Συνεντοπισμός της P4 στους Μονοαμινεργικούς Νευροδιαβιβαστές και η Επίδρασή της στη Συμπεριφορά Генетικά Τροποποιημένων Μυών

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Διατμηματικό Πρόγραμμα Μεταπτυχιακών Σπουδών στη Βιοϊατρική Τεχνολογία

ΠΑΤΡΑ 2010
To my parents,

my lifetime companions Aris and Terry

and my beloved Maho.
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Σύνοψη
(Abstract in Greek)

Στο Κεντρικό Νευρικό Σύστημα υπάρχουν διακριτές περιοχές με αμιγώς αισθητική λειτουργία κι άλλες με αμιγώς κινητική. Υπάρχουν ωστόσο και περιοχές, των οποίων η λειτουργία είναι να συντονίζουν ολόκληρο το νευρικό σύστημα. Σημαντικό ρόλο σε αυτόν το συντονισμό έχουν οι χολινεργικοί και οι μονοαμινεργικοί νευρώνες, οι οποίοι με χαρακτηριστικές προτιμήσεις από τις πιο περιπλοκές λειτουργίες του ΚΝΣ (μνήμη, δραστηριοποίηση, διάθεση, libido και πολλές άλλες).

Η P4 είναι μια πρωτεΐνη που ανακαλύφθηκε μόλις το 2004. Ανήκει στην οικογένεια των μεταφορέων SLC10 και μοιράζεται αρκετά κοινή δομή με τα υπόλοιπα μέλη, παρόλο που εκφράζεται σε εντελώς διαφορετικές περιοχές από αυτά. Η μοναδικότητα της P4 έγκειται στο ότι είναι ένας διαμεμβρανικός νευροδιαβιβαστής που επιτόπιται κάθετα στους χολινεργικούς και στους μονοαμινεργικούς νευρώνες γεγονός που την καθιστά τον μόνο νευροδιαβιβαστή με διτή παρουσία και στα δύο αυτά συστήματα. Ωστόσο, δεν υπάρχουν ακόμη πληροφορίες για τον ακριβή εντοπισμό της σε αυτούς τους νευρώνες όταν γίνεται το πρόβλημα το οποίο μεταφέρει.

Προηγούμενα πειράματα έδειξαν ότι η P4 σχετίζεται με την έκκριση της ντοπαμίνης. Η αρχική μας υπόθεση είναι ότι αυτό προκύπτει μέσω κάποιας αλληλεπίδρασης της P4 και της VMAT2, η οποία είναι υπέυθυνη για την απελευθέρωση ντοπαμίνης από τα συναπτικά κυστίδια. Μια τέτοια σχέση, θα μπορούσε να δικαιολογηθεί αν οι δύο αυτές πρωτεΐνες επιτόπιταν στο ίδιο συναπτικό κυστίδιο. Προκειμένου να επαληθεύσουμε αυτήν την υπόθεση, χρησιμοποιήμας τη μέθοδο της Ανοσοκατακρήμνησης. Τα αποτελέσματα μέχρι στιγμής είναι ενθαρρυντικά και με λίγες βελτιστοποιήσεις, η μέθοδος αυτή θα μπορούσε να επαληθεύσει αυτήν την υπόθεση.

Προκειμένου να διερευνηθεί περαιτέρω η λειτουργία της P4, μελέτης συμπεριφοράς καθώς και φαρμακολογικές μελέτες πραγματοποιήθηκαν σε γενετικά τροποποιημένα ποντίκια, τα οποία δεν μπορούν να συνθέσουν P4. Τα αποτελέσματα έδειξαν ότι η P4 δεν επηρεάζει τη λειτουργία της μνήμης, ωστόσο ίσως σχετίζεται με την ανάληψη πρωτοβουλίας και την επιθυμία ανταμοιβής. Παράλληλα, οι δοκιμές εμποδίζουν έκδοση ντοπαμίνης από την έκκριση της ντοπαμίνης. Μελέτες με φλουοξετίνη έδειξαν πιθανή επίδραση της P4 στην έκκριση σεροτονίνης, η οποία ένας τόσος δεν ήταν αρκετά ξεκάθαρη προς το παρόν. Τα πειράματα αυτά αποκάλυψαν ακόμη παράγοντες οι οποίοι μπορούν να επηρεάσουν την έκβαση των δοκιμών σε γενετικά τροποποιημένα ποντίκια και οι οποίοι θα πρέπει να λαμβάνονται υπόψη κατά το σχεδιασμό μελλοντικών πειραμάτων.

Οι ιδιότητες της P4 δεν έχουν κατανοηθεί ακόμα πλήρως και απαιτούνται πολλά επιπλέον πειράματα προκειμένου αυτές να αποκαλυφθούν. Νέες φαρμακολογικές μελέτες έχουν ήδη σχεδιαστεί, ενώ παράλληλα σύγχρονες μοριακές τεχνικές έχουν επιστρατευθεί ώστε να ρίξουν φως στην ακριβή της τοποθεσία.
ABSTRACT

This master thesis summarizes the efforts to identify the location and the behavioural effect of P4, a recently discovered protein. P4 belongs to the family of sodium-dependent bile acid transporters (SLC10), whose first two members are expressed in the gastrointestinal system. However, P4 itself shows a wide expression pattern in various areas of the Central Nervous System. According to electron microscopy, in situ and immunohistochemistry studies, P4 is expressed specifically in both the cholinergic and the monoaminergic terminals, two systems that are responsible for various neurologic and psychiatric disorders. Previous cocaine provocation in KO animals indicated a possible effect of P4 in the release of dopamine. Since VMAT2 is responsible for the transfer of dopamine into the synaptic vesicles, the relation between P4 and VMAT2 needs to become clear. Using Immunoprecipitation, we tried to distinguish if P4 and VMAT2 share the same synaptic vesicle. Moreover, studies in animal models were used in order to unravel the function of P4: the Radial Arm Maze assessment showed no influence of P4 on memory but a possible effect in the reward system, amphetamine provocation showed that KO animals are more affected by amphetamine, while fluoxetine provocation indicated a possible effect of P4 in serotoninergic neurons. Tests in animals with different genetic origin revealed several factors that can affect the final outcome of these studies (age, weight and housing). All this data will help us design our future studies and cast more light in the properties of P4.
INTRODUCTION

1.1 Modulatory Systems in the Brain

Human brain can be divided in certain regions according to functional criteria. Some areas are purely sensory, while others are purely motor. There are also areas whose role is fundamental, even though they cannot be classified as sensory or motor areas; these are the modulatory regions of the brain, which are part of neural circuitries underlying complex behaviors. Brain’s modulatory systems influence motivation, memory and emotion; they can affect reward and attention and, thus, interact with the sensory and motor systems.

Neuromodulation can affect large areas of the nervous system, due to neurotransmitters which are able to have an effect in various neurons. Typical examples of neuromodulators are acetylcholine, norepinephrin, dopamine and serotonin. Neurons that use acetylcholine as a neurotransmitter are classified as cholinergic neurons and those who use norepinephrin, dopamine and serotonin are classified as monoaminergic neurons. In the following paragraphs, the basic characteristics of these two neural systems are described.

1.1.1 The Cholinergic System

The term cholinergic system refers to all the neurons that use acetylcholine (ACh) as a neurotransmitter. The parasympathetic part of the autonomic nervous system is completely cholinergic and all the preganglionic neurons of the sympathetic system are also cholinergic. Moreover, the vertebrate neuromuscular junctions, the basal forebrain and brain stem complexes are cholinergic as well. In particular, there are numerous cell bodies in the nucleus basalis which synthesize ACh and they have many projections in the cerebral cortex.

ACh is constructed from Acetyl CoA and choline, through an enzymatic reaction which is catalyzed by choline acetyltransferase. Nervous tissue is not able to construct choline; thus this must be provided with the diet and then through the blood stream it is delivered to neurons. Acetyl CoA is a common substrate in many metabolic pathways and is not restricted in the cholinergic neurons. Vesicular acetylcholine transporter (VACHT) carries ACh into synaptic vesicles. The function of VACHT consists on exchanging protons which are located in the synaptic vesicles, with ACh which is located in the cytoplasm [47]. There is no reuptake mechanism for ACh. Once ACh is binded to the targeted receptor and the signalling is over, ACh is hydrolysed into choline and acetate. However, there is a reuptake mechanism for choline; the high-affinity choline transporter (CHT) can make choline once again available for the synthesis of ACh [31].

In the peripheral nervous system ACh excites muscle contraction, apart from being responsible for the neurotransmission in the autonomic nervous system. When ACh is connected to the nicotinic receptors of a skeletal muscle, it affects the balance of sodium ions in the muscle cell, causing as a final response the contraction of this muscle. On the other hand, when ACh is connected to the muscarinic receptors of the cardiac muscles, the final response is inhibition of the cardiac muscles’ contraction. In the central nervous system, ACh is strongly related to neural networks that affect plasticity (especially the process of learning and the working memory), arousal and reward. Ach is also proven to impact the process of waking up and sustaining attention [4]. Judging from the diverse functions of ACh, one can easily apprehend that when the cholinergic neurons are not functioning properly, many disorders could occur. Typical examples of such disorders are myasthenia Gravis [48] and Alzheimer’s disease [35].
1.1.2 The Monoaminergic System

The term monoaminergic system refers to all the neurons that use monoamines as neurotransmitters. Serotonin (5-HT) and catecholamines belong to this group of amines, whose common characteristic is the fact that they are all derived from aromatic amino acids. The catecholamines – dopamine (DA) norepinephrin (NE) and epinephrine – are all synthesized from the same amino acid, tyrosine, under a common mechanism. On the other hand, serotonin is synthesized from the amino acid tryptophan, under a different pathway.

Cell bodies of serotonergic neurons are located in and around the midline raphe nuclei of the brain stem and their projections are widely distributed throughout the brain and the spinal cord. 5-HT is implicated in depression, the major disorder of the mood, and is also involved in appetite regulation [7].

There are four major dopaminergic tracts: three of them (the nigrostriatal pathway, the mesolimbic tract and the mesocortical tract) are located in the substantia nigra of the midbrain and the fourth one originates in the arcuate nucleus of the hypothalamus. The nigrostriatal pathway is crucial for the control of movement and when its function is disturbed, certain disorders arise, like Parkinson disease, Huntington’s disease and hemiballismus [49]. The mesolimbic and mesocortical tracts’ influence affect emotion and motivation and are affected in schizophrenia [37]. The fourth dopaminergic tract projects to the pituitary gland and it regulates secretion of hormones, like vasopressin and oxytocin.

Neurons located in the locus ceruleus, a nucleus of the brain stem with many complex modulatory functions, use NE as a neurotransmitter. Although these neurons might be limited in number, they project widely throughout the cortex, cerebellum and spinal cord. In the peripheral nervous system, the postganglionic neurons of the sympathetic nervous system use NE as a neurotransmitter [40]. Besides its role as a neurotransmitter, NE is also a prominent stress hormone [62].

5-HT, DA and NE have unique transporters in order to be transferred from the synaptic cleft into the cellular cytosol. The 5-HT transporter (SERT) is responsible for 5-HT, norepinephrin transporter (NET) is responsible for NE and the dopamine active transporter (DAT) is responsible for DA. They are all Na+ and Cl− dependent transporters with twelve transmembrane domains [46]. DAT and NET do not have high affinity and they can therefore transport both DA and NE, while SERT is highly specific for 5-HT [68]. The vesicular monoamine transporters 1 and 2 (VMAT1 and VMAT2) are responsible for transferring all these neurotransmitters from the cellular cytosol into the synaptic vesicles. The two isoforms have a similar structure but different expression patterns. VMAT1 is primarily expressed in the adrenal medulla, while VMAT2 is expressed in the monoaminergic neurons of the central nervous system [68].

There is strong evidence that monoamines are able to regulate each other through complex interactions. Dopaminergic input can up-regulate 5-HT and down-regulate NE. Increased serotonergic activity is able to inhibit the activity of NE and DA, while increased activity of NE can suppress DA and affect 5-HT in both directions [22, 24]. All these interactions take place in the ventral tegmental area, the locus ceruleus and the dorsal hippocampus [22]. Apart from the interactions between all monoamines, it is also proven that GABA and glutamate have a bidirectional regulatory ability on monoamines [26].
1.2 P4: A Recently Identified, Orphan Transport Protein in the Monoaminergic and Cholinergic Neurons

P4 was identified as a cholinergic marker during a screening for potential candidates [15]. Moreover, electron microscopy showed that P4 is located in the synaptic vesicles, in-situ studies proved that P4 is also expressed in monoaminergic neurons and further immunohistochemistry staining with VACH-T and VMAT2 showed that P4 is exclusively expressed in cholinergic and monoaminergic synaptic terminals (Emilsson, Hörnberg, Rogoz, Limbach and Kullander, unpublished data). Up to date, P4 is the only protein that is exclusively expressed in both these systems. Cholinergic and monoaminergic neurons are responsible for normal behavior as well as for several neurologic and psychiatric disorders. By understanding the function and the properties of P4, we could possibly get a step closer to comprehend the mechanisms behind these disorders and, thus, discover new ways to treat them more efficiently.

P4 is a member of the sodium bile acid co transporter family, which is also known as SLC10. However it has a unique expression pattern in humans that discriminates it from the other members of this family, since it is expressed in the Central Nervous System. It is still an orphan protein with most of its functional properties to be clarified in the future.

1.2.1 The SLC10 Family

The so-called solute carrier (SLC) families consist of passive transporters, ion-coupled symporters and antiporters in the plasma membrane and other cellular membrane compartments. Among them, solute carrier family 10 (SLC10) is known as the sodium bile cotransporter family and it contains over 50 members in animal, plant and bacterial species [25]. In humans, until 2003 this family comprised only two members: Na+/taurocholate cotransporting polypeptide (SLC10A1 or NTCP) and apical sodium-dependent bile acid transporter (ASBT or SLC10A2). NTCP consists of 349 amino acids and ASBT consists of 348 amino acids. They both have an extracellular N-terminus, an odd number of transmembrane helices (sever or nine) and a cytoplasmic C-terminus. NTCP is expressed in the liver and in the pancreatic acinar cells, while ASBT is expressed at high levels in terminal ileum and at lower levels in renal proximal tubules and biliary epithelium. These two members of the SLC10 family are components of the enterohepatic circulation of bile salts and they are not present in the CNS [27].
Since 2004, five new members of the SLC10 family have been discovered and they are known as P3 (SLC10A3), P4 (SLC10A4), P5 (SLC10A5), sodium-dependent organic anion transporter (SOAT or SLC10A6) and P7 (SLC10A7). The functional properties of P3, P4, P5 and P7 have not yet been identified. P3 consists of 477 amino acids and P3 cDNA was cloned from placenta and teratocarcinoma cDNA libraries. P5 consists of 434-438 amino acids and expression data indicates that it is expressed in the liver, kidney and intestine, which is similar to the expression pattern of ASBT. SOAT consists of 370-377 amino acids, which is quite close to the sequence size of NTCP and ASBT. However, SOAT is not a bile acid transporter; instead it transports steroid sulfates under a highly sodium-dependent mechanism. Its expression is identified in testis, placenta and pancreas [22]. P7 is the most recently identified member; experimental data cannot provide sufficient information about P7.

After P3, P4, P5, SOAT and P7 were discovered, it became obvious that this family does not consist uniquely of bile acid transporters. Even though these new members share a significantly similar sequence with the original SLC10 members (Table 2), their expression pattern is not exclusively located in the gastrointestinal area (Table 1).

### 1.2.2 P4: Structure and Expression

The structure of P4 is similar to the structures of the other members of the SLC10 family; amino acid similarities are highest with NTCP, ASBT and SOAT (Table 2). However, it lacks an ALGMMPL motif and its N-terminal is much longer than that of NTCP, ASBT and SOAT [21]. Nevertheless, it shares the same transporting motif with the other family members and therefore it is characterised as a putative anion transporter. Despite the fact that there have been experiments [20] trying to identify what P4 is transporting, P4 is still an orphan transport protein. Transport assays with bile acid, steroid sulphates and choline showed no affinity between P4 and these substrates [20]. Likewise, the transporting abilities of P3, P5 and P7 are not yet specified.

As Table 1 indicates, the members of the SLC10 family do not share the same expression pattern. P4 is primarily expressed in the central nervous system, placenta and liver. In conclusion, P4 has still an unspecified transporting ability and it shows strong dissimilarities in the expression pattern, compared to the other family members. This may suggest that P4 has an entirely unique function.

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**Table 2:** Amino acid sequence identities and similarities among the six human members of the SLC10 family. Values are percentages.

<table>
<thead>
<tr>
<th></th>
<th>NTCP</th>
<th>P4</th>
<th>ASBT</th>
<th>SOAT</th>
<th>P3</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NTCP</strong></td>
<td></td>
<td>29.7</td>
<td>34.9</td>
<td>33.4</td>
<td>19.4</td>
<td>22.0</td>
</tr>
<tr>
<td><strong>P4</strong></td>
<td>53.5</td>
<td></td>
<td>29.1</td>
<td>28.7</td>
<td>23.0</td>
<td>21.7</td>
</tr>
<tr>
<td><strong>ASBT</strong></td>
<td>62.8</td>
<td>54.4</td>
<td></td>
<td>41.8</td>
<td>18.8</td>
<td>22.0</td>
</tr>
<tr>
<td><strong>SOAT</strong></td>
<td>62.6</td>
<td>51.5</td>
<td>69.7</td>
<td></td>
<td>21.1</td>
<td>20.1</td>
</tr>
<tr>
<td><strong>P3</strong></td>
<td>39.2</td>
<td>44.9</td>
<td>38.8</td>
<td>36.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P5</strong></td>
<td>44.6</td>
<td>48.7</td>
<td>43.4</td>
<td>41.3</td>
<td>60.9</td>
<td></td>
</tr>
</tbody>
</table>

[21]
1.3 Memory, Locomotor Behavior and Motivation

In the following paragraphs, some of the behaviors and cognition processes, which are connected to the cholinergic and the monoaminergic neurons are presented. The neural networks behind memory, locomotion and motivation are complex and in some cases not fully understood. However, since P4 is located in both the cholinergic and the monoaminergic neurons, it could possibly affect these brain's functions. Moreover, experiments focused on these functions could indicate possible roles of P4 in the cholinergic and monoaminergic neurons.

1.3.1 Memory

Memory is a complex task for the brain, involving different regions and diverse neurons in order to store and then recall various information. There are several ways to categorize memory, but the most common one is the division between working memory (short-term) and reference memory (long-term).

Two major components constitute working memory: short-term storage (on the order of seconds) and executive processes that operate on the information which has been stored. According to the nature of the information, different regions are activated in order to store or recall memories. Regions that participate in the working memory are the prefrontal cortex, the posterior parietal cortex, the thalamus, the caudate, and the globus pallidus. It is proven that acute stress can affect the efficiency of working memory; this effect is thought to result from the supraoptimal levels of catecholamines in the prefrontal cortex, after a stressing incident.

Working memory can adapt to reference memory through the process of rehearsal and meaningful association. As in the case of working memory, two major components constitute reference memory: implicit and explicit memory. All the information about how to perform a skill is stored through the implicit memory, a memory that is recalled unconsciously. Factual knowledge of people, places and things and the meaning of these facts are referred to as explicit memory, a memory which is recalled by deliberate and conscious effort. Implicit and explicit memory involves completely different neuronal networks. Explicit memory involves the temporal lobe system, while implicit memory requires the cerebellum and amygdala in addition to the specific sensory and motor neurons recruited for the task being learned.

1.3.2 Locomotor Behavior

The ability to move from one place to another is crucial for all animals. Locomotion is manifested in various forms, depending on the animal: stepping, running, swimming, crawling and flying are some typical examples. The main characteristic of locomotion is its rhythm and the presence of one or more appendages that allow the animal to move. In order for locomotion to be effective, several muscles need to be co-ordinated to contract synchronically and with the appropriate strength. Lower levels of the nervous system are able to control locomotion, without any intervention by higher centres. Nonetheless, it is still important for these regulatory neurons to be able to modify locomotion, according to the different environments in which it could take place.

Stepping, for instance, is regulated by neuronal circuits that are located entirely within the spinal cord. These circuits can be activated by tonic descending signals from the brain and they do not require sensory input, in order to function. However, they are strongly regulated by input from limb proprioceptors. The central neural network that generates a rhythmic pattern of motor activity is known as central pattern generator (CPG) and up to date CPGs have been identified and analyzed for more than fifty different rhythmic motor systems, controlling among others walking, swimming and respiration. CPGs are able to function even without afferent...
feedback signalling from peripheral receptors and they are extremely flexible.

Complex neurochemical interactions occurring in the circuitry of the basal ganglia regulate locomotor activity. Normal locomotor behavior depends on a critical balance between striatum and pallidum. The striatal output pathways are affected by dopamine. The dopaminergic input lead to a reduced inhibition of the thalamocortical neurons and thus it facilitates movements initiated in the cortex. In other words, the dopaminergic action is responsible for paired movement. Both hypo- and hyperkinetic disorders can be explained by specific disturbances in the basal ganglia – thalamocortical motor circuit, including the depletion of the dopaminergic input [36].

Locomotor activity can be affected by various factors, including heat, stress or drugs that are blocking the function of the CPGs [45]. Under a stress reaction, high levels of adrenocorticotropic-hormone, corticosterone, epinephrine and norepinephrin are observed in the plasma [14 and 22]. Locomotor activity is proved to be increased during panic attacks induced by pentyleneterazol [54], exposure to psychological stressors [60] and electrical stimulation of the dorsal periaqueductal grey nuclei [29]. Benzodiazepines can reduce the reaction to all these stressors, which could mean that the increased locomotor activity could be interpreted as a high anxiety and stress. Frontal cortex activation is evident in both anxiety and stress [63].

1.3.3 Disorders of Mood and Motivation

Major depression disorder (MDD) is one of the most studied disorders of the mood and yet its pathogenesis remains complex and not completely unravelled. Abnormalities that are responsible for depression begin on the gene level and they expand up to the structure and the function of the central nervous system and the autonomic nervous system.

As far as it concerns the structural alternations of the central nervous system, which are observed in MDD, they are quite diverse. The ventromedial prefrontal cortex demonstrates increased activity [16], while the lateral orbital prefrontal cortex is most likely involved by suppressing maladaptive and preservative emotional responses [42]. The dorsolateral part of the prefrontal cortex seems to show a decreased activity which influences the compromised

**Figure 1:** Sites of action of antidepressent drugs at noradrenergic neurons.

I) Stimulation of release of NE at nerve terminals (i.e. amphetamine),
II) Stimulation of receptors (i.e. clonidine),
III) MAO Inhibitors: Inhibition of enzyme that oxidizes NE (i.e. pargyline),
IV) Tricyclics: Inhibition of reuptake (i.e. desipramine),
V) Inhibition of the enzyme that inactivates NE (i.e. tropolone)
15

working memory, impaired sustained attention and executive dysfunction that characterize MDD [30]. The subgenual anterior cingulate cortex demonstrates an increased metabolism in MDD [67]. Moreover, there are imaging findings that suggest a reduced volume of the hippocampus [18] and increased activity in amygdala [55]. Increased thalamic activity [8] and abnormalities in the connections between limbic and paralimbic prefrontal areas [58] complete the plot of structural pathogenic alternations in the central nervous system, that accompany MDD. On the other hand, the autonomic nervous system, demonstrates an increased tone of the sympathetic part together with a decreased tone of the parasympathetic part [41].

There are several evidences indicating participation of the monoaminergic system in MDD. Several studies indicate alteration in the NE and 5-HT receptor density in the cortical and limbic formations of depressed patients [50]. In a PET imaging study, dopamine type-1 receptors were found significantly reduced in MDD [12]. DAT has a 15% lower binding capacity in MDD, while SERT shows a higher binding capacity during a depressive episode [44]. All these indication can explain why monoamine oxidase inhibitors (MAO), tricyclic compounds and selective serotonin reuptake inhibitors (SSRIs) are widely used as treatment to MDD (Figures 1 and 2). However, if monoamines were the major abnormality in MDD, it is difficult to explain why 50% of the patients failed to respond in the first line SSRI treatment, 65% did not achieve remission and more than 50% of those who did still had two or more residual symptoms, during the STAR*D trial [61].

Therefore, monoamines are not the only neurotransmitters that are related to MDD. GABA, glutamate, galanin and substance-P play an active role in mood regulation and thus they are also responsible for MDD. In summary, there is a complex dysregulation of interrelated neurotransmitter systems and distributed neural networks in brain, that can affect the mood and under elaborate mechanisms they can cause depression disorder.

1.3.4 Investigating Brain Function in Animal Models

Although animals cannot be used in order to examine complex human behaviors, like language, they do have similar basic functions. Non-human primate brains are very close to human brain. However, rodents have a simpler nervous system, compared to humans, they are genetically more homogeneous than non-human primate brains, they are less expensive to

![Figure 2: Sites of action of antidepressent drugs at serotonergic neurons.](image)
maintain, they provide us with greater numbers of experimental animals and much more is known about their neurobiology than primates. Moreover, it is easier for a researcher to control certain variables such as diet, environment and learning history.

Various behavioral and pharmacological studies can be performed in rodents, in order to study certain brain functions or the effect of various drugs on these functions. For example, memory can be tested through the Radial Arm Maze, while motivation can be tested through the Forced Swim Test. Pharmacological studies were originally designed, in order to examine pharmacokinetics and pharmacodynamics of new agents. In Neuroscience, when we know the properties of a drug, we can use it in order to block or activate a specific neurotransmitter and then investigate its effect in genetically modified mice of our interest. For the purposes of this study, two agents were used to study locomotor behavior and motivation: amphetamine and fluoxetine.

Amphetamine is a psychostimulant. It is used widely to treat several disorders, such as attention deficit, narcolepsy and obesity. It has a significant effect in the dopaminergic neurons, increasing the levels of DA in the synaptic cleft. It has the ability to bind to DAT and thus it is transported inside the neuron. Inside the cell amphetamine forces VMAT2 to pump out more DA from the synaptic vesicle and as a result, it causes an excess of DA in the neuron, which forces DAT to work in reverse and transport DA towards the synaptic cleft (Figure 3) [17]. Moreover, amphetamine interacts with monoamine oxidase both in the cytosol and in the synaptic cleft, preventing the breakdown of DA. Even though amphetamine has the highest affinity to DA, it can also affect serotonergic and noradrenergic neurons in a similar way as with DA neurons (Figure 2), but its effect on dopaminergic neurons is more dominant.

The behavioral effect of amphetamine depends on the dose: higher levels of amphetamine results in higher levels of DA. This could be possibly explained by the fact that different receptors are activated by different levels of amphetamine. At low doses, it is shown that more D2 receptors are activated compared to D1, possibly because amphetamine has a higher affinity for D2 [17]. In higher doses, both receptors are activated. In our experiments, amphetamine was used in order to measure its effect in locomotor activity before and after drug application.

Fluoxetine (Prozac) was generally considered for many years as the first SSRI, even though historically it is zimelididine that introduced this type of antidepressant drugs. This fact reveals the strong influence of fluoxetine in modern society over the years; even though nowadays there are many new agents against depression, its popularity is still high. Since fluoxetine belongs to SSRIs, its effect is specific only for the serotonergic neurons. As shown in Figure 1, it increases the levels of 5-HT in the synaptic cleft by inhibiting the uptake of 5-HT. The anti-depressant effect of fluoxetine on laboratory animals is dose-dependent [13]. Fluoxetine was used in two experiments: i) in the Forced Swim Test, in order to observe how fluoxetine can affect the depression-like effect that is developed during the test and ii) in locomotion measurements, in order to observe if fluoxetine can cause any variations in the locomotor behavior of KO mice.
AIMS OF THIS STUDY

Electron microscopy, in situ and immunohistochemistry studies have showed that P4 is located in the monoaminergic and the cholinergic neurons, and more specifically in synaptic vesicles at the synaptic terminals. The overall aim of this study is to identify the synaptic vesicles where P4 is located and moreover to increase the understanding of P4’s function in the CNS. Previous pharmacological studies with cocaine showed an increase of locomotion in KO animals. Since DA has an evident effect in locomotor behavior and VMAT2 is responsible for the storage of DA into the synaptic vesicles, our initial hypothesis is that P4 and VMAT2 share the same synaptic vesicle.

Specific aims:

• To develop an Immunoprecipitation protocol that could test if P4 and VMAT2 are co-localized in the same synaptic vesicle.

• To examine if P4 affects Working or Reference Memory.

• To investigate the effect of amphetamine provocation in locomotor behavior in animal models.

• To investigate the effect that fluoxetine provocation has in motivation in animal models.

• To examine the impact of genetic origin in the outcome of studies in animal models and explore other possible factors that could alter their aftermath.
MATERIALS AND METHODS

2.1 Animals

The P4 knockout mice (KO) were ordered from TIGM and they were generated from a 129/SvEvBrd strain. According to studies on brain tissue, spinal cord and neurons, these animals do not show any structural abnormalities or abnormalities in the distribution of key molecular markers, such as VMAT2, VACHT and Synaptophysin (Emilsson et al., unpublished data). Moreover, electrophysiological studies proved that there are no differences as far as it concerns the signaling in the spinal cord (Emilsson et al., unpublished data). KO mice appear normal during all the stages of development, they are born with the same Mendelian frequency and their phenotype is indistinguishable from their wild type (WT) littermates during adolescence and adulthood.

All animals are kept in a specially designed facility inside the laboratory premises. There are breeding cages (two females and one male, all older than 7 weeks) of heterozygous and homozygous animals. All litter is housed together separated by gender. As for the males, only males who are coming from the same breeding are housed together. All animals are housed under a 12 hour light-dark cycle (lights on at 0600 hours, lights off at 1800 hours) with ad libitum access to food and water except during behavioral tests. All animal procedures were approved by the local ethical committee in Uppsala, and guidelines from European Communities Council Directive (86/609/EEC) were followed.

2.2 Genotyping

When mice were approximately 4 weeks old, they were tagged with a unique number and weaned in a new cage, together with their siblings of the same gender. At the same time, a tail clip of 2mm was acquired, in order to be used for genotyping. In each tail clip, 75 μl of Buffer 1 (250 mM NaOH, 2 mM EDTA) were added. Then the samples were boiled for 45 minutes at 96°C and after that they were put on ice for 2 minutes. Afterwards 75 μl of Buffer 2 (400 mM TRIS HCl pH 8.0) were added and the samples were kept in 4°C [65].

To genotype the mice, 1 μl from the tail samples containing genomic DNA was added to 15.82 μl H2O for the WT primers and 10.42 μl H2O for the KO primers, 2 μl 10 x PCR Buffer (Fermentas), 1.2 μl 2 mM MgCl (Fermentas) for the WT primers and 1.5 μl 2 mM MgCl (Fermentas) for the KO primers, 0.15 μl Primer 1, 0.15 μl Primer 2, 0.5 μl dNTPs and 0.18 μl Hot Start Taq (Fermentas) to a total volume of 20 μl. Amplification was performed in a Biorad Ms Mini© programmed with an initial denaturation at 95 °C for 2 minutes, 30 cycles at 95 °C for 0.3 minutes, 62 °C for 1.2 minutes, and 72 °C for 1.15 minutes, followed by 72 °C for 6 minutes and 20 °C for 2 minutes. Both KO and WT primers were from Eurofin MWG Operon (primer sequence on Table 3).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO P4 2F</td>
<td>5’-CGACGCTTGACCAGACAGA-3’</td>
</tr>
<tr>
<td>KO P4 2R</td>
<td>5’-AGACGGCAATATGGTGGAAA-3’</td>
</tr>
<tr>
<td>WT P4 2F</td>
<td>5’-GGAAAGACATGGCTGACTGCTTG-3’</td>
</tr>
<tr>
<td>WT P4 1R</td>
<td>5’-CACCCGGTTGTATTTGTAGC-3’</td>
</tr>
</tbody>
</table>

Table 3: Sequence of the primers which were used for genotyping
2.3 Vesicle purification

In order to isolate vesicles, I used the protocol that was developed by Huttner et al [66] with some modifications. One brain was dissected and then put in a glass tube with 10 ml Homogenization Buffer (320mM sucrose, 4mM HEPES, 1mM phenylmethylsulfonyl fluoride, 1:1000 protease inhibitor cocktail for mammalian tissue, pH adjusted to 7.4 using KOH). The brain was then homogenized by 10 strokes at 900 rpm, using a Eurostar Digital (IKA-WERKE). The solution was put into a Beckman tube and centrifuged for 10 minutes at 1300g at 4 °C, using a JA 17 rotor, in a Beckmann centrifuge. The supernatant was collected and centrifuged again for 15 minutes at 14000g at 4 °C using a JA 17 rotor, in a Beckmann centrifuge. Then, the supernatant was discarded and the pellet was lysed both osmotically and mechanically. In order to do so, the pellet was resuspended in 10ml Lysis Buffer (10mM HEPES, 1mM phenylmethylsulfonyl fluoride, 1:1000 protease inhibitor cocktail for mammalian tissue, pH adjusted to 7.4 using KOH) and then it was shocked with 3 strokes at 2000 rpm, with a Eurostar Digital (IKA-WERKE).

The final solution contained lysed crude synaptosomes (isolated terminals of neurons), which means that all different types of vesicles were included and not only synaptic vesicles. This solution was used immediately for immunoprecipitation or was stored at -20 °C for further use. However, it should be noted that after freezing the synaptosomes, all vesicles were broken and this solution was not efficient for immunoprecipitation anymore.

Before these synaptosomes were further used, a measurement of protein concentration was applied, using the Bradford method. 5μl of synaptosomes were mixed with 500μl of dye (1:4 Coomassie Brilliant Blue G-250 diluted in distilled water) and the concentration was measured in a spectrophotometer.

2.4 Immunoprecipitation

Immunoprecipitation (IP) is a fast and efficient method in order to isolate a specific protein, by using a targeted antibody. The principle of this isolation is illustrated in Figure 4. The primary antibody, which can target a specific protein is either added to the tissue sample (indirect technique) or pre-coated onto the magnetic beads (direct technique) before the cell isolation. The magnetic beads are then mixed with the tissue sample in a tube. After a short incubation, the beads will bind to the proteins of interest and this complex will be separated by a magnet. In the present protocol, Dynabeads Protein G (Invitrogen) was used. These beads are precoated with Protein G and they can be used with every antibody that is able to bind to Protein G.

In the indirect method, 1.4μg of antibody was mixed with 150μg of synaptosomes in 700μl of Buffer A (2mM EDTA, 5% bovine serum albumin in PBS). This solution was incubated overnight at 4 °C, in rotation.

**Figure 4:** Basic principle of cell isolation using the direct and the indirect method of Immunoprecipitation
The next day, 75μl of Dynabeads were washed 2 times for 5 minutes in Buffer 1 (0.1% bovine serum albumin in PBS), using the magnet in order to separate the beads from the washing solution. After the washing, the beads were mixed with 300 μl of Buffer A and then with the antibody-synaptosomes mix. This solution was incubated for 1 hour in room temperature, in rotation. When the incubation was over, the tube was placed in the magnet and the supernatant was removed. The complex beads-antibody-vesicles was then washed 3 times in 200μl of Buffer A and subsequently 3 more times in 200 μl of Buffer 1. When this step was done, the complex was ready to be loaded on a gel for Western Blot analysis.

In the direct method, 75 μl of Dynabeads were washed 2 times for 5 minutes in 1 ml Buffer 1, using the magnet in order to separate the beads from the washing solution. Afterwards, 1.4 μg of antibody was added in the beads and they were all resuspended in 700 ml Buffer A. 150 μg of synaptosomes were adjusted into 300 ml of Buffer A and then they were mixed in a tube with the solution containing the beads and the antibody. This tube was incubated overnight at 4°C in rotation. The next day, the tube was placed in a magnet and the supernatant was removed. Then, the complex beads-antibody-vesicles was washed 3 times in 200μl of Buffer A and subsequently 3 more times in 200 μl of Buffer 1. When this step was done, the complex was ready to be loaded on a gel for Western Blot analysis.

2.5 Western Blot

According to earlier publications [68], before loading a sample on a gel, this sample needs to be mixed with loading buffer and then to be boiled. Pre-casted Tris-HCl gels, 10% or 12.5% (Bio-rad Criterion Tris-HCl gels) were used and as a ladder the PageRuler Prestained Protein Ladder (Fermentas). In general 20 μl of total sample were loaded in each well of the gel (unless it is stated differently). 15μl of sample was mixed with 5 μl of loading buffer (200 mM Tris, 8% SDS, 40% glycerol, 0.8% Bromophenol blue, pH 6.8) and 0.25μl β-Mercaptoethanol (Bio-Rad). The samples were then boiled for 5 minutes at 96°C, in order for the proteins to be denatured.

The gel was carefully placed into a Criterion Electrophoresis Buffer tank and loaded with all the boiled samples and the protein ladder. The tank was filled up to the indicated point with Electrophoresis Buffer 1x (5x Electrophoresis Buffer contains 15gr Tris, 72 gr glycine, 50 ml 10% SDS and water in a total volume of 1 litre). The gel was then electrophoresed at 130 Volts for 90 minutes, accordingly. When the electrophoresis was done, the blot was transferred in a nitrocellulose membrane by semi-dry transfer. Filters, blot and membranes were soaked into Transfer Buffer (3.02 gr Tris, 14.4 gr Glycine, 200 ml Methanol, 10 ml 10% SDS and water in a total volume of 1 litre) at least 10 minutes prior to the transfer. The acquired blot was then blocked for 1 hour in 5% skim milk diluted in TBS-t 1x (1 litre of 5x TBS-t contains 60.5 gr Tris, 87.6 gr NaCl, 5 ml Tween-20, water, pH 7.5 adjusted with HCl). After blocking, the blot was incubated overnight at 4°C in agitation, with primary antibody.

The next day, the blot was washed several times (2 instant washes, followed by 4 washes of 5 minutes each) in TBS-t and then it was incubated with a secondary antibody (HRP that could detect the primary antibody) for 1 hour in room temperature, at shake. Afterwards, the blot was washed again several times in TBS-t (2 instant washes, followed by 4 washes of 5 minutes each). Finally, the blot was incubated with 10 μl of luminous reagent A (68mM p-coumaric acid C-9008, Sigma diluted in DMSO), 1 ml of luminous reagent B (1.25mM Luminol A-8511, Sigma diluted in 0.1M Tris, pH 8.5) and 3 μl of H2O2 inside a plastic membrane, which was carefully put inside a cassette. This cassette was transferred in the dark room, where X-ray film (General Electric) was exposed to the blot prior to development. The exposed film was then developed in Agfa CP1000 Developing Machine. The antibodies that were used in each experiment, the exposure time of the film and any other alternation of the basic protocol that
was presented in this paragraph will be stated together with the results of each experiment.

2.6 Radial Arm Maze

In order to observe any differences in the working memory and the reference memory between the WT and the KO population, we used the assessment of the Radial Arm Maze. The 8-arm radial maze consists of an octagonal central platform (50 cm in diameter) with eight identical radial arms (65 cm long, 12 cm wide and 10 cm high) extending outwards. Each arm has a plastic food cup approximately 3 cm from the end. The whole structure is elevated 70 cm high above the floor.

Eighteen adult animals participated in the study, nine WT mice (three males and six females) and nine KO mice (three males and six females), all of them between 7 and 20 weeks old. To eliminate any deviations between the two groups due to the different breeding origin, for each WT mouse its KO sibling from the same breeding was used. All animals were weighed before the study started as well as daily before the trials, in order to ensure that there were no differences in the metabolism of the two populations. A food deprivation schedule was carried out during the trials; animals had no access to food from 1800 until 1300 of the next day, when the trial was over. However, animals were allowed to drink water freely during the whole study. The trials lasted for five days and the schedule was the following:

Day 1 (Acclimation day): Food pellets (rewards) were placed in four arms (arms 1, 2, 4 and 7), while the remaining arms were empty. This food pellet placement was adopted in order to avoid that mice developed a “stimulus-response” strategy (for example turn right at every arm or every other arm) to obtain the food. Each animal was placed in the centre of the octagonal platform and allowed to explore the maze for 10 minutes. The purpose of this first day was for every mouse to explore all the arms. As a result, no mouse was removed from the maze in case this mouse succeeded on finding and eating all the rewards before 10 minute had elapsed, unless it had already visited all eight arms.

Days 2 to 5: Food pellets (rewards) were placed again in the same arms as on day 1. Each animal was placed in the centre of the octagonal platform and allowed to explore the maze for 10 minutes. Only if the animal succeeded on eating all the rewards, it was removed from the maze before 10 minutes had elapsed.

During every trial, the entries in each arm were recorded, by an observer blind to the genotype of each mouse. Every day, the trial started at 0900 and it was concluded by 1300. After each trial was concluded, all mice returned into cages with food supply (until 1800).

2.7 Amphetamine Provocation

Eighteen adult animals participated in these trials, nine WT mice (3 males and 6 females) and nine KO mice (3 males and 6 females), all of them between 7 and 24 weeks old on the first day of the trial. Three doses of amphetamine were tested: low dose (0.5 mg/kgr), medium dose (1.5 mg/kgr) and high dose (3 mg/kgr). As it is stated above, for each WT animal its KO sibling
from the same breeding was chosen to participate in the study. Before the first trial, all animals were weighed, in order to determine the appropriate amount of amphetamine that they should receive.

The study was designed according to previous locomotion studies performed in the lab [9, 39 and 65]. All animals were divided randomly into four groups and each group received a different dose of amphetamine or 0.9% saline on each trial. As a result, there have been four trial days in total, in order for all animals to receive all the different treatments. Amphetamine was dissolved in saline and aliquoted in eppendorf tubes and then it was frozen in -20°C. Saline was aliquoted and frozen as well. One hour before each trial all doses of amphetamine and saline were thawed and then they were injected to the corresponding group of animals intraperitoneally. Immediately after an animal was injected, it was placed inside an automated locomotion cage. Its locomotion, rearing activity and total activity was recorded by automatic photoreceptors during 90 minutes in dim light. Between each trial day, there has been a time interval of seven days, in order to ensure that the previous dose of amphetamine would be completely removed from the mice. This part of the study lasted four weeks.

Two weeks after the first part of the study was completed, new trials were performed, under a slightly different pattern. During this part of the study, only saline and the medium dose were tested. In the beginning of the trial, each animal spent 40 minutes inside the automated activity cage in order to acclimate. Then, the animal was injected intraperitoneally with saline or the medium dose of amphetamine and returned in the locomotion cage for 60 more minutes. Locomotion, rearing activity and total activity were recorded again for 100 minutes. On the first trial day, animals were treated with saline and two days the animals were treated with the medium dose of amphetamine.

The whole study lasted six weeks and during this period all animals had ad libitum access to food and water.

2.8 Fluoxetine Provocation

Forty eight animals participated in this study in total. Among them, sixteen adult animals coming from Het/Het breeding, eight WT mice (six males and two females) and eight KO mice (six males and two females), all of them between 10 and 26 weeks old. For each WT mouse, its KO sibling from the same Het/Het breeding cage was chosen. Additionally, thirty eight adult animals coming from WT/WT breeding or KO/KO breeding cages participated in the study, sixteen WT mice (twelve males and four females, all siblings) and sixteen KO mice (twelve males and four females, all siblings). These animals were between 8 and 15 weeks old. The effect of fluoxetine provocation on these mice was detected with two methods: The Forced Swim Test and the activity record in automate locomotion cages. Fluoxetine was purchased from LKT laboratories.

- **Forced Swim Test:**

  The Forced Swim Test (FST) was originally developed by Porsolt [51] and is up to date the most used tool for screening antidepressants. Many factors could affect the sensitivity and the variability of FST; among them the type of administration (acute or chronic), the age of mice, the gender, the environmental conditions and the observer could decrease the variability, while the structure of the assessment can decrease the sensitivity [6]. The original Porsolt’s method consists of direct immersion of animals after injecting drugs. However, for the current study, we chose a modification of this method, based on recent publications [1 and 28]. All animals were
subjected to a pre-test trial, in order to avoid variations and for maintaining consistency in the immobility time between the two different groups (WT and KO).

During the pre-test trial, each animal was placed for 6 minutes in a glass cylinder (19 cm in diameter, 35 cm high) containing fresh water up to a height of 20 cm, whose temperature was 25±3°C. When the trial was over, the animal was dried with a towel and it was allowed to dry in a cage placed under a source of heat for 10 minutes, before it returned to its home cage. Twenty four hours later (DAY 1), each animal was injected intraperitoneally either with 20 mg/kgr of fluoxetine (test group), or with vehicle (control group). Fluoxetine was dissolved in the vehicle (0.9% saline) every morning before the trials and it was used fresh. Thirty minutes after injection, each animal was placed in the same cylinder in order to swim in similar environment for a period of 6 minutes. One week later (DAY 2), the trial was repeated; the animals that were treated with saline before, they were treated with fluoxetine this time and those who were treated with fluoxetine before, they were treated with saline this time. The behaviour of all mice was videotaped. Later on, the videotape was analyzed by an observer who was blind to the genotype of each animal, by the aid of AniTracker version 1.2 (rsUtils). All animals were divided randomly in the two different treatment groups.

The main purpose of this study was to measure the time of immobility for each mouse. The first 2 minutes inside the cylinder were characterized by a vigorous swimming activity, followed by a period of immobility. The mouse was considered to be immobile if it ceased struggling and remained floating passively, making only the necessary moves to keep its nose above water. The last 4 minutes are used in order to record the immobility, but in this study, we used 1 min intervals for the whole 6 minutes, in order to measure the immobility period \([56]\). Since fluoxetine has an antidepressant effect, it was expected to reduce the immobility time, giving us the opportunity to compare the effect between the two different populations.

• **Locomotor Behavior and Fluoxetine:**

In order to examine locomotion behaviour, automated locomotion cages were used, following the same method described earlier. Briefly, each animal was placed inside an activity cage for 30 minutes in order to acclimate. After that, the animal was injected intraperitoneally either with saline (DAY 1) or with 20 mg/kgr fluoxetine (DAY 2) and it was placed back into the activity cage, where its locomotion, rearing activity and total activity was recorded by automatic photoreceptors during 90 minutes in dim light. During the last 60 minutes of this trial, the animal was considered to be under the influence of fluoxetine.

The fluoxetine provocation study lasted in total six weeks and during this period all animals had ad libitum access to food and water, apart from the testing period. The animals that were originated from a Heterozygous breeding participated first in the FST and then in the activity cages. For the FST, a time interval of seven days was chosen between DAY 1 and DAY 2, in order to be sure that fluoxetine would be removed from their system. The animals that were originated from a Homozygous breeding were divided into two groups, based into their genotype and gender. Sixteen of them participated in both the FST and the activity cages and the rest sixteen participated only in the activity cages. This pattern will allow us to compare how the animals from Heterozygous and Homozygous breedings behave and at the same time to observe if there was any effect in the locomotion on the animals that were firstly involved into the FST.

**2.9 Statistical Analysis**

For all behavioural and pharmacological studies, all data are expressed as mean±SEM
(standard error of the mean). The effects of genotypes, time and genotype \( x \) time interaction were determined by two-way ANOVA. Statistical significance between the two genotypes was analysed with Student \( t \)-test and differences were considered as significant at \( p<0.05 \). Statistical analysis and graphs were made with Prism 5.0b for Mac OS X (GraphPad Software, Inc.).

In all graphs, when the difference between the two curves is significant (\( p<0.05 \)), it is marked with one star (\(*\)); when the difference is very significant (\( p<0.01 \)), it is marked with two stars (\(**\)); when the difference is extremely significant (\( p<0.005 \)), it is marked with three stars (\(***)).
RESULTS

3.1 Assessment of the co-localization of two different proteins in the same synaptic vesicle

In order to prove that P4 and VMAT2 are co-localized, we needed an efficient protocol to isolate synaptic vesicles and an Immunoprecipitation (IP) protocol able to target the vesicles of our interest.

Two protocols were tested for the purification of synaptic vesicles: i) a protocol whose outcome was crude synaptosomes and ii) a protocol whose outcome was an enriched fraction of synaptic vesicles. The second one includes one additional step, during which a sucrose gradient is applied on the crude synaptosomes, in order to isolate the fraction of synaptic vesicles. Both protocols are efficient in the isolation of synaptic vesicles (Figure 7). The final concentration of synaptic vesicles is higher when using the second protocol; this is stated by a stronger band in the Western Blot analysis (the same amount of protein was loaded in the gel for both protocols). Moreover, the first protocol was tested in lysates isolated from a whole brain and half of a brain, in order to test its sensitivity; it is proved that it works efficiently in both cases (note: the same amount of protein from each sample was loaded on the gel).

In order to precipitate and/or detect VMAT2 in a blot, we used a self-made antibody donated by professor Salam El Mestikawy. Crude synaptosomes and enriched synaptic vesicles were loaded on a gel and the blot was incubated with this antibody (Figure 8). The VMAT2 band is stronger in the crude synaptosomes than in the enriched synaptic vesicles.

To test the IP protocol and verify if it was efficient in order to isolate the vesicles of our interest, we used Synaptophysin. Fresh synaptosomes were isolated from a WT and a KO animal and they were precipitated with magnetic beads and Synaptophysin. The final outcome of the IP, together with crude synaptosomes from the WT and the KO animals as controls, were loaded on two gels and the blots were incubated either with Synaptophysin or with VMAT2. We can observe (Figure 9) that no synaptic vesicles were left in the supernatant that was collected after the IP; the band that corresponds to the synaptic vesicles is evident only in the precipitated fraction of WT and the KO vesicles and in the WT and KO synaptosomes (controls). When the blot was incubated with VMAT2, we could not observe the VMAT2 band in the immunoprecipitated vesicles.

At the same time, an IP with VMAT2 was performed, using fresh vesicles from both a WT and a KO mouse. The precipitated vesicles were loaded on a gel and the blot was incubated with
Synaptophysin (Figure 10). The Synaptophysin band is missing from the immunoprecipitated vesicles and we can only observe two bands around 55 and 35 kDa, which correspond to the heavy and the light chain of the VMAT2 antibody that was used in the IP. Synaptophysin is only evident in the controls. Another VMAT2 antibody was tested (Millipore), but the result was the same (data not showed here).

In general, IP is an effective protocol, in order to prove that two proteins are co-localized. Two factors are critical for the final outcome of this protocol: the antibodies and the vesicles. The success of IP is strongly related to the affinity of the antibodies that are tested. Moreover, the vesicles need to be intact when they are incubated with the antibodies and the beads. From all the results that are stated until now, we can assume that further optimization is needed, before we can test our initial hypothesis.

3.2 Memory Test of P4 between WT and KO animals

The Radial Arm Maze focuses on spotting the differences in the Working and the Reference Memory, between two different populations of mice. When a mouse is revisiting a reward arm (an arm where there is food), after it has eaten the reward during a previous visit there, it is considered as an error of the working memory. The total number of revisits in reward arms, after these rewards were taken, consists the Working Memory Errors. On the other hand, visiting arms where there is no reward counts as error of the reference memory and the total number of visits in non-reward arms consist the Reference Memory Errors rate.

No significant difference was noted in the Working and Reference memory between the WT and the KO mice (Figure 11 A-B). However, there is a significant difference in the reaction of mice against the rewards (Figure 11C). Even though both WT and KO mice visited reward arms in an equal rate (data not shown here), we can see that the WT mice were eating all the rewards that they could find. Especially after the second day, the WT mice were able to retrieve almost all four rewards that were placed in the maze. As far as it concerns the KO population, they didn’t eat all the rewards, despite the fact that they were able to locate the rewards as well as the WT mice.

In conclusion, there is no significant difference in the Working or the Reference Memory between WT and KO mice. On the other hand, KO mice seemed more reluctant on getting their reward.
3.3 Amphetamine Provocation Increases Locomotion in KO mice

Animals had been treated with three different doses of amphetamine and the vehicle (saline), before the estimation of activity in the locomotion cages. Injection with amphetamine causes an immediate effect of higher activity. This effect starts dropping gradually in time, as amphetamine is getting out of the system of the treated animal. The results of all doses are presented in Figure 12 (A-D).

When the animals were treated with saline (Figure 12A), there was no significant difference in locomotion between the two populations. After the animals were introduced to the new environment, locomotion dropped in both the WT and KO mice, following in general the same pattern. In the low dose (Figure 12B), we can observe that there is a significant difference between the two populations (p<0.005). The KO mice are more affected by amphetamine and as a result they are much more active than the WT mice. This difference becomes even more evident, for the animals that were treated with the medium dose of amphetamine (Figure 12C); in this case it is obvious that the KO mice are more hyperactive than the WT ones.

In the high dose of amphetamine, there is no significant difference between the two curves (Figure 12D). However, we can see that the curve which corresponds to the KO mice, progresses under a very different pattern in this dose. While the WT mice achieve a higher activity in the high dose compared to the medium one, the KO mice begin with a similar activity as in the medium dose only to start dropping immediately. After the first forty minutes, the KO mice seem to be less active than the WT mice; however, judging by the SEM, we can remark this fact as a tendency and not as a significant difference.

Figure 11: Results of the Radial Arm Maze. All data are presented as mean±SEM.
During the second trials of the amphetamine provocation, the animals were allowed to acclimate for forty minutes into the cages before they were injected with saline or the medium dose of amphetamine. The results are following the same pattern as before (Figure 13). We can observe that there is a significant difference in locomotion even when the animals were treated with saline (Figure 13A). In the medium dose, the difference is significant again (Figure 13B). In this graph, it is stated even more clearly how amphetamine affects the two populations, as it
becomes easier to compare the status of all animals before and after the injection.

The outcome of this study is the fact that amphetamine affects in a higher rate all mice that lack expression of P4. This effect is dependent on the dose: the medium dose increases locomotion more than the low dose does. However, the high dose reverses the phenomenon: KO mice have a tendency to decrease locomotion faster than the WT mice.

3.4 Fluoxetine Affects Motivation of WT and KO mice Differently

Since fluoxetine has an anti-depressent effect, its application in mice is expected to increase the motivation of animals to escape from a stressful environment (in this case, the stressful factor was the Forced Swim Test). In previous trials of FST, animals were tested without the application of any substance. All animals were tested with the same protocol as in the fluoxetine trials for two days in a row. The analysis of the results showed no significant difference in the immobility between the WT and the KO mice during the first day (Figure 14A). However, during the second day the KO animals were less motivated to swim and thus they spent more time immobile than the WT animals (Figure 14B).

In the fluoxetine trials, we can notice that the mean values of immobility for both the WT and the KO are not significantly different in any part of the FST (Figure 16); on the trial day, during which all animals were naïve and not injected with any substance, as well as on the following days, when animals were treated either with saline or with fluoxetine, the mean values of immobility for both populations are quite similar to each other. However, we can observe a
tendency for the KO to be less depressed than the WT when they are treated with saline and more depressed than the WT, when they are treated with fluoxetine. This observation can be verified when the results are expressed in comparison with time (Figures 16C and 16E). The only significant difference in these two graphs concerns the timeline analysis of saline, where the KO mice are much less immobile than the WT.

In Figure 15, we can observe the effects of both treatments in the different populations. As it is expected, fluoxetine reduces the depression-like behavior for the WT mice (Figure 15A, p<0.05). However, fluoxetine has the exact opposite effect in the KO mice; animals who lack P4 manifest an unexpected behavior under the effect of an antidepressent drug and their activity is reduced when they are treated with fluoxetine (Figure 15B, p<0.05).

To summarize, both WT and KO animals have a similar motivation during the first day of the test. When animals are treated with saline, KO animals tend to be more motivated than the
WT ones, but there is no difference in their total immobility. When animals are treated with fluoxetine, KO animals tend to lose their motivation to swim compared to the WT mice who become more motivated; however, in this treatment as well, there is no difference in total immobility between WT and KO mice.

3.5 Effects of Fluoxetine Provocation in Locomotor Behavior

The effect of fluoxetine in locomotion was also studied. The analysis of the data allowed us to observe if fluoxetine causes any differences in locomotion in animals that lack the expression of P4. All animals acclimated for 30 minutes in the locomotion cages; after the acclimation, each animal was injected with fluoxetine and returned in the locomotion cages for 90 minutes.

When animals were treated with saline, KO mice were more active (Figure 17A). On the other hand, when animals were treated with fluoxetine, there was no significant difference in activity between the populations (Figure 17B). WT mice were more active only forty minutes after the injection with saline; after the 80th minute there is a peak in activity for the animals that were treated with fluoxetine (Figure 17C). This fact is in accordance with the expected effect of an antidepressant drug. KO mice were significantly more depressed when they were treated with fluoxetine and they were more active when they were treated with saline (Figure 17D).

In conclusion, fluoxetine is not responsible for any significant variation in the locomotor behavior between WT and KO mice. It tends to increase the locomotor behavior 40 minutes after injection in the WT mice. However, it doesn’t seem to have a similar effect in the KO mice. Moreover, saline treatment showed that KO mice are more active than the WT ones.

*Figure 17: Fluoxetine Provocation - Locomotor Behavior. The effect of saline (A) and fluoxetine (B) is plotted together with the comparison of the effect of each treatment for WT (C) and KO (D) mice. The white arrow indicates the time of injection. All data are expressed as mean value±SEM*
### 3.6 Genetic Origin of Animals Affects the Outcome of Research in Animal Models

Until now, all research in animal models included animals coming from Het/Het Breedings: for every KO mouse its WT sibling also participated in the study as a control. In order to examine if genetic origin can affect the result of these studies, fluoxetine provocation was also tested in animals coming from Homozygous Breedings (KO/KO and WT/WT Breeding). WT and KO mice were not siblings in this case. The motivation of these animals was tested in the Forced Swim Test and their locomotor behavior in the locomotion cages with the same protocols as for the animals from Het/Het Breedings.

In the FST, the mean value of immobility was not different between KO and WT, when the animals were treated with saline or fluoxetine (Figure 20D and 20F). Nevertheless, during the trial day, the KO mice were less motivated than the WT (Figures 20A and 20B). Moreover, WT animals were more motivated than the KO ones when they were treated with saline (Figures 20C and 20D) and tended to be less motivated when they were treated with fluoxetine (Figures 20E and 20F).

In **Figure 18** we can observe the effect of each treatment in the different populations. WT mice tended to be less motivated when they received fluoxetine, while KO mice tended to reduce their immobility period when they were treated with it. Nevertheless, we can only observe this fact.

**Figure 18:** Forced Swim Test – Fluoxetine Provocation – Animals from Homozygous Breedings. Comparison of the effect of each treatment between WT and KO mice. All data are expressed in mean value±SEM.
as a tendency, since none of these differences is significant.

In the results of the locomotor behavior, we can observe that when animals were treated with saline, WT mice were more active than KO mice (Figure 19A). On the other hand, when animals were treated with fluoxetine, WT mice were less active than the KO ones (Figure 19B). In both cases, it seems as the main difference between the two populations occurred during the acclimation period. Moreover, there is no significant difference between the two different treatments for WT and KO mice (Figures 19C and 19D).

In conclusion, there is not a difference in motivation between WT and KO mice when they are treated with fluoxetine in the FST. However, the tendencies that these animals show are different from the tendencies of animals from Het/Het Breedings. By comparing the results of
fluoxetine provocation between animals from Het/Het Breedings and animals from Homozygous Breedings, we observe that genetic origin has an impact on the final outcome.
DISCUSSION

4.1 Discovering the exact location of P4

Previous results from electron microscopy, in situ and immunohistochemistry studies showed that P4 is located in synaptic vesicles at the synaptic terminals of monoaminergic and cholinergic neurons. Cocaine provocation showed a higher increase in locomotion in KO animals compared to WT. Cocaine affects dopamine which can regulate locomotion. Since VMAT2 is responsible for storing dopamine into the synaptic vesicles, the initial hypothesis of this study is that P4 and VMAT2 share the same synaptic vesicle.

In order to prove that P4 and VMAT2 are co-localized, the Immunoprecipitation (IP) technique was optimized. According to this protocol fresh synaptosomes are incubated with magnetic beads and one antibody able to detect one of these proteins (i.e. P4). The outcome of the IP is loaded on a gel for Western Blot analysis and the final blot is incubated with an antibody able to detect the other protein (i.e. VMAT2). Generally, Immunoprecipitation is an efficient method to prove that two proteins are located in the same vesicle. Nevertheless, its success depends on the quality of the tissue and the affinity of the antibodies that are used.

The vesicles need to be intact; otherwise, IP will isolate only the epitope that corresponds to the used antibody and the final blot will not give any results when incubated with the second antibody. In our experiments with Synaptophysin, we could observe the Synaptophysin band in the final blot, but not the VMAT2 band. Likewise, when VMAT2 was used for IP, we couldn’t observe the Synaptophysin band. One possible explanation for this could be the fact that the vesicles broke during their preparation. Many factors can influence vesicles’ integrity, such as temperature, pH and reagents that are used. Most of these factors were controlled and kept constant during the experiments.

Furthermore, the antibodies that are used can affect the final outcome. There are data supporting the efficiency of the Synaptophysin and the VMAT2 antibodies which we used for Western Blot analysis. However, there are no published experiments that used these antibodies for IP. In the case of VMAT2, experimental data are even fewer, since this antibody is “home-made”. Until recently there were no commercial P4 antibodies in the market and thus an IP with P4 was not tested so far. Nonetheless, we currently possess a new P4 antibody with promising results on Immunohistochemistry and Western Blot analysis. Our next goal is to test this antibody in IP.

Certainly, there are also other methods that could be used in order to identify the exact location of P4 in the synaptic vesicles; some of them are more complex than IP, while others could expedite the current protocol. For example, a cross-linking protocol could create a bond between P4 and VMAT2, in case they are located close to each other. This is one way to augment the chances of precipitating the part of the vesicle’s membrane which contains the proteins of interest. Immunoprecipitation is still a promising protocol and with certain optimizations it could prove the initial hypothesis. Other techniques that could help towards this direction are Electron Microscopy and Stimulation Emission Depletion (STED) microscopy.

4.2 Memory is not Affected by P4. Is the Reward System Affected?

Since P4 is located in monoaminergic and cholinergic neurons, it is possible that its presence affects various behaviours that are connected to these neurons. In the present study,
we tested how it could influence the working and the reference memory; the results proved that P4 is not responsible for any dominant alternation in the process of memory. In this test, there was not any tendency that could discriminate WT from KO mice.

However, there was a difference in the reaction of mice against the rewards. Since the same kind of reward was used for all mice, this observation is hard to interpret. The weight of all mice was observed during the study and there was no metabolic difference between the two populations (Figure 21). As a result, differences in the metabolism can not explain this fact. Possible reasons to explain this difference could lie in the sensory system (different apprehension of smells or images) or in the reward system. The mesolimbic and the mesocortical pathway are responsible for the reward system and dopamine has a dominant role in both these pathways [37]. New studies, focused in this system could clarify if P4 has a significant role in it.

### 4.3 Function of P4 in the Dopaminergic Neurons

Apart from the behavioral studies, another way to test P4’s function is by provoking animals with various drugs that can block or activate targeted neurotransmitters. Pharmacological studies can reveal possible connections between neurotransmitters.

In this case, the amphetamine trials showed that mice that lack P4’s expression are more active when they are treated with amphetamine, compared to control mice. In previous trials, where cocaine was used to provoke both WT and KO animals, the results were similar to the ones that we retrieved from the amphetamine trials (data not presented in this study). KO mice react in a more vigorous way when they are treated with amphetamine, compared to WT mice; their locomotion reaches higher levels. This effect is equivalent to the dose of amphetamine that is injected, until the dose of 1.5 mg/kg. When the dose reaches 3 mg/kg, the KO mice start reducing the hyperactivity and after 40 minutes they become less active than the WT ones. This could be due to the oversaturation of the dopaminergic system with amphetamine, which happens faster when P4 is missing.

Amphetamine increases the levels of dopamine in the synaptic cleft, by triggering VMAT2 to release more dopamine from the synaptic vesicles and by forcing DAT to work in reverse and pump dopamine in the synaptic cleft. Cocaine has the same final outcome by interfering in the function of DAT. The lack of P4 makes the effect of these two drugs more evident in animals. Reserpine is an antipsychotic and antihypertensive drug that is able to block VMAT2. Future research on the effect of reserpine in the locomotor behavior of KO mice could provide us with more information about the function of P4 and indications if P4 can influence VMAT2.

### 4.4 Function of P4 in the Serotonergic Neurons

Fluoxetine inhibits the reuptake of serotonin and thus it augments the levels of serotonin in the synaptic cleft. It is a widely used anti-depressent and its application is expected to increase motivation. Fluoxetine trials of this study might have not indicated any evident...
difference between WT and KO animals, but they revealed a tendency for the KO mice to behave differently. For instance, KO mice from Het/Het Breedings seemed to have an expected reaction: when they received fluoxetine, they became less motivated to swim. However, the trials of animals from Homozygous Breedings showed an opposite tendency and thus it is difficult to draw any safe conclusions at the moment.

Future studies, where more animals will participate, evenly distributed between female and male mice can cast more light in the function of P4 in the serotoninergic neurons. This is a way to augment the sensitivity and specificity of our study and at the same time to observe possible differences between the two genders. Another aspect that could improve the final outcome is the dose of fluoxetine that is used; higher doses cause a more significant result in the tested animals [6]. Finally, it would be interesting to test how animals would react, if they were treated in a chronic basis instead of an acute dose. It is proved that subchronic and acute effects of antidepressent drugs are increased by chronic administration [6].

It is important to mention that the locomotion cages illustrated that the peak of fluoxetine’s effect in our mice occurred around forty minutes after the injection. Taking this into account, we can assume that the results of the FST could be different if all animals were tested one hour after injection instead of thirty minutes.

### 4.5 Factors That Can Affect Studies in Animal Models

Fluoxetine trials aimed to indicate a possible function of P4 in the serotoninergic neurons. Moreover, the design of this study could point any possible factors that can affect the final outcome of pharmacological studies. Our main interest was to observe if the breeding origin can alter the behavior of mice. For this reason, we tested separately animals coming from Heterozygous and Homozygous Breedings. The final results reveal more than one factors that could influence the final outcome.

The age and the weight of the animals can change significantly the results of the Forced Swim Test. More particularly, it is proved that many antidepressent drugs are more active in young animals than in older ones; the same observation stands also for animals with a low weight compared to those that weigh more [6]. Another crucial factor that can affect the response of animals to antidepressents is their housing; animals that are isolated or kept in cages with few animals, tend to be less active and more immobile when they are treated with antidepressents [6].

In all experiments presented in this study, all mice shared a cage with their siblings of the same gender. All the animals from Heterozygous Breedings were kept in cages of two mice, while the mice coming from Homozygous Breedings were kept in cages with at least three animals in them (in some cages there were even six animals in the same cage). Moreover, the animals from Heterozygous Breedings had a much wider range of age compared to the other group; the younger one was 10 weeks old on the first trial day, while the oldest one was 26 weeks old. The mice from Homozygous Breedings were between 13 and 15 weeks old during the first trial day. Likewise, the animals had equivalent differences in their weights (data not presented here). In order to test how significant the effect of age is on our results, all data will be analyzed again with the ANCOVA method, by calculating the covariance between treatment, genotype and age. Unfortunately, there is no way to calculate the variations that are caused by the different properties of housing, since there was not a constant number of mice per cage for the animals from Homozygous Breedings.

Moreover, it is known that retesting the animals in the FST has an effect in the final
outcome [6]. During the first day, animals are naïve to this stressful experience; on the second day they can recall this experience and their reaction depends on their motivation. There are two reasons explaining why we chose to apply a trial day, during which all animals were naïve: i) in order to normalize their reaction during the following days, when they were treated, ii) previous studies showed no difference between the two genotypes during the first day, but a difference in motivation during the second day. However, it could be interesting to observe the effect of fluoxetine when all animals are naïve.

Consequently, it is now apparent that genetic origin has a significant influence in research in animal models. Animals from Homozygous Breedings are easier to maintain (larger production of litters of the same genotype, ability to house more litters in the same cage) and thus they looked appealing for behavioral and pharmacological studies. However, from now on only animals from Heterozygous Breedings will be tested. It may be harder to retrieve WT and KO siblings, but they are able to cancel the factors that were mentioned above: any genetic variations due to their ancestors are cancelled and thus the final outcome will be normalized. Moreover, WT and KO siblings share the same age (thus approximately the same weight as well) and the same cage.

Pharmacological studies (as well as the behavioral ones) demand many factors to be taken into account in order for them to be designed, they are time-consuming and they require a generous financial support. However, they can provide us with invaluable information about the role of P4 and, thus new pharmacological studies are currently under design (i.e. reserpine provocation).

4.6 Conclusions

From all the molecular, behavioral and pharmacological studies that were realized until now, it is evident that P4 is a unique protein: it is the only transporter evident in both the monoaminergic and the cholinergic system. Since both these systems’ malfunction is responsible for various neurologic and psychiatric disorders (i.e. Parkinson disease, schizophrenia, attention-deficit hyperactivity disorder and depression), it is really important to comprehend its exact location as well as the signal that it is transporting. Several steps have already been done towards this direction; we now know the neurons were it is located, several behaviors that can affect and the fact that it has an impact in the dopaminergic and serotoninergic neurons, when they are provoked by drugs. However, there are still many steps left, until we will be able to fully comprehend its properties. By achieving that, we might be able to understand more the mechanisms behind several disorders and maybe even manage to treat them more efficiently. Therefore, more studies need to be designed and new techniques need to be exploited.
ACKNOWLEDGEMENTS

This master thesis was conducted in the Unit of Developmental Genetics, Neuroscience Department, Uppsala University in Sweden, from June 2009 until May 2010. Two people made this study possible: Klas Kullander and Lina Emilsson. I will be always obliged to Klas for giving me the chance to be introduced in the amazing world of research. I will always admire his ability to co-ordinate diverse projects and to be helpful, accessible and supportive under all circumstances. Lina is the best supervisor I could ever imagine! Thanks to her I became able to think scientifically and work independently. Her remarks helped me understand how to plan an experiment from scratch and got me a step closer to write and present as a scientist.

I am grateful to the Co-ordinator of the MSc in Biomedical Engineering in Greece, Nikolas Pallikarakis who accepted this joint master thesis work between Uppsala University and University of Patras. I have no words in order to thank enough Eleni Panoutsopoulou, the administrator of the MSc in Patras. Without her I would still be lost in bureaucracy; she was always available and supportive. I wish all the administrators could become half productive as she is! I would also like to thank my supervisor in Greece Zoi Lygerou, for all her support and her remarks that made this thesis looking much more comprehensive. Last but not least, I am thankful to Theofilos Madamadiotis and Stavros Taraviras, members of the examination committee.

Two scientists helped a lot the Immunoprecipitation protocol to evolve: Johannes Friedrich Zander shared his invaluable experience on isolating synaptic vesicles and optimizing the IP, Salam El Mestikawy donated us the VMAT2 antibody.

I am more than thankful to all these people for making my life in the lab and in Uppsala much easier and entertaining: Cecilia Yates for all her help with paperwork and (hard-to-find-in-Sweden) accommodation, Martin Larhammar for all his help in the FST, Kasia for helping me on finding my way in the lab and finding lost chemicals at the back side of the shelves, Christiane for her help with the confocal microscope and her suggestions about IP, Malin for taming the Zygous monster for me, Nadine for making me feel comfortable for being a tidiness freak, Sofia for being an inspiring professor in Swedish, Casey, Lena, Emma, Nadine, Eduardo, Fernando for making lunch time an unforgettable experience and for sharing a beer in the cold winter nights or the bright summer ones.

I am also thankful to Phoebe Petroulias for designing the cover of this thesis: as an excellent architect, you did an excellent job! Special thanks go to my friends in Stockholm Valentina and Joao. I hope our paths will never stop crossing each other. All my friends in Greece deserve my gratitude for the endless conversations we had on Skype.

I am not sure if I should thank Uppsala or the Japanese exchanges for bringing Maho Kawai in my life. You made my life full again, you filled my future up with dreams, you help me to become better every single day. これほんの始まりだって私にはわかってる。愛してる。

Finally, I want to honor the people who influenced my life and my personality more than anyone: my parents Athena and Polychronis and my precious brother and sister Aris and Terry. Η πίστη σας σε εμένα, οι συμβουλές σας, μα πάνω απ’ όλα η αγάπη σας θα είναι για πάντα η κινητήρια μου δύναμη. Σας ευχαριστώ για όλα!
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