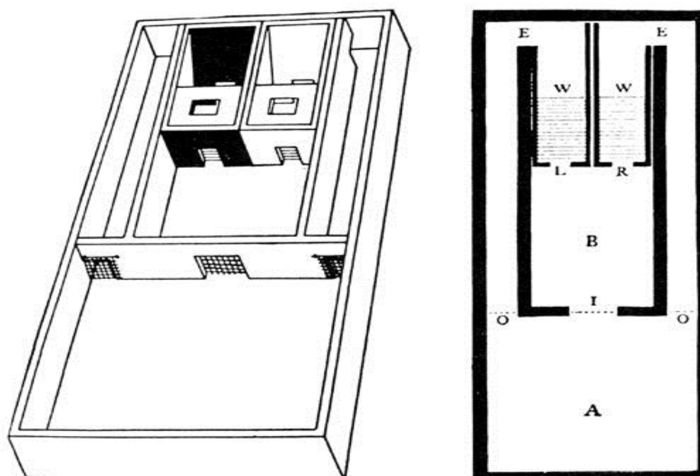
A retinal cell type is defined by a set of specific features, for example shape, size, density over the retina surface, molecular signature and the level of stratification in the inner plexiform layer. The cells have also been divided into horizontal and vertical cells. The photoreceptors, and the bipolar and the ganglion cells are traditionally seen as vertical and as a part of the retinofugal pathway. The horizontal and amacrine cells are seen as horizontal cells, and are considered as modulatory interneurons, which means that they act as linking neurons between other cells. (Strettoi & Parisi 2014, 5–6.)

Intrinsically photosensitive retinal ganglion cells (ipRGCs) were quite recently discovered. They have direct communication with the brain and help to synchronize circadian rhythms with the solar day. They are also part of the papillary light reflex and other behavioural and physiological responses to environmental illumination. (Berson 2003, 314.)

4.4.2 Mouse vision testing and the histology preparations of the eye

Because the visual system is responsible for many tasks, there is not only one behavioral test that is able to perform as a comprehensive test of visual function. Requirements for the animal to perform well on behavioral tests of visual function, the basic visual apparatus, and the motor apparatus should be intact and function well. There are many options for a behavioral test of visual function, for example maze-based tests and measuring light-induced changes in the phase of the circadian rhythm. (Pinto & Enroth-Cugell 2000, 533.)

The principle of maze-based tests is to train the mouse to move toward a visual stimulus. Then the visual stimulus is manipulated, and then it is observed whether the animal still moves towards the stimulus. In training, the animal is motivated either by food or by forcing the animal to swim to a hidden escape platform, near the “correct” stimulus. These tests give information about the visual guided behavior of many inbred strains, and the visual acuity of individual mice. (Pinto & Enroth-Cugell 2000, 533.) The Yerkes’ discrimination box (picture 7) is one example of a maze-based test for visual discrimination in animals. All possible extra hints to the mouse must be minimized and controlled precisely. The mouse is placed on a “nest box” where it leaves and enters the choice area. In the choice area mouse must choose its entry of the left or right chamber. The experimenter provides visual clues for the mouse to decide the right way. (Fuller & Wimer 1966.)



PICTURE 7. Yerkes' discrimination box: A) nest box, B) choice area (Fuller & Wimer 1966).

Measuring the light-induced changes in the phase of the circadian rhythm requires a clear circadian pattern of activity. This can be done, for example, with a computer-monitored activity wheel. When administering the phase-shifting stimuli, the animal must be kept in darkness while handling. About two weeks is needed to establish a constant pattern of activity, and one week to evaluate each phase shift. This kind of test is suitable for comparing different strains, but usually not for screening individual mice in a mutagenesis program. (Pinto & Enroth-Cugell 2000, 534.)

There are many techniques in sampling the eye and the associated intraorbital glands. The whole head can be fixated with the eyes still detached to it. Sectioning can be made across the head before decalcification. The eyes can also be enucleated before decalcification. During necropsy it is technically easier to section the eyes while they are attached to the head. Problems might arise when trying to get a complete section through the globe and the optic nerve. The eye may not fixate properly if a standard formalin fixative is used. Alternatively the head can be bisected longitudinally after decalcification. The eyes should be sectioned in vertical longitudinal plane through the optic nerve. Because the distribution of the cone photoreceptor cells varies between dorsal and ventral parts it is necessary to mark nasal, dorsal or ventral points so that both sides of the retina are examined. (Scudamore 2014, 195–199.)

The eye is very prone to artefacts during the handling, dissection or fixation and processing. In some cases the retina might be thinner, or have a loss or disorganization of the cellular layers, than it usually would be. This suggests atrophy which is common in certain strains of mice, particularly those based on C3H and FVB stock. In albino strains the light-induced retinal atrophy is common. The retinal atrophy means a variable loss of the photoreceptor layers, the outer nuclear and outer plexiform layers. (Scudamore 2014, 195–199.)

4.5 Hormone measuring methods

Measuring hormones is somewhat of a challenge because of their extremely low concentrations, typically in pico- or nanomolars. They also appear in an environment with closely related and potentially interfering compounds thus demanding great sensitivity

and specificity from the measuring method. The most commonly used methods are immunoassays, particularly for most of the clinically important hormones. Also gas chromatography mass spectrometry (GCMS) and high performance liquid chromatography (HPLC) are frequently used in measuring especially small hormones. Biological methods are used in research work and as a reference method for standardising some polypeptidhormones. (Stenman & Hämäläinen 2010; Wallace 2011, 45.)

Radioimmunoassays (RIA) are nowadays mainly replaced by methods using non-radioactive labels but they are still frequently used in research work. Small hormone measuring methods are simplified these days by removing the liquid and solid phase elutings and the chromatographic cleansing point. That way for example the immunoanalysis can be done straight away from a serum sample. Analysing polypeptides has become quicker and more sensitive, and its clinical usability has been improved. With some hormones you may have to change the analysing method due to different situations to get the best results. (Stenman & Hämäläinen 2010.)

4.6 Chromatography

In a chromatographic analysis the sample is divided into components that are identified, and their concentrations are measured. Chromatography is based on balance. The sample is separated either in a chromatography column or a sheet where there are two phases, the stationary phase and the mobile phase, which are unable to dissolve in each other. The two phases are in interaction with each other. Compounds move in chromatography when they are in the mobile phase. (Jaarinen & Niiranen 2005, 139–140.)

If a compound adheres weakly into the stationary phase, it moves quickly, and a compound that is strongly adhere into the stationary phase moves slowly with the mobile phase. The absorption and the partition features of the compounds effect their movement in the phase. By choosing the right chromatography you can separate two very similar compounds from each other. (Jaarinen & Niiranen 2005, 139–140.)

There are many different types of chromatography to choose from. The mobile phase can either be a liquid or a gas. The stationary phase is either a liquid or a solid material. The idea is that the stationary phase does not move with the mobile phase. If the stationary

phase is liquid, it can be made very viscose or bound chemically into a carrier-substance so that it becomes a motionless layer. Chromatography types are accordingly divided by the mobile phase into either gas- or liquid chromatography. (Jaarinen & Niiranen 2005, 139–140.)

4.6.1 Gas chromatography mass spectrometry

Gas chromatography separates volatile compounds from each other. In the gas chromatography the mobile phase is gas, and the stationary phase is a high boiling liquid. In the gas chromatography the sample is drawn up into a syringe with a needle. The sample is then injected through a hot injector port. The temperature of the injector is higher than the boiling point of the compounds so that the compounds evaporate into the gas phase inside the injector. The mobile phase gas can be for example helium. The mobile phase gas flows through the injector and pushes the compounds of the sample onto the column. In the column the compounds are separated and partitioned between the mobile phase and the stationary phase. (Wake Forest College Department of Chemistry 2015.) Before the compounds exit the column, they pass through a detector that records the different compounds. (Jaarinen & Niiranen 2005, 183).

The gas chromatography combined with the mass spectrometry is a quick and reliable way to separate and identify compounds. This technique is suitable for compounds that evaporate enough and tolerate the temperatures of the gas chromatography. In this application the gas chromatography is connected straight to the mass spectrometry. The compounds separated by the gas chromatography travel from the column to the ionizator of the mass spectrometry that ionizes the incoming gas. From the ionized gas, mass spectres are measured using a computer. (Jaarinen & Niiranen 2005, 183.)

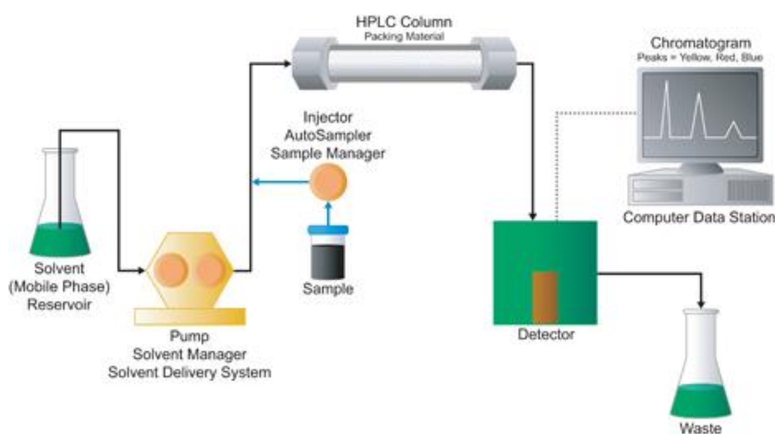
4.6.2 High performance liquid chromatography

The high performance liquid chromatography (HPLC), also known previously as a high pressure liquid chromatography, is a very common chromatography type. It can be used to analyse inorganic and organic compounds. The HPLC makes it possible to analyse several compounds at the same time. The liquid chromatography is an important method

in analysing large organic molecules, for example proteins and drugs. The HLPC can be applied to almost every situation on the condition that the sample dissolves in some solvent. The sample is not usually changed during detection, so it can be salvaged for possible further analyses. (Jaarinen & Niiranen 2005, 153–155.)

The liquid chromatography is built from an injector, a pump, a column, a detector, a capillary that links the parts together, and a printing device (picture 8). The sample volume is about 10 µl and it is injected through the injector to a high pressured mobile phase that moves in narrow capillaries. The sample proceeds with the eluent into the column, which is filled with the stationary phase. The stationary phase incorporates small particles, and the separation of the compounds is more effective when the particles are smaller because that means the active surface is larger. (Jaarinen & Niiranen 2005, 153–155.).

The eluent must proceed through the column in an even flow which is why the pump must pump the eluent against the high pressure without pulsating. The compounds of the sample are separated when it proceeds through the column. The compounds go to the detector, and the last compounds have a balance similar to the stationary phase. The function of the detector is to measure a signal from the compound and check against the measured time, and this forms the chromatogram. (Jaarinen & Niiranen 2005.)



PICTURE 8. HPLC system (Waters Corporation 2015).

Even though the HPLC is a relatively safe chromatography method, toxic solvents can cause pulmonary irritation from the stationary phase. A good ventilation in the working area is an essential safety matter because small particles of less than 5 µm have access to lungs. Amorphous Silica is commonly used in the stationary phase, and it is as far as is

known safe to use, but it is not recommended to be inhaled. Any stage that involves possible escape of the stationary phase dust in the air, must be done in a fume cupboard. (Meyer 2010, 8–11.)

The liquid chromatography is safer to use than the gas chromatography. The compressed liquid does not produce imminent danger like a compressed gas does. The liquid from the joint hoses can spill, which causes greater danger to the worker. The compressed liquid can expose human skin to serious injuries. (Meyer 2010, 8–11.)

4.6.3 Reverse-phase chromatography and ion-pair chromatography

The reverse-phase chromatography is a HPLC mode that has a polar mobile phase and a non-polar stationary phase. In the normal-phase HPLC the stationary phase is polar, usually silica, and the mobile phase is non-polar (hexane). Because the reverse-phase HPLC has such a broad applicability, it is used for approximately 75% of all HPLC methods. (Waters Corporation 2015.)

Even though water has often been described as the strongest elution medium for chromatography, this is only true for the absorption processes. The problem with using water is that it can interact strongly with the active centers in silica and alumina. This causes the absorption of the sample molecules to come highly restricted, and they quickly elute as a result. (Meyer, 2010, 173.)

In the reverse-phase chromatography exactly the opposite applies, as water cannot wet the non-polar and hydrophobic alkyl groups and does not interact with them at all. This makes water the weakest mobile phase in the reverse-phase system. The sample elution takes time, and the more water used in the eluent, the longer the retention time. (Meyer, 2010, 173.)

Most protocols use water mixed with a miscible polar organic solvent, for example acetonitrile or methanol, as a mobile phase. A water/solvent-mixture ensures the right interaction of the analyte with the non-polar hydrophobic particle surface. (Waters Corporation 2015.) The most popular type of the reverse-phase HPLC packing is the C18-bonded silica, also known as octadecylsilane (ODS), which is an alkane with 18 carbon atoms. (Meyer 2010, 173; Waters Corporation 2015.)

The ion-pair chromatography is used when a sample contains ionic components that are too polar for the reverse-phase chromatography (Dolan 2008). The ion-pair chromatography is an excellent choice when it is needed to separate mixtures of acids, bases and neutral products under certain circumstances (Meyer 2010, 217). For example when the pH of the sample is low, it might be polar enough to go unretained by the reverse-phase mode (Dolan 2008). The reverse-phase chromatography can only separate weak acids or bases. The ion-pair chromatography extends the reverse-phase mode by adding an organic ionic substance to the mobile phase. (Meyer 2010, 217.)

The organic ionic substance is called the ion-pair reagent. The reagent is similar to soap and this chromatography mode was called the “soap chromatography” in the beginning. The ion-pair reagent consists of an ionic end and a nonpolar tail, for example such as in hexane sulphonic acid. The reagent is added to the mobile phase, and it balances out with the column. The nonpolar stationary phase, for example C18, strongly holds the nonpolar end of the ion-pair reagent, and the charged functional group sticks out into the mobile phase. This enables the ionic species of the opposite charge of the sample to get retained to the stationary ion-pair reagent, and enables the chromatographic retention. (Dolan 2008.)

In the ion-pair chromatography you can influence the selectivity by choosing a certain ion-pair reagent. Also if analytes are both either acid or base, the ion-pair mode is a good way to separate them. (Meyer 2010, 218.)

4.7 Immunoassays: immunometric and competitive immunoassays

The main principle of the immunoassay is to generate a signal from a small amount of analyte by using reagents. The immunoassay is founded on a natural antibody-antigen binding that forms for example in a human body when something outside the body (antigen) comes to contact with the defence mechanics of the body (antibody). The antibody or the antigen works as kind of a magnet to which their selective target, the antigen or the antibody, is attached to. Because of such a small amount of analyte, it is important to use another reagent to generate the signal from the attached target. The level of the signal tells us how much target analyte there is in the sample. The immunoassays are used in a very wide range. For example the antibodies can bind natural and man-made chemicals, biomolecules, cells and viruses, and have an exceptional specificity. The binding is very strong between the antibody and its target. (Wild 2013, 7.)

Antibodies used in immunoassays can be either monoclonal or polyclonal. The monoclonal antibodies are produced from only a one B-cell line while the polyclonal antibodies include different B-cell line antibodies. The polyclonal antibodies bind into different epitopes of the same antigen. To produce antibodies, one must use immunogen to elicit an immune response in an animal. (Liddell 2013, 245–265.) A general recommendation is that the species being immunised are phylogenically distinct from the immunogen carrier, so that the immunogen has the most immunogenic effect (Copley, Law & Jenner 2002, 48–50; Liddell 2013). The monoclonal antibodies are usually used for large molecules in two-site assays and the polyclonal antibodies are more suitable in small molecule immunoassays (Copley et al. 2002, 48–50).

The choice of animal for the production of the antibodies is constricted by considerations of what is available and how much antibody is required (Liddell 2013, 245–265). Some favour, for example, rabbits that are the most common species used on research scale, because they have low maintenance costs, and produce appropriate amounts of antibodies (Copley et al. 2002, 48–50).

For monoclonal antibodies, practical choices are rodents, particularly mice (Copley et al. 2002, 48–50). It is preferable to use several animals to produce the desired amount of antibodies, because there can be significant differences between the antibodies. For obtaining the optimal amount of antibodies, it is recommended to use at least four animals

with each immunogen. With one immunogen the minimum number of animals should be at least six. (Copley et al. 2002, 48–50; Liddell 2013, 246). The antibodies have differences even if they are genetically identical and when using outbred animal strains the differences are intensified (Copley et al. 2002, 48–50).

The signal reagent is important in discovering the amount of analyte in the sample. There are many available reagents for signalling in the immunoassays. Radioactive isotopes were the first used labels, and these days they have been mainly replaced by very popular enzyme labels. The idea of the label is to conjugate it onto the antibody about to be labelled. The radioactive isotopes produce a radioactive signal that is strong and easily detected. They also expose the workers to radiation, and that is why they are mostly substituted with safer label reagents. (Stenman & Hämäläinen 2010.)

Enzymes are proteins that bind to specific targets and also catalyse specific reactions. An enzyme needs a substrate to start the reaction, and with the appropriate substrate enzyme labels can generate colour, or create fluorescent or luminescent end products. These end products can be measured by optical and electronical equipment. A molecule of an enzyme can use many molecules of substrate if the signal generation system is sensitive enough. This is called the enzyme-linked immunosorbent assay (ELISA). (Wild 2013, 8.)

The immunoassays are divided into two main types: the non-competitive and the competitive immunoassay. In the competitive immunoassay the analyte is a non-labelled antigen and the assay measures the ability of the analyte to compete with the labelled antigen. In the one-step competitive assay the non-labelled and labelled antigens compete in binding onto a limited amount of antibodies. The non-competitive immunoassay binds the analyte antigen between two antibody reagents. Depending on what stages are featured in the assay it is also named heterogeneous or homogeneous. (Wild 2013, 8.)

In the heterogeneous assays the binded antibody-antigen complexes are separated from the free, labelled antigens. Homogeneous assays do not need this kind of separation because the labelled component only generates a signal when binded onto the analyte. (Wild 2013, 8.)

The immunometric assay is the simplest type of an immunoassay. It can also be called a non-competitive immunoassay. Its core idea is to immobilize an antibody onto a surface, for example plastic, and this antibody captures the test analyte from the sample. Then a different labelled antibody is binded into the analyte molecule to generate a signal. For example a radioactive isotope can be used in labelling an antibody. If the assay is heterogeneous, the excess unbound labelled antibody is washed away after all the antibodies and antigens have incubated for a while. In the homogeneous assay this kind of separation does not happen. The signal tells the concentration of the analyte in the sample. (Wild 2013, 7.)

The labelled component is sometimes called the tracer, and the efficient removal of an unbound labelled component is critical in a heterogeneous immunometric assay. This stage is called separation. The antibody is binded into a material that is called the solid phase. The term “sandwich assay” is often used to describe immunometric assays because the antibodies form a kind of a sandwich around the analyte. The sandwich assay is used when you have a big enough antigen to bind two antibodies. (Stenman & Hämäläinen 2010; Wild 2013, 7.)

The sensitivity of the sandwich method grows, and the reaction gets faster when the antibody concentration is enlarged. Compared to RIA this method is faster and more sensitive and the best sensitivity is achieved by using fluorescing and luminating labels. Using a great amount of antibodies is not a problem because the monoclonal antibodies are easy to produce in large amounts. This advantage of the method is its wide measure range especially when the hormone concentration in the sample varies. (Stenman & Hämäläinen 2010.)

The competitive immunoassays are based on binding inhibition, and that is why they are called competitive methods. In the competitive immunoassay only one antibody is used, and the amount of it is limited. Other reagent, the tracer, is made from the target analyte, for example a hormone, and labelled with proper signal generation material for example a radioisotope or an enzyme. The tracer and the target analyte compete in binding onto the antibodies. The amount of the antibody and the labelled analyte is critical in the competitive immunoassay. They are also known as a reagent limited method. (Stenman & Hämäläinen 2010; Wild 2013, 8.)

The signal of the label indicates the amount of the analyte in the sample. If the signal is strong it means there is not a lot of analyte in the sample and vice versa. The result is then compared to a standard diagram. Depending on which label is used, the name of the immunoassay varies. The label can be for example an enzyme, a fluorescent or a luminescence compound. The radioimmunoassay (RIA) was the first one but other applications have come up after it, for example the fluoroimmunoassay (FIA) and the enzymeimmunoassay (EIA). (Darwish 2006, 217–235; Stenman & Hämäläinen 2010.)

The sensitivity and specificity of the inhibition methods are mostly reliant on the affinity of the antibody. The most sensitive assays use polyclonal antibodies that have a greater affinity than the monoclonal antibodies from mice. The sensitivity of an inhibition method can be improved by reducing the antibody concentration and extending the reaction time. The most sensitive radioimmunoassays are slow, and their reaction time is usually over 12 hours. The size of the molecules also matters. Small molecule hormones, such as steroids, react faster than bigger ones. Commercial systems usually aim for a compromise that makes the method faster and simpler, but at the expense of sensitivity. (Stenman & Hämäläinen 2010.)

5 PROCESS OF THE STUDY

5.1 Choosing the subject

We got the subject for this study in September 2014. We formed a draft of the subject and started to collect material for the study. The topic remained the same from the start. The draft was presented to the teachers in November 2014, and the edited version of the plan was submitted to the working life representative in December 2014. We got the final permission in December 2014.

The idea for this study came from our client contact. They were interested in getting to know more about the possible assay methods used in measuring melatonin and dopamine concentrations from the mouse retina. They provided us with some previous research articles that concerned this subject.

5.2 Data acquisition

Material used in this thesis was collected by using different search engines and databases. The data consists of source material and material used in the literature review. The data acquisition for the study was done during the spring 2015. After that we went through all the data and chose which articles where essential for our study. The premise of our research frame was to outline the research topic to involve measuring methods of melatonin and dopamine in the mouse retina.

We used following concept groups as search terms: “dopamine AND melatonin AND retina”; “dopamine AND melatonin AND retina AND circadian rhythm”; “dopamine AND melatonin AND mouse AND retina”. The criteria for the data acquisition was that the research articles must be in article form, and the articles should occur in well-known, reliable and peer-reviewed publications. We also defined the search to articles issued in 2005 – 2015, and the articles had to be written in English.

We carried out the research with chosen search terms using Pubmed, EBSCO, ScienceDirect, Springer Link, Wiley Online and Web of Science databases. If an article had an appropriate headline and it was available in a Full text –format, it was accepted to the next stage.

First we went through the search results based on their headlines and chose the ones that seemed to be suitable for the purpose of our study. We went through several articles. The ScienceDirect database gave us the most search results, 979 results on one search term group. The results of the different database searches are marked on the worksheet (table 1). Most of the articles in the databases were the same ones, so there was a lot of overlapping which can be seen in the search results. Then we read all abstracts of the chosen articles and picked the ones that were still closest to our study. At that point we were left with 35 articles (table 1).

TABLE 1. The original search results

<table>
<tr><th>Search terms</th><th>Database</th><th>Search results</th><th>Chosen by headline</th><th colspan="2">Chosen by abstract</th><th>Chosen by research methods</th></tr>
<tr><td rowspan="6">dopamine/melatonin/retina</td><td>Pubmed</td><td>41</td><td>13</td><td>11</td><td rowspan="18">In total 35 articles</td><td rowspan="18">3 articles (+ 1 published before year 2005)</td></tr>
<tr><td>EBSCO</td><td>28</td><td>7</td><td>7</td></tr>
<tr><td>Science Direct</td><td>979</td><td>33</td><td>0</td></tr>
<tr><td>Springer Link</td><td>304</td><td>34</td><td>34</td></tr>
<tr><td>Wiley Online</td><td>453</td><td>20</td><td>17</td></tr>
<tr><td>Web of Science</td><td>83</td><td>20</td><td>19</td></tr>
<tr><td rowspan="6">dopamine/melatonin/retina/circadian rhythm</td><td>Pubmed</td><td>25</td><td>10</td><td>10</td></tr>
<tr><td>EBSCO</td><td>14</td><td>7</td><td>7</td></tr>
<tr><td>Science Direct</td><td>580</td><td>14</td><td>2</td></tr>
<tr><td>Springer Link</td><td>212</td><td>18</td><td>18</td></tr>
<tr><td>Wiley Online</td><td>318</td><td>19</td><td>17</td></tr>
<tr><td>Web of Science</td><td>51</td><td>15</td><td>15</td></tr>
<tr><td rowspan="6">dopamine/melatonin/mouse/retina</td><td>Pubmed</td><td>5</td><td>3</td><td>3</td></tr>
<tr><td>EBSCO</td><td>14</td><td>7</td><td>7</td></tr>
<tr><td>Science Direct</td><td>690</td><td>21</td><td>8</td></tr>
<tr><td>Springer Link</td><td>231</td><td>13</td><td>13</td></tr>
<tr><td>Wiley Online</td><td>391</td><td>21</td><td>21</td></tr>
<tr><td>Web of Science</td><td>26</td><td>10</td><td>10</td></tr>
</table>

5.3 The intake of research articles

The chosen 35 articles were then read through. The articles had a lot of information concerning our study, but very few of them had a topic that fitted with our literary review scheme. Of the articles provided by the client contact we chose one for our study, even though it was not written in the years we based our search on. This article was the basis of our descriptive literature review.

After reading all the articles we had three research articles and the extra one (table 1). Although two of the articles used different animals than we had in mind in their study, we chose to accept them because the research aspects were similar with each other. Even though we included melatonin in the research, only one article mentioned the melatonin assay. Some of the articles we chose based on the abstract did measure melatonin, but the research aspect did not fit our study.

We made additional searches from the chosen databases to direct our search on the melatonin assay. The search terms were “melatonin AND assay AND method”; “melatonin AND retina AND assay”. This did not bring any new information for our study.

6 REVIEW OF RESEARCH ARTICLES

From the selected articles (appendix 1) we gathered information concerning what kind of animals they used, how they processed the eyes and prepared the samples, and what assays did they use. All the articles we chose had a different objective for their study. Doyle et al. (2002) became our main article because they set out to examine the role of dopamine and melatonin in the circadian rhythm in mice's eyes (Doyle et al. 2002, 593). Pozdeyev et al. (2008) concentrated on dopamine and its effect on the rhythms of protein phosphorylation in photoreceptor cells (Pozdeyev et al. 2008, 2691). Other two research articles used rats in their studies, but they focused on different aspects. Chanut et al. (2006) evaluated dopamine and its metabolites, serotonin and amino acid level variations from the retina of the epileptic NER rat compared to normal rats (Chanut et al. 2006, 56). Sakamoto et al. (2005) set out to investigate the control of melanopsin expression in the rat retina (Sakamoto et al. 2005, 3129).

6.1 Animal handling and retina sampling

Doyle et al. (2002) used C3H and C57 mice. The C3H mice produce melatonin rhythmically when measured from the pineal gland. The C57 mice, on the other hand, have a mutation that affects the gene encoding AA-NAT (the enzyme aralkylamine N-acetyltranferase, regulator of circadian rhythm) that results in the lack of measurable melatonin. The C3H mice were raised in the research laboratory and the C57 mice were bought from Charles Rover Laboratories Inc., in Wilmington, MA. (Doyle et al. 2002, 594.)

Doyle et al. (2002) housed the mice for the dopamine study in individual cages when they were 6 to 8 weeks old. The cages were light tight and had a constant level of temperature and humidity. The light cycle was 12 hours of light and 12 hours of dark (LD12/12), and a group of rmice was killed every three hours beginning one hour after the lights went on. (Doyle et al. 2002, 594.)

Pozdeyev et al. (2009) used three different strains of mice: C57B1/6J mice that were a wild type, Drd4$^{-/-}$ mice that lack dopamine D4 receptors (and have a C57B1/6J background) and C3H/f$^{+/+}$ mice that are unaffected by genetical retinal degeneration. The animals were kept in a LD12/12 and were euthanized by cervical dislocation. (Pozdeyev et al. 2009, 2692–2693.)

Chanut et al. (2006) used Epileptic rats (originated from a group of CRJ:Wistar rats) and Wistar Rats. The rats lived in 22-23 degrees Celsius and had a free access to water and food. The rats were kept under a strict LD12/12. The rats were 2, 6 or 12 months old and there were both female and male rats. The rats were killed either at 11:00 a.m. or 11:00 p.m. (after four hours of light being either on or off). At least 12 rats were used per group (same sex, age and type) at one time. The rats were killed by decapitation. (Chanut et al. 2006, 62.) Sakamoto et al. (2005) used 8 to 10-week-old male rats from the Fisher strain that were bought from Charles River Laboratories. The rats were kept in LD12/12 and killed in groups with an overdose of halothane four times a day. (Sakamoto et al. 2005, 3129–3130.)

In all studies the eyes of the animals were dissected rapidly after the sacrifice. In all of the studies the dissection was done in dim, almost infrared light when the animals were killed during the dark cycle. If the sacrifice happened during the light, cycle, the dissecting was done in a normal light. (Chanut et al. 2006, 62; Doyle et al. 2002, 594; Pozdeyev et al. 2009, 2692–2693; Sakamoto et al. 2005, 3129–3130.)

Chanut et al. (2006) and Sakamoto et al. (2005) froze the retinas immediately with dry ice and stored them at -80˚C (Chanut et al. 2006, 62; Sakamoto et al. 2005, 3129–3130). In Doyle's et al. (2002) study the retinas were first sonicated into 100 µl of ice-cold 0.1 M perchloric acid containing 20% acetonitrile and after that frozen with dry ice and stored at -70˚C (Doyle et al 2002, 594). Pozdeyev et al. (2009) do not mention this phase in their article (Pozdeyev et al. 2009, 2692–2693).

6.2 Sample preparations

Before analysing the compounds in the retina samples, they must be blended thoroughly to achieve a homogenized sample. The retinas are usually homogenized in an 80 µl solution of 0.2 M perchloric acid (HC_lO_4) which contains 0.1% sodium metabisulfite ($Na_2S_2O_5$) and 0.1% EDTA (Chanut et al. 2006, 62). Volume of the perchloric acid can change to 100 µl containing only 0.01% sodium metabisulfite and 25 ng/ml internal standard 3,4-dihydroxybenzylamine hydrobromide, without the EDTA. (Pozdeyev et al. 2009, 2694–2695). Even a smaller amount of perchloric acid has been used for homogenization. Sakamoto et al. (2005) used 0.2 ml of 0.2% perchloric acid that contained 0.1% of sodium metabisulfate and 100 ng/ml of internal standard 3,4-dihydroxybenzylamine hydrobromide. (Sakamoto et al. 2005, 3130.) The retinas can also be sonicated to achieve a homogenized form (Chanut et al. 2006, 62).

After the homogenization the retina samples are centrifuged usually at 15,000 g for 5 – 10 minutes (Chanut et al. 2006, 62; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130). Lower 11,000 g for 10 minutes has also been used in centrifuging homogenized retinas (Doyle et al. 2002, 594). The centrifuging can also be performed at 4 °C and the supernatants stored at -80 °C before performing the assay (Chanut et al. 2006, 62). They can be also filtered through nylon filters before injecting them into the assay system (Doyle et al. 2002, 594). Another option is not to use any filtering before the assay (Chanut et al 2006, 62; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130).

6.3 Measuring dopamine

Among other things high performance liquid chromatography (HPLC) system can be used to measure concentrations of dopamine, 3,4.dihydroxyphenylacetic acid (DOPAC) and homovanillac acid (HVA) from sample solutions (Chanut et al. 2006, 62). An application of HPLC is to use ion-pair reverse-phase in determining dopamine and DOPAC levels (Doyle et al. 2002, 594; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130).

Sample volume of supernatants varies from 20 to 80 µl (Doyle et al. 2002, 594; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130). Commonly used reverse-phase

chromatography device is Ultra-sphere ODS C18 with 5µm column (Chanut et al 2006, 62; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130). Doyle et al. (2002) used Microsorb^{TM} Short-One C18 reverse-phase column and automatic injector Jasco AS-950-10 during injections (Doyle et al. 2002, 594).

For the mobile phase of HPLC there are a lot various forms. For example water/methanol (92:8 v/v) containing 0.1 M KH_2PO_4, 0.01 mM EDTA and 5 mM heptane sulphonic acid, pH 3.85 is one possibility (Chanut et al. 2006, 62). Another variation is to use a compound of 75 mM phosphate buffer, pH 3, containing 25 mM EDTA, 1.7 mM 1-octanesulfonic acid, 0.01% triethylamine and 7-7.5% acetonitrile (Doyle et al. 2002, 594). Modification from this compound is 0.1 M sodium phosphate, 0.1 nM EDTA, 0.35 sodium octyl sulphate, 5.5% acetonitrile, pH 2.7 (Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130). Flow rate applied to HPLC system can vary from 0.9 ml – 1.0 ml/min (Chanut et al. 2006, 62; Doyle et al. 2002, 594).

For detecting different compounds from the samples an electrochemical detector is usually operated with HPLC. Amperometric electrochemical detection at working potential of 0.78 V which is relative to Ag/AgCl electrode is one choice of electrochemical detector. (Chanut et al 2006, 62; Sakamoto et al. 2005, 3130.) Doyle et al. (2002) performed the detection using ESA Coulochem II electrochemical detector. The detector was fitted with a 5020 guard cell set at +400mV, and a 5011 glassy carbon analytical cell with a screening electrode set at -75 mV and the measuring electrode set at + 250 mV. (Doyle et al. 2002, 594.)

External standards are needed when quantifying dopamine and DOPAC concentrations in HPLC (Chanut et al 2006, 62; Doyle et al. 2002, 594; Pozdeyev et al. 2009, 2694–2695; Sakamoto et al. 2005, 3130). To be ensured of the precision of the assay, standard mix containing known concentrations of dopamine, DOPAC and HVA should be used after every eight samples. The peaks of the substance were identified and concentrations calculated by comparing retention times and peak areas with those of known external standards (Doyle et al 2002, 594.)

6.4 Measuring melatonin and melatonin injections

Doyle et al. (2002) were only ones to measure melatonin. They also injected melatonin-deficient mice with melatonin to observe its effect on the dopamine rhythms. They trained C57 mice into 12 hour light and 12 hour dark cycle, and then put them in 10 days of constant darkness (DD). At the first day of DD the mice were given intraperitoneal injections of melatonin. Melatonin dozes were determined by measuring 1mg/kg and it was issued with 0.9% NaCl containing 0.1% ethanol. Other mice got ethanolic-saline solutions instead of melatonin. The injections were administrated once a day for 10 days at circadian time 21. After ten days the animals were killed at three hour intervals. As described earlier, one retina went to the HPLC assay. The second retina was sonicated into 100 µl of phosphate buffer that contained 0.9 NaCl and 1% gelatine (PBS-gel) and frozen to dry ice. The injections and sacrifice were performed under near-infrared lights. (Doyle 2002, 595.)

Melatonin must be extracted from the retinal homogenates before the assay. This is done by dissolving retinas overnight at 4 °C with 1 ml of chloroform. The extract is then washed with 200 µl of 0.1 M sodium carbonate buffer with pH 10.15. Then 700 µl samples are dried under nitrogen gas and re-suspended after that in 25 ml of PBS-gel. Doyle et al. (2002) performed radioimmunoassay as in Tosini and Menaker (1996) and did not write more specifically about the assay and we did not attain the original article. (Doyle et al. 2002, 595.)

6.5 The results

Doyle et al (2002) came to the conclusion that healthy mice have a circadian rhythm and a dopamine rhythm. The mice that are incapable of producing melatonin have neither but can produce them when injected with melatonin. (Doyle et al. 2002, 593.) Pozdeyev et al. (2008) proved their hypothesis right that dopamine has an effect on the rhythms of protein phosphorylation in photoreceptor cells (Pozdeyev et al. 2008, 2691).

Chanut et al. (2006) got the result that in epileptic rats the levels and the retinal physiology was different compared to normal rats (Chanut et al. 2006, 56). Sakamoto et al. (2005) discovered that dopamine controls melanopsin expression in the rat retina. (Sakamoto et al. 2005, 3129).

Although two of the articles, Chanut et al. (2006) and Sakamoto et al. (2005), used rats in the studies, we accepted them, because the assay methods were similar with other articles (Chanut et al. 2006, 56; Sakamoto et al. 2005, 3129). Even though Doyle et al. (2002) was out of our year limit, we chose to include it, because it became our main article to which we compared other articles and their methods. It was also the only study that measured melatonin concentration. (Doyle et al. 2002, 593.) We tried to search more articles concerning melatonin measurement, but could not find any. It seems to be a standard in many retina related studies to only concentrate on the quantification dopamine and other cathecholamines than melatonin. We decided to include the only melatonin assay found in our search and to revise also their use of melatonin injections.

7 REFLECTION

In our study we used descriptive literature review as our research method. Although we were familiar with its general principles from our earlier school assignments, for us this was a new way of using these principles to process excessive amounts of data to construct logical descriptive results. Descriptive literature review is a useful method when there is a lot of research data and it is not too limited by methodological rules.

We knew about melatonin and dopamine based on our previous studies in the biomedical laboratory science programme. We also had basic knowledge of anatomy particularly concerning the eye. However we were not that familiar with the use of laboratory mice in the eye research nor the assay methods of hormones. We knew that melatonin was secreted during the night, but we did not know that there was melatonin also in the eye. The part of dopamine in the circadian rhythm was previously an unknown subject to both of us. It was interesting to find out that there is an independent circadian system in the eye, too.

7.1 Evaluating the study

We tried to choose suitable databases to search articles from, so that we would get as much reliable and extensive data as possible. Because the subject of this study was relatively unknown to us, we used an article we had before we begun the search to guide us in the right direction in choosing the review articles. Even though we were not absolute in limiting our review articles, we chose to limit the articles to regard mammals only, and preferably mice. The mice were a natural subject of the study because our client uses them in their research.

We used search term groups that were fairly similar to each other. We could have used differing search term groups to avoid the large overlapping of the search results, but we wanted to make sure that we did not miss any articles concerning melatonin, dopamine and circadian rhythm. Even though we could not use most of the articles in our review, they worked well as an additional reference material.

During the process of this study we realized that we needed additional searches to cover the assay field more specifically. Even though the final amount of articles was not that big and at first we doubted if they would even be enough, we noticed that the information concerning melatonin and dopamine assays we got from them was enough for our study.

7.2 Reliability

The reliability of our study was an important part of the process. We tried to choose the search databases that would accomplish the most reliable results for our review. The article was considered respectable if it was found in a reliable database and it had been published in a journal of this field. Even though we cannot safely say that all of the review articles are reliable, because we are not experts in this field, we believe that they are.

At first we decided to use the systematic literature review as a method for this study, but at some point we noticed that with this literature material we would not able to go through our data with such a systematic and relentless way that the systematic method demands. Luckily our teachers suggested that we should change the method from the systematic review to the descriptive one, so that we had more freedom processing our literature material, and we were able to select articles that gave us relevant information concerning the subject.

We have also brought up the references we used clearly and featured them in the reference list. The study could have been even more detailed and extensive because of the subject. Anyway we did our best considering our resources and this study coincided with its original purpose to gather some compact information on this subject. One reference was from 1966, but we decided to use it anyway because the information in it was still relevant.

7.3 Arised problems

Our biggest problems with this work were finding the right articles and going through the massive amount of them. We also had trouble when some of the articles we chose were not precisely using the same kinds of animals or methods. But then we realised that this was a part of the process of the study to accept that sometimes you cannot find perfect

answers. We had worked together in previous school projects so we knew each other's working methods. Thus it was easy to work together throughout the whole thesis project. However, no project goes without any problems, and that was true for this project also. The biggest stress factor was to try and fit the study together with other school work and work practice.

Even though we tried to schedule our work and stick to it, there were a few slip-ups. This means that the writing happened mostly in cycles rather than continuously. It would have been preferable to work constantly during the process so that every step would not feel like a restart. We also could have scheduled the work even more precisely in order to avoid a final spurt. On the other hand, we were able to finish even before the due date so we were satisfied with that.

7.4 Learning experiences

During the writing of this study, we gained deeper knowledge of what is the best way to collect proper information, and how to separate literature based on a specific task. This work gave us more information about laboratory mice, the retina and the assays. Even though the simple basis of chromatography was a previously familiar subject, the theoretical context of this study helped us to deepen the former knowledge.

The actual writing of this thesis was at some point challenging, mostly because of the language, as we had not done an entire paper in English before. Although we use and read English almost daily in our contemporary society, using it in a scientific way was new to us. We noticed that it was a bit slower to write in English than it would have been to write in Finnish but at some point the writing speed improved.

We imagined that it would be fairly easy to find dopamine and melatonin related articles and the material was easy to come by at first. We also knew that a lot of work had to be done sorting the collected data. Nevertheless, the amount of work surprised us because we had to go through such a considerable amount of articles in the first elimination phase, even though it was by abstract only.

When we started going through the research articles, we realized that only a few articles answered our assignment questions. This is where we got a little despaired about the amount of articles, but we got over it when we realized that there simply was not any new information for our study in those articles we went through.

Overall our knowledge of this subject grew and we gained a lot of experience concerning a literature review and writing a study in English. There is always room for improvement and this study could have been larger. But we think that overall we managed to achieve the goals we set out to get. We also hope that Professor Petri Ala-Laurila's research laboratory sees this review as useful information for their work.

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APPENDICES

Appendix 1. Research chart

Article	The objective of the research	Methods	Subject of research	Results
Chanut et al. (2006). Variations of dopamine, serotonin, and amino acid concentrations in Noda epileptic rat (NER) retina	Evaluate dopamine, its metabolites, serotonin (5-HT), and amino acid level variations in retina from NER, at two different nycthemeral periods (11 a.m. and 11 p.m.)	HPLC	Epileptic rats, different ages (2, 6, and 12 months)	Retinal physiology is affected by the epileptic status.
Doyle et al. (2002). Circadian rhythms of dopamine in mouse retina: The role of melatonin	A circadian rhythm of retinal dopamine content in the mouse retina - examine the role of melatonin in its control	HPLC (for dopamine), radioimmunoassay (for melatonin)	C3H+/+ mice (which produce melatonin) and C57BL06J mice that are genetically incapable of melatonin synthesis	1. Mice have a circadian clock that generates a rhythm of dopamine. 2. Mice that do not have melatonin do not have circadian rhythms or a dopamine rhythm. 3. A dopamine rhythm can be generated by melatonin injections.
Pozdeyev et al. (2008), Dopamine modulates diurnal and circadian rhythms of protein phosphorylation in photoreceptor cells of mouse retina	Test the hypothesis that dopamine regulates rhythms of protein phosphorylation in photoreceptor cells	HPLC	Wild type C57Bl/6J mice ; congenic mice lacking dopamine D4 receptors on a C57Bl/6J background (Drd4−/− mice); C3H/f+/+ mice, a C3H sub-strain that is unaffected by the retinal degeneration (rd1) mutation	Dopamine regulates the rhythms of protein phosphorylation in photoreceptor cells
Sakamoto et al. (2005), Dopamine regulates melanopsin mRNA expression in intrinsically photosensitive retinal ganglion cells	Investigate the mechanisms that control melanopsin expression in the rat retina	HPLC	Male rats (8-10 weeks old)	Dopamine is involved in regulating melanopsin and its expression.