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The localization and function of the synaptic vesicle carrier P4

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Abstract

P4 is a Solute Carrier with high expression pattern in the brain, especially in monoaminergic and cholinergic areas. The presence of P4 in both these areas is highly unusual and interesting for the research regarding many neurological disorders.
In-Situ hybridisation, immunohistochemisty and PLA experiments were performed to investigate the presence of P4 in the brain, in neuromuscular junctions and in adrenal glands, the colocation between P4 and VAChT respectively P4 and VMAT and the possibility that P4 is involved in the transport of acetyl choline and monoamines.

Keywords

P4, transport, monoamines, acetyl choline, VMAT, VAChT, immunohistochemistry, neuromuscular junctions, neurological disorders

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The localization and function of the synaptic vesicle carrier P4

Jeanna Högberg

Populärvetenskaplig sammanfattning

P4 är ett protein som är starkt uttryckt i hjärnan. Intressant nog så är det uttryckt i både områden som producerar monoaminer, så som seretonin och dopamin, och i områden som producerar acetylcholin, som får våra muskler att röra sig. Detta gör att det är ett mycket intressant protein för forskning på neurologiska sjukdomar där ofta både monoaminer och acetylcholin är inblandade såsom i Parkinson och Alzheimers sjukdom.

Man har funnit att P4 är lokaliserat till små synaptiska vesiklar och i detta examensarbete undersöker vi om P4 sitter i samma vesiklar som de kända transportproteinerna VAChT och VMAT2. Den ena, VAChT, transporterar acetylcholin och den andra VMAT2, monoaminer. Det är också intressant att ta reda på vad som finns i de vesiklar som P4 sitter i för att få en bättre uppfattning om vad P4 transporterar. Därför vill man rena fram stora mängder av vesiklar med P4 i och därefter även jämföra med vesiklar i vävnader utanför hjärnan där P4 är starkt uttryckt.
I detta arbete fick vi inga tydliga indikationer på om P4 och VAChT respektive P4 och VMAT2 är lokaliserade till samma vesiklar, däremot fann vi att proteinet P4 är starkt uttryckt i både binjurar och i neuromuskulära kopplingar (”neuromuscular junctions”).

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List of content

Abbreviations

Ab	Antibody
ACh	Acetylcholine
CNS	Central Nervous System
DAT	Dopamine transporter
GABA	gamma-Amino butyric acid
Gp	Guinea pig antibodies
Gt	Goat antibodies
NMJ	Neuromuscular Junction
NT	Neurotransmitter
PFA	Para-Formaldehyde
PLA	Proximity Ligation Assay
Rb	Rabbit antibodies
SLC	Solute Carrier
SSV	Small Synaptic Vesicles
SSC	Saline-sodium citrate
SYP	Synaptophysin
TBS-T	Tris Buffered Saline-Tween
VAChT	Vesicular Acetyl Choline Transporter
VMAT	Vesicular Monoamine Transporter

1 Introduction

Signals between neurons in the brain are transmitted either by electrical synapses or chemical synapses. In electrical synapses, a passive flow of ions transport electric current from one neuron to another. In chemical synapses signals are transported by small molecules called neurotransmitters. The major transmitter systems are the monoaminergic, cholinergic, GABAergic and the glutaminergic systems. These systems affect for example our mode, behavior, cognition, learning and memory. Imbalance between neurotransmitter systems are involved in neurological disorders such as bipolar disease, schizophrenia and Alzheimer´s disease. Therefore investigating neurotransmitter systems are important for understanding both normal and pathological human behaviors.

1.1 P4 localization

With the human genome project completed, the setup of human genes are now mapped. However, the functions of many genes are still unknown. Such a gene is the solute carrier P4 that was identified by Jorgensen *et al.*, in 2005. *In–situ* hybridization experiments have shown that P4 is expressed in monoaminergic as well as in cholinergic associated areas. Monoamiergic brain regions include locus ceruleus, substantia nigra and raphe nuclei. These areas are responsible for the production of noradrenalin, dopamine and serotonin. Cholinergic expressing brain areas are on the other hand responsible for the production of acetylcholine.
P4 has been localized to symmetrical synaptic vesicles with immunoflourescent histochemistry and electron microscopy (Emilsson *et al.*, unpublished data). Further, P4 has been localized to synaptic vesicles in monoaminergic and cholinergic neurons by co-staining P4 with vesicular monoamine transporter 2 (VMAT2) and vesicular acetylcholine transporter (VAChT) in adult mouse spinal cord sections. However, no co-expression with P4 and vesicular glutaminergic transporter 1 (VGLUT1) was identified (Emilsson *et al.*, unpublished data).
The exclusive participation of a specific protein in the monoaminergic and the cholinergic neurotransmitter systems makes P4 in to a unique protein since no other protein, molecule or drug, specifically addresses these neuronal populations and circuitries. Furthermore, the participation of both these neuronal systems in mental disorders and motor-diseases does not only make this possible carrier in to a new exiting protein in neuroscience but also in research regarding diseases affecting higher brain functions.

1.2 P4, a member in the SLC10 family

P4 is one of the members in a group of solute carrier proteins, the SLC10 family, formerly called the "sodium/ bile acid co-transporter family".[1] The expression pattern of P4 shows high expression in brain, placenta and pancreas (Splinter *et al*., 2006). The high expression of P4 in the brain was unexpected because P1 and P2, two other members of the SLC10 family, are bile acid transporters of hepatic and intestinal origin with important roles in liver physiology. P6, another member in the SLC10 family, is a sodium-dependent organic anion transporter and is considered to play an important role for the cellular delivery of prehormons to testes, placenta, adrenal gland and probably other peripheral tissues.[2] There are two other members in the SLC10 family with unknown function, P5 and P7 and none of those are highly expressed in the brain.
The rat P4 protein consists of 437 amino acids estimated to be a seven transmembrane domain topology (N_{exo}/C_{cyt}). Compared to the other members in the SLC10 family P4 has a longer N-terminal and lacks an "ALGMMPL"-motif.[3] These topology differences might be the reason why P4 is highly expressed in the brain whereas the other members in the SLC10 family are not.

1.3 Functional studies on P4 knock out mice compared to wild type mice

Functional studies, performed to investigate P4 have been done by behavioral studies on P4 knock out (P4 KO) mice. These studies have shown that P4 KO mice are less motivated compared to the wild type mice, when tested in the Porsolt forced swim test. Lack of motivation is moreover a behavioral associated to depression-like behavior in humans. In locomotion tests KO mice present an increased activity. Motor behavior tests revealed increased motor coordination in the Rotarod test and a possibly increased learning capability (Emilsson *et al.*, unpublished data). The P4 KO mice also have an increased sensitivity to mechanical stimuli in the Von Frey test, which indicate that P4 transports a molecule or component that has effects on the sensation of pain. In summary, P4 seems to affect mood, coordination and the sensation of pain.

The behavioral characteristics that the P4 KO mice show are similar to effects expected if the serotonergic and the dopaminergic systems are altered, these include depression-like behavior, increased activity and altered coordination of movements. One hypothesis is that P4 is a transporter that is involved in the transport of monoamines such as serotonin and dopamine into vesicles.Where serotonin participates in the regulation of emotional states, hormone secretion, control of movement, sensation of pain and higher brain functions such as cognition. Dopamine on the other hand plays an important role in the reward system, function of the memory and coordination of movements. Pathologically, serotonin is related to anxiety, depression, schizophrenia and feeding disorders[4]. Dopamine is known to be

involved in diseases such as schizophrenia, depression, Parkinson's disease and drug addiction[5]. In some diseases such as depression, both serotonin and dopamine function are altered, shown by increased association between depression and the genes for the serotonin transporter (SERT) and the dopamine transporter (DAT)[6].

1.4 Chemical synapses

In chemical synapses signals between neurons are transmitted over the synaptic cleft by neurotransmitters (NT) or neuropeptides. The neurotransmitters are stored and transported in small synaptic vesicles (SSV) and the neuropeptides in large dense-core vesicles (LDV) in the presynapses. When an electrical signal reaches the end of a presynaps, the vesicular membrane of the SSV fuses with the presynaptic membrane and the transmitters are released into the synaptic cleft where they can bind to postsynaptic receptors. The binding of a neurotransmitter to a receptor results in signal transmission through an opening of ion permeable channels or by activating intracellular signalling cascades e.g. phosphorylation processes. After a signal transmission the neurotransmitters has to be degraded or removed from the synaptic cleft and packed into presynaptic vesicles again. Different kinds of proteins participate in this process depending on which neurotransmitter that are being degraded or again taken back into the vesicle.[7]

The reuptake and transport across the presynaptic membrane are often a sodium-depended transport mediated by transporter proteins of the solute carrier family. Members in the SLC1- and SLC6-family are for example transporter proteins of dopamine, 5-HT, norepinephrine, glutamate, GABA and glycine.[8] Ion coupled transport of a substrate, in this case neurotransmitters, against their electrochemical gradient allow accumulation of a substrate inside a membrane body, and this is the principle for other solute carriers as well.
One type of members of the SLC families are proteins acting for example as passive transporters, channels where ions can pass the membrane without energy consumption, or ion-coupled symporters, two or more ions transports across the membrane using the electrochemical gradient, also called co-transporters. The proteins are situated in plasma membrane and other cellular membrane compartments. [9]

1.5 Acetylcholine

Acetylcholine (ACh) was the first neurotransmitter to be identified. It is synthesized in the nerve terminals from acetyl coenzyme A and choline, catalyzed by cholin acetyltransferase. The degradation of ACh is carried out by acetylcholinesterase. One molecule of acetylcholinesterase degrades about 5000 ACh molecules per second. Acetylcholinesterase is present in high concentration in the synaptic cleft to ensure fast degradation of the neurotransmitter.[10]

ACh is present in both the central and peripheral nervous system. In the peripheral nervous system it activates muscles and it is a major neurotransmitter in the (parasympathetic) autonomic nervous system.[11] Vesicular Acetylcholine transporter (VAChT) is responsible for transporting acetylcholine into small synaptic vesicles in the presynaps. VAChT loads approximately 10,000 molecules of ACh into one cholinergic vesicle.[12] P4 has been co-localized with both VAChT and acetylcholinesterase.[13]

1.6 Monoamines

Monoamine transmitters are derived from aromatic amino acids. Examples of monoamines are serotonin, dopamine, epinephrine (commonly called adrenaline), norepinephrine (noradrenalin) and histamine among others.

Vesicular Monoamine Transporter (VMAT) carries monoamines into small synaptic vesicles. Most of the monoamines have separate transporters for the reuptake of the monoamines from the synaptic cleft for example the dopamine transporter (DAT) and the specific serotonin transporter (SERT). Addictive drugs such as amphetamine and cocaine have, among other effects in the synaps, their psychotropic effect by inhibiting those transporters. The transporters are also targets for pharmaceutics. Selective serotonin reuptake inhibitors (SSRI) are antidepressant drugs that increase the amount and time of serotonin in the synaptic cleft. This pharmacological provocation results in increased signalling of serotonin. Monoamine oxidase (MAO) and catechol *O*-methyltransferase (COMT) degrades monoamines and is target for antidepressant drugs as well.[14]

1.7 Adrenal Glands

The adrenal glands release hormones responsible for the maintenance of internal fluids, sodium and potassium levels and responses to stress. The adrenal glands are positioned in the thoracic abdomen superior of the kidneys. The glands consist of an inner medulla and outer cortex. The cortex synthesizes and secretes corticosteroid hormones, for example cortisol. Chromaffin cells in the adrenal medulla release the catecholamines epinephrine (adrenaline), norephinephrine (noradrenalin) and dopamine.[15] Beyond the function mentioned earlier the catecholamines also function as hormones in the autonomic nervous system, commonly called the “fight or flight”-system. The catecholamines are released directly to the blood stream as a response to preganglionic sympathetic fibers that fire tonically at low frequencies and release acetylcholine to the chromaffin cells in the adrenal medulla. Acetylcholine activates nicotinic receptors at chromaffin cells, which as a response releases the

catecholamins.[16] When investigating the function of P4, the adrenal glands might be of specific interest since these glands release both acetylcholine and catecolamines, which means that both the cholinergic and the monoaminergic system participate in the function of these glands.[17]

1.8 Neuromuscular junctions

The synaptic cleft between a motor neuron and the muscle fibers is called neuromuscular junction. Muscular contraction is executed by motor neurons that release acetylcholine at the neuromuscular junction due to an electrical signal causing Ca^{2+}-influx. Motor neurons from the spinal cord project to the muscle fibers in skeletal muscles. One motor neuron branches and innervates many muscle fibers in the same muscle.[18] The small branches end in a folded depression on the surface of the muscle fiber. The postsynaptic receptors are located to the folded muscle membrane.[19] The endplate of the motor neuron and the folded postsynaptic membrane results in a characteristic appearance of the NMJ.[20]

Alpha-Bungarotoxin is a ligand to the acetylcholin receptor in the postsynaps. Alpha-bungarotoxin conjugated with a fluorescent labelled mouse antibody can be used to detect the synaptic membrane at the neuromuscular junction.

1.9 The overall aims of the research regarding P4

Solving the following questions is the main part of the research regarding P4:

-What does the vesicle carrier P4 transport and what cellular functions does this protein participate in?

-What are the factors that makes P4 a part of the function in both cholinergic and monoaminergic neurons?

-What phenotypic changes are detectable in a P4 knock-out mouse?

The present degree project was divided in to three parts (1-3) with different aims and strategies, as presented below:

1) Aim: Verify that the P4-gene is not expressed in our genetically modified mouse, the P4 KO mice line.

Strategy: P4 KO mice verification was conducted by *In situ* hybridization and immunohistochemistry studies on cryo tissue sections from wild type and KO mice spinal cord.

2) Aim: Find structures from which we can purify the P4 protein
Strategy: To be able to investigate the properties of a P4 vesicle, the function of P4 within the vesicle and most of all identify the hypothesized substrate of P4 we need to purify vesicles with the P4 protein in high quantities. One possibility to obtain large amounts of vesicles is to purify vesicles from synapses between the motor neurons and the muscle fibers, the neuromuscular junctions (NMJ). We hypothesize that there are vesicles in the NMJ that include P4 due to the large content of acetylcholine vesicles. The adrenal gland is responsible for secretion and storage of catecholamine (Serotonin, dopamine and noradrenalin) vesicles.[21] Cholinergic neurons innervate the adrenal gland, which means that both the cholinergic and the monoaminergic system are present here. We have chosen to do immunohistochemical studies to investigate if P4 is expressed in these structures.

3) Identify in which specific vesicles P4 is located by the Duolink assay.
Strategy: Duolink is a proximity ligation assay (PLA) that can detect protein interaction within a range of 40 nm, which is about the size of a small synaptic vesicle. With this method we will investigate the hypothesis that P4 is located to the same vesicles as VMAT2 and VAChT respectively. If we can verify that they are expressed in the same vesicles it will give increased information to provide hypothesis about the function of this carrier and the substrate that it is transporting. To begin with the PLA experiments have to be optimized for our specific antibody P4.

Solving these questions is only a small part of the research around P4. Purifying vesicles containing P4 for mass spectrometry, preparing the tissue samples for ISH, IHC and PLA and functional studies is done parallel to this work.

2 Material and Methods

TECHNIQUES

2.1 In situ Hybridization

In situ Hybridization is a method used to localize mRNA sequences in a tissue sample or in cell preparations. A complementary strand (probe) of the mRNA sequence of interest is hybridized to the mRNA in the sample. The complementary oligonucleotide is labelled either with a modified ddNTP at the 3' or the 5'-end of the probe or with a 3'-tail with 5-50 modified nucleotides. For visualization we have used digoxigenin (DIG)-labelling which is nonradioactive 3'-labeling method.

Experimental procedure: Slides with spinal cord cryo sections (40 nm) were dried for 20 minutes in autoclaved cuvettes. The tissue were fixed in 4% Para formaldehyde for 10 minutes in room temperature and washed in 1xPBS 3x5 minutes, the tissue were then treated with Proteinas K (0.15 µl/1 ml 10 mM Tris-HCL, pH 8.0) for 8 minutes and refixed in 4% Para formaldehyde for 5 minutes. Washed 2x5 minutes in 1xPBS, acetylated for 10 minutes in a beaker, with stirring, in 197 ml depc-water, 2.7 ml triethaolamine, 330 ul 37% HCl and 500ul acetic anhydride added right before the slides were placed in the beaker. To permeabilize the tissue a solution of 1% Trition and RNAse free PBS were added to the slides and the slides were incubated for 30 minutes in room temperature in a humidified chamber with 50% form amide and 5xSSC. Prehybridization buffer were added and slides were incubated for 2 hours in 65°C. The hybridization probes (SLC10A4 Probe (Pkk296) 193ug/ul) were heat denatured in hybridization buffer at 80°C for 5 minutes, cooled on ice for 5 minutes and then pre-warmed to 65°C. Prehybridization buffer were exchanged to the hybridization buffer with the probes and covered with a cover slip and incubated overnight (ON) in 65°C. Next day the slides were placed in a beaker with pre-warmed 5xSSC(65°C), cover slip were removed and the slides moved to a beaker with pre-warmed 0.2xSSC and incubated in 65°C for 1 hour. 0.2xSSC were added to the slides at room temperature for 5 minutes and then TBS for 5 minutes. The slides were incubated for 1 hour with blocking solution containing B1 buffer and blocking mix (1:10). The slides were incubated ON in (4°C) in the same blocking solution plus anti-digAP antibody (1:5000). The third day the slides were washed in 50 mL TBS-T with 100 mL 1000xLevamisol 3x5 minutes then in NTMT with levamisol (10ul 1000x Levamisol/5ml NTMT) in humidified chamber 3x5 minutes. Developed in 37°C in 50 ml PVA (5%), 0.25 ml $MgCl_2$ (1M), 86ul NBT, 86ul B-CIP and 50ul 1000xLevamisol. When signal intensity were optimal slides were washed in water, 50% glycerol were added and cover slips placed on top of the sample and fixed with nail polish.

2.2 Immunohistochemistry

Immunohistochemistry (IHC) is a method used to localize protein expression in a tissue sample. Antibodies are used that are produced to bind specific antigens in a combination with flourofor-labelled secondary antibodies targeting the primary

antibody. IHC targeted proteins are then visualize in an epi-fluorescent or confocal microscope.

To identify P4s expression in mice spinal cord tissue sections, the polyclonal P4 antibody produced in rabbit was used together with a secondary fluorophor-conjugated goat anti rabbit polyclonal antibodies (Table 1).

Table 1. Immunohistochemistry, antibodies and dilutions

Adrenal gland	**Primary ab (conc)**	**Secondary ab (conc)**
	P4 (1:500)	Cy 3 (1:100)
	VAChT (1:500)	Cy 5 (1:200)
		DAPI (1:1250)
	Negative control	Cy 3 (1:100)
		Cy 5 (1:200)
		DAPI (1:1250)

Neuromuscular junctions	**Primary ab (conc)**	**Secondary ab (conc)**
	VAChT (1:500)	Cy 3 (1:100)
	P4 (1:500)	Cy 5 (1:200)
		Bungaro toxin (1:1000)
		DAPI (1:1250)
	Negative control	Cy 3 (1:100)
		Cy 5 (1:200)
		Bungaro toxin
		DAPI (1:1250)

Spinal cord VAChT	**Primary ab (conc)**	**Secondary ab (conc)**
	VAChT (1:100)	Cy 3 (1:100)
		DAPI (1:1250)
	VAChT (1:250)	Cy 3 (1:100)
		DAPI (1:1250)
	VAChT (1:500)	Cy 3 (1:100)
		DAPI (1:1250)
	VAChT (1:1000)	Cy 3 (1:100)
		DAPI (1:1250)
	Negative control	Cy 3 (1:100)
		DAPI (1:1250)

Spinal cord VAChT, SYP	**Primary ab (conc)**	**Secondary ab (conc)**
	VAChT (1:1000)	Cy 3 (1:100)

	SYP (1:400)	Alexa 488 (1:200) DAPI (1:1250)
	VAChT (1:2000)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	VAChT (1:3000) SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	VAChT (1:4000)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	Negative control SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)

Spinal cord P4, SYP	**Primary ab (conc)**	**Secondary ab (conc)**
	P4 (1:250) SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	P4 (1:500) SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	P4 (1:1000) SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)
	P4 (1:2000) SYP (1:400)	Cy 3 (1:100) Alexa 488 (1:200) DAPI (1:1250)

Experimental procedure: Mouse tissue from spinal cord, adrenal gland and neuromuscular junctions were dried and marked with Pap pen, the tissue were washed 3x1 min in 1xPBS, and blocked for one hour with blocking solution containing triton (0.3%), Horse serum (2%) and BSA (1%). Primary antibodies were added according to table 1. Primary antibodies were incubated ON, 4 °C. Antibodies were added in antibody solution containing triton (0.01%) H. Serum (2%) and NaAc (0.01%). The Slides were washed 3x5 min in 1xPBS, Slides with Synaptophysin were incubated with Synaptophysin (1:400) ON in 4 °C. The slides were washed 3x5 min in 1xPBS. The Secondary antibodies (according to table 1) were added in antibody solution and incubated for 1h in room temperature. Slides were washed 3x10 min in 1xPBS, mounted with DIG mount medium, nail polish were used to hold the glass.

2.3 PLA DuolinkTM By Olink Bioscience

Duolink is a technique that can detect protein interactions with a resolution of 40 nm, the approximate size of a SSV. In comparison, the resolution of IHC is between 200-

900 nm. Hence, Duolink makes it possible to detect if P4 and VAChT respective P4 and VMAT2 is localized to the same vesicles or not, knowledge that will provide information regarding what kind of vesicles that P4 is located to.
Duolink™ is an in situ PLA-method developed by Olink Bioscience. The method enables localization, detection and quantification of individual proteins, protein interactions and protein modifications. The method can be used in tissue samples on glass slides or in fixed cells.[22]

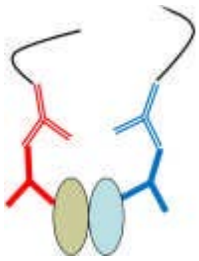


Figure 1. The primary antibodies binds to an antigen on the target protein, secondary antibodies binds to the primary antibodies. An oligonucleotide is attached to the secondary antibodies.

The PLA method is based on primary antibodies that attaches to the antigen presented on their target proteins that in a next step interact with secondary antibodies with attached PLA probes. The PLA-probes have unique oligonucleotides that are coupled to each other if they are in proximity (i.e. within 40 nm) (Figure 1). When ligated, the oligonucleotides form a circle with the PLA probes. One of the PLA probe oligonucleotides is constructed as a primer and when polymerase and nucleotides are added, a rolling-circle amplification can take place. For visualization a fluorofor is added and binds specifically to a repetitive sequence on the DNA that is produced in the rolling-circle amplification. The signals can be visualized in fluorescent microscopes and will give high spatial resolution.[23]
To analyse the results image analysis softwares are used, e.g. Blobfinder, software optimized for image analysis of individual PLA signals developed by the Centre for Image Analysis, Uppsala University.

Experimental procedure: Tissue from mouse spinal cord were dried and marked with Pap pen, the tissue were washed 3x1 minute and blocked for one hour with blocking solution from Olink. The P4 antibody were added in antibody solution from Olink and incubated over night or 48 hours in 4 °C. Then the slides were washed 3x5min and incubated ON with primary antibody, including anit-rabbit P4 polyclonal antibody (obtained from J. Geyer Justus-Liebig University of Giessen) anti-guinea pig VAChT (Millipore), anti-mouse Synaptophysin (SYP) (Sigma)), Bungarotoxin, alexa 488 conjugated (Invitrogen), according to table 2. The slides were washed 3x5min and PLA probe solution were added in antibody solution from Olink and the slides were incubated for 2 hours. PLA probe solution was tapped off and the slides were washed in 1xTBS-T 3x5min. The slides were incubated for 15 minutes with Duolink Hybridization solution and washed for 1 minute. Duolink Ligase (1:40) in Ligation solution were added to slides and incubated for 15 minutes. Washes 2x2 minutes. Polymerase (1:80) added in amplification solution and slides were incubated for 90

minutes. Washes 2x2minutes. Detection solution and Fitch anti mouse 488nm (1:200) added to the slides and incubated for 60 minutes. The last wash is done in five steps:
1) 2x SSC for 2 min
2) 1x SSC for 2 min
3) 0.2x SSC for 2 min
4) 0.02x SSC for 2 min
5) 70% EtOH for 1 min

Before visualization 7 droops of Duolink Mounting Medium were applied on the sample slide and a cover slip placed on top of the sample. Analyze were done in fluorescence microscope.

F nothing else is stated, are all incubations performed in humidified chambers in 37°C and all solutions from Olink are stocks diluted (1:5) in autoclaved water and all slides were washed in chambers with TBS-T under gentle agitation. Dilutions and antibodies used are visualized in table 2.

Table 2. **PLA Antibodies** Scheme over primary and secondary antibodies that were used in each experiment, concentration of primary antibody, which PLA probes and dilutions of PLA probes.

Experiment 1	**Antibody**	**Conc**	**Sec Ab**	**Type**	**Comment**
Slide 1	P4	1;500	Rb+/Rb-	Optimization	Our blocking solution
Slide 2	P4	1;500	Gp+/Rb-	Negative control	Our blocking solution
Slide 3	VMAT2	1;50	Gt+/Gt-	Optimization	Our blocking solution
Slide 4	VAChT	1;1000	Gp+/Gp-	Optimization	Our blocking solution
Slide 5	P4	1;500	Rb+/Rb-		Duoblock
Slide 6	P4	1;500	Gp+/Rb-		Duoblock

Experiment 2	**Prim Ab 1**	**Prim Ab 2**	**Conc (Ab1/Ab2)**	**Sec Ab**	**Type**
Slide 1	P4	VAChT	1;500/1;250	Gp+/Rb-	Optimization
Slide 2	P4		1;500	Gp+/Rb-	Negative control
Slide 3	VAChT		1;250	Rb-/Gp+	Negative control
Slide 4	VAChT		1;250	Gp+/Gp-	Positiv control

Experiment 3	**Prim Ab 1**	**Prim Ab 2**	**Conc (Ab1/Ab2)**	**Sec Ab**	**Type**
Slide 1	P4	VAChT	1;500/1;100	Rb+/Rb-	Optimization
Slide 2	P4		1;500	Rb+/Gp-	Negative control
Slide 3	VAChT		1;100	Gp+/Gp-	Positive control
Slide 4	VAChT		1;100	Rb+/Gp-	

Experiment 4	**Prim Ab 1**	**Prim Ab 2**	**Conc**	**Sec Ab**	**Type**
Slide 1	VAChT		1;100	Gp+/Gp-	Positive control

Experiment 5	Prim Ab 1	Prim Ab 2	Conc	Sec Ab	Conc
Slide 1	P4	VAChT	1;500/1;100	Rb+/Rb-	Optimization
Slide 2	VAChT		1;100	Gp+/Gp-	Positive control
Slide 3	VAChT		1;100	Rb+/Gp-	Negative control

Experiment 7	Prim Ab 1	Prim Ab 2	Conc	Sec Ab	PLA probe dilution (Ab+/Ab-)	Type
Slide 1	P4		1;500	Rb+/Rb-	(1;5/1)	Optimization
Slide 1b	P4		1;500	Rb+/Gt-	(1;5/1)	Negative control
Slide 2	P4		1;500	Rb+/Rb-	(1/1;5)	Optimization
Slide 2b	P4		1;500	Rb+/Gt-	(1/1;5)	Negative control
Slide 3	P4		1;500	Rb+/Rb-	(1;5/1;5)	Optimization
Slide 3b	P4		1;500	Rb+/Gt-	(1;5/1;5)	Negative control
Slide 4	P4		1;500	Rb+/Rb-	(1/1)	Optimization
Slide 4b	P4		1;500	Rb+/Gt-	(1/1)	Negative control
Slide 5	VAChT		1;1000	Gp+/Gp-		Positive control
Slide 6	VAChT		1;1000	Gp+/Gt-		Negative control

Experiment 7	Prim Ab 1	Prim Ab 2	Conc	Sec Ab	PLA probe dilution (Ab+/Ab-)	Type
Slide 1	P4		1;500	Rb+/Rb-	(1;5/1)	Optimization
Slide 1b				Rb+/Rb-	(1;5/1)	Negative control
Slide 2	P4		1;500	Rb+/Rb-	(1/1;5)	Optimization
Slide 2b				Rb+/Rb-	(1/1;5)	Negative control
Slide 3	P4		1;500	Rb+/Rb-	(1;5/1;5)	Optimization
Slide 3b				Rb+/Rb-	(1;5/1;5)	Negative control
Slide 4	P4		1;500	Rb+/Rb-	(1/1)	Optimization
Slide 4b				Rb+/Rb-	(1/1)	Negative control
Slide 5	VAChT		1;1000	Gp+/Gp-		Positive control
Slide 6	VAChT		1;1000	Gp+/Gt-		Negative control

Experiment 8	Prim Ab 1	Prim Ab 2	Conc (Ab1/Ab2)	Sec Ab	PLA probe dilution (Ab+/Ab-)	Type
Slide 1	P4	VAChT	1;1000/1;1000	Rb+/Gp-	(1;5/1;5)	Optimization
Slide 2	P4		1;500	Rb+/Rb-	(1;5/1;5)	Optimization
Slide 3	P4		1;1000	Rb+/Rb-	(1;5/1;5)	Optimization
Slide 4	P4		1;2000	Rb+/Rb-	(1;5/1;5)	Optimization
Slide 5	P4		1;500	Rb+/Gt-	(1;5/1;5)	Negative control
Slide 6	P4		1;1000	Rb+/Gt-	(1;5/1;5)	Negative control
Slide 7	P4		1;2000	Rb+/Gt-	(1;5/1;5)	Negative control
Slide 8	VAChT		1;1000	Gp+/Gp-	(1;5/1;5)	Positive control
Slide 9	VAChT		1;1000	Gp+/Gt-	(1;5/1;5)	Negative control

2.4 Ethical considerations

Animal care, breeding, and experimental procedures were conducted according to instructions from the local ethical committee at Uppsala University and guidelines from European Communities Council Directive (86/609/EEC) were followed.
P4 heterozygote mice are from Texas A&M Institute for Genomic Medicine bred from the 129/SvEvBrd strain.

3 Results

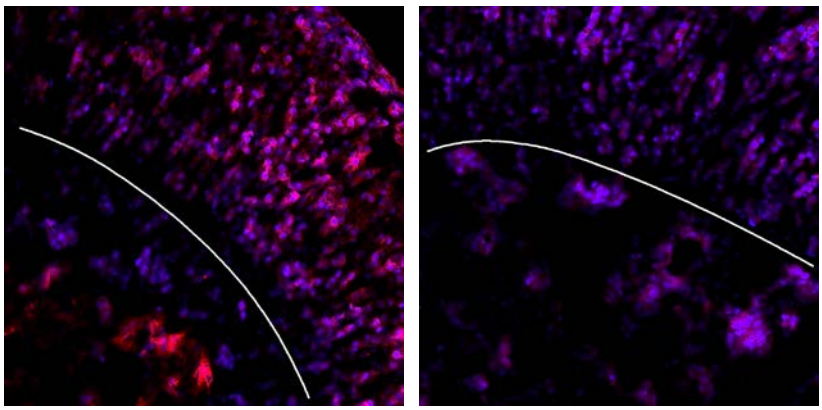
3.1 In situ hybridization results

The *In situ* hybridization was done to verify the genetically modified P4 mouse. The spinal cord sections of the P4 KO mouse showed no gene expression of P4. Positive control showed expression in the region of the motor neurons. IHC experiments showed that P4 is expressed in the Adrenal gland as well as in neuromuscular junctions.

3.2 Immunohistochemical results

3.2.1 Adrenal Gland KO WT

Positive P4 signals were detected in the medulla and cortex of the adrenal gland. Red staining is P4 signals, blue are nuclear staining. Signals in the medulla are a little stronger than signals in the cortex, while P4 knockout tissue had no P4 expression**.**

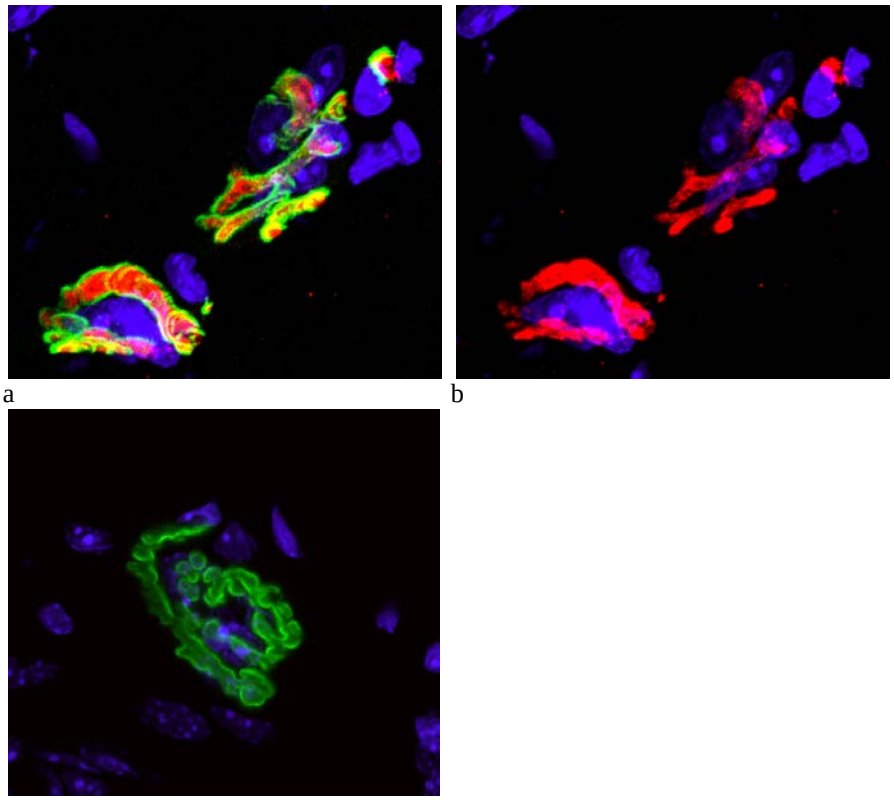


a b

Figure 2. Adrenal gland
P4 signals in the medulla of the adrenal gland. Red dots are P4 signals, blue dots are nuclear staining.
(a) P4 signal in the medulla of the adrenal gland (WT), and less signals or background signals in the cortex of the adrenal gland
(b) Negative control, P4 KO, shows no P4 signals.

3.2.2 Neuromuscular junctions

P4 signals were detected in the in neuromuscular junctions, P4 signals are indicated in red. Alpha-Bungarotoxin that binds ionotophoretically to the acetylcholin receptor in the postsynaptic membrane is visualized with green colour. Blue colour is nuclear staining.



a b c

Figure 3. Neuromuscular junctions.
P4 signals in Neuromuscular junctions. The red staining is P4 signals. The blue staining makes nuclears visible, the green staining is acetylcholine receptors in the synaptic membrane.
(a) Neuromuscular junction with alpha-bungarotoxin that stains the acetylcholine receptor.
(b) Neuromuscular junction without alpha-bungarotoxin.
(c) Negative control with no P4 antibody.

3.3 PLA

P4 has previously been shown to co-localize with VAChT (Geyer *et al., 2008*) and P4 was located to motor neurons in the spinal cord of mice. The purpose of using PLA was to investigate if P4 were located to the same synaptic vesicles as VAChT and VMAT2. To evaluate the technique, several experiments were performed (See Table 2). Images were done with a confocal microscope.

The first image in every row (Figure 4) is red P4-protein staining and blue nuclear staining. The second image is green synaptophysin staining. Synaptophysin stains the synaptic membrane and differentiates between grey matter and white matter. This means that in these pictures we can distinguish the grey matter in the ventral horn (light green staining) from the white matter (dark green) and the motor neurons (small black holes). The motor neurons have the shape of small slices of garlic (black) in the grey matter (light green). The third image in every row is a merged image of the first and the second image, which makes it possible to locate the P4-signals in the ventral horn. P4 signals are expressed around the motor neurons in the ventral horn.

VAChT signals (Figure 4c) are very clear, of motor neuronal character and similar to earlier IHC results. A high background signalling (Figure 4b) is visible in the first and third image of VMAT2, especially in the white matter (the right upper corner). VMAT2 signals around motor neurons are not distinguishable from background signals. The P4 sample (Figure 4a) has, similar to VMAT2, high background signalling. Weak P4 signals appear around motor neurons (marked with arrows). No signals are visible in the negative control (Figure 4d).

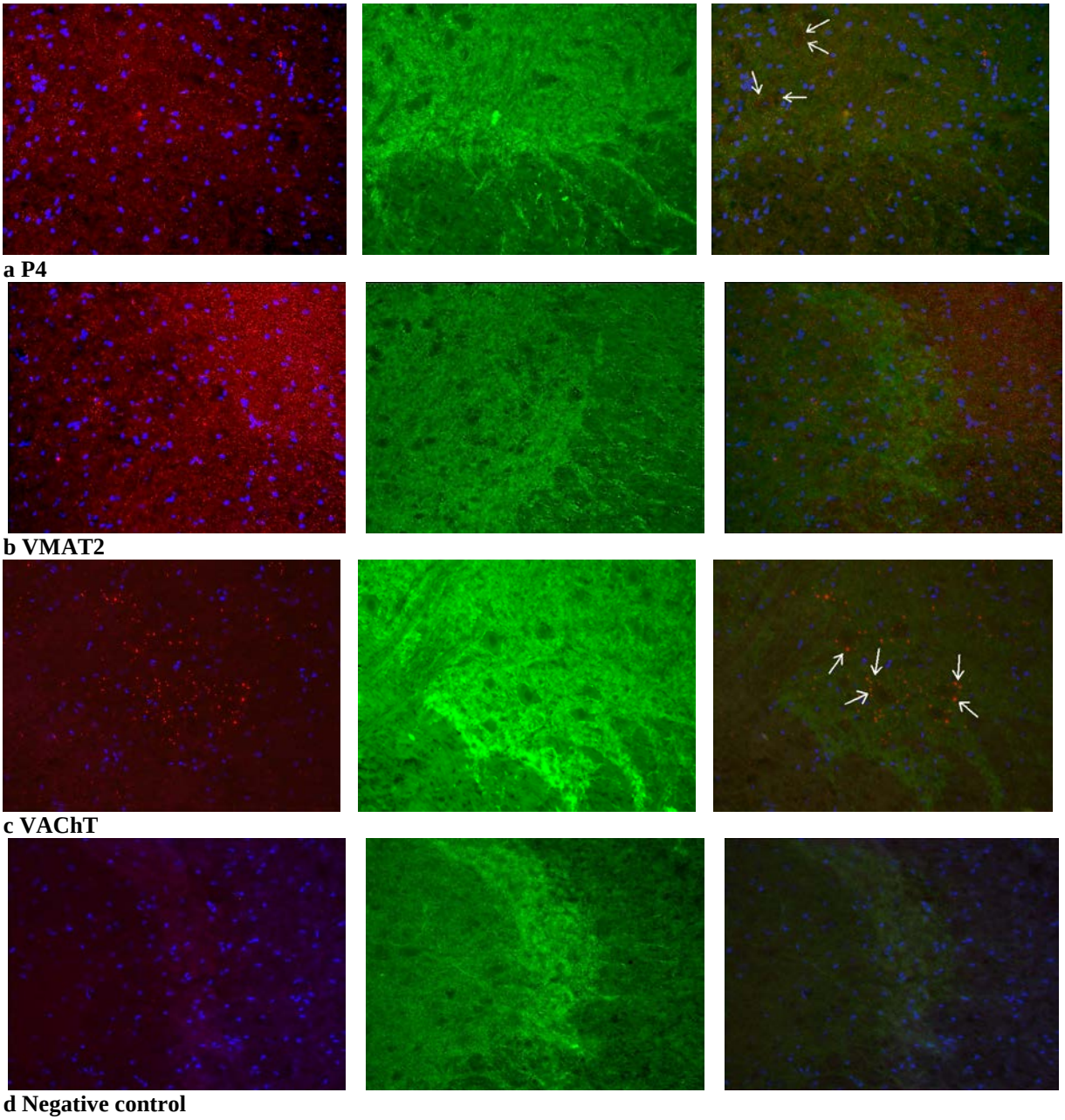


Figure 4. P4, VMAT2 and VAChT signals in the ventral horn of the spinal cord of mice.
First image in every row is red P4-protein staining and blue nuclear staining. The second image is green synaptophysin staining and the third image is a merged image of the first and the second picture.

(a) P4 signals are spread evenly over the tissue with high background signalling, a few signals are distinguishable around motor neurons (marked with arrows) in the merged image.
(b) VMAT2 signals with high background signalling. Signals around neurons are not distinguishable from background signals.
(c) VAChT signals have motor neuronal character (marked with arrows).
(d) Negative control with no P4 signals.

In the first experiment, both secondary antibodies were directed against one single protein, P4 (Figure 4a), VMAT2 (Figure 4b) and VAChT (Figure 4c) respectively, to evaluate the technique. The negative control (Figure 4d) was a sample with mismatching secondary antibodies.

In the second experiment, we tried two different primary antibodies, P4 and VAChT, and two different secondary antibodies that each attach to one of the primary antibodies. This was done to evaluate if the technique is more suitable for detecting protein interactions or proximity between two proteins than detecting single proteins. A few positive signals were found. In the next experiment, the VAChT antibody was diluted less (from 1:250 to 1:100) in an attempt to increase the positive signals and decrease the background signals. The results were not significantly different, which was why we did one more experiment where we tried to verify the positive control. This experiment was successful and we could confirm that the positive control gave a good signal. The standard protocol was tried again but positive signals were still difficult to distinguish from background signals. Secondary antibodies were diluted (1:5) to investigate if those were the reason to the high back ground signals. The samples with diluted secondary antibodies had little less background. In the last experiment, we tried the P4-VAChT interaction again with diluted secondary antibodies (1:5) and a serial dilution of the P4 antibody (from 1:500 to 1:2000). The result was difficult to interpret since signals sometimes appear inside neurons. This happened in all concentrations and in both KO and WT which is why we believe that it was a false signal. The results are summarized in table 3 below.

Table 3. **Results of PLA** Experimental performance. Detailed information about antibodies and dilutions can be found in Tabel 2PLA antibodies.

Exp	**Type of experiment**	**Primary antibodies**	**Secondary antibodies**	**Result**
1	**PLA standard protocol**			
	P4, VAChT and VMAT2 were used as single protein targets to test PLA standards for each antibody separately.	P4 VAChT VMAT2	Rb+/Rb- Gp+/Gp- Gt+/Gt-	VAChT signals were clear and strong. Signals in P4 and VMAT2 were spread over the sample tissue and not concentrated around the motor neurons.
2	**Protein interaction**			
	P4 and VAChT were tried together to see	P4-VAChT P4	Rb+/Gp- Rb+/Rb-	A few positive signals were found in the P4-

	if the technique is more suitable for detecting protein interactions than single proteins.	VAChT	Gp+/Gp-	VAChT.
3	**VAChT dilution**			
	The VAChT antibody was less diluted to see if that affected the signals from the protein interaction or decrease the background signalling.	P4-VAChT P4 VAChT	Rb+/Gp- Rb+/Rb- Gp+/Gp-	The signals were not significantly stronger or more distinguishable.
4	**Positive control**			
	Verify the positive VAChT control.	VAChT	Gp+/Gp-	Positive results.
5	**Protein interaction**			
	P4 and VAChT were tried together again to check the protocol and working procedure.	P4-VAChT VAChT	Rb+/Gp- Gp+/Gp-	Signals in P4-VAChT were hard to distinguish from background signals and not comparable to the signals in the positive control.
6	**Secondary antibody dilution**			
	The secondary antibodies were diluted to decrease background signals.	P4 VAChT	Rb+/Rb-(diluted from 1:1 to 1:5) Gp+/Gp-(diluted from 1:1 to 1:5)	The samples with diluted secondary antibodies 1:5 gave less background signals.
7	**Protein interactions + Primary antibody dilution**			
	One protein interaction between P4 and VAChT is tried with diluted secondary antibodies (1:5). Dilution of primary antibody P4 was also tried	P4-VAChT P4 (diluted from 1:500 too 1:2000)	Rb+/Gp- Rb+/Rb-(diluted from 1:1 to 1:5)	Signals inside neurons in both KO and WT and in all concentrations. As well as in the P4-VAChT interaction sample.

4 Discussion

The *In situ* hybridization showed that the P4-gene was not expressed in the KO animals and verified the genetically modified mouse. The results from the IHC experiments showed that P4 is present in the synapses of neuromuscular junctions and in the medulla of the adrenal gland. The signals in the cortex of the adrenal gland can be background signals, alternatively there are P4 proteins even in the cortex but in smaller amounts than in the medulla.
Very strong signals in the NMJ probably means that the P4 protein is participating in the release, the reuptake or co-working with VAChT in transporting acetylcholine into vesicles.

The purpose of using Duolink PLA was to investigate if P4 and VAChT are located to the same vesicles. These experiments cannot confirm that they are, neither can we eliminate the possibility that they are co-located to the same vesicles. The technique has to be further optimized. Parameters to change can be: new antibodies targeting other antigens of the protein, incubation time, incubation temperature, antibody dilutions and including a blocking enhancer to the protocol.
Due to the high background and unspecific binding of the P4 antibody it was very difficult to interpret the results. Another problem was that the signals sometimes appeared inside the neurons when they are expected in or close to the synaptic membrane. This could be due to signals from above or under focus in the sample during microscope examination. Signals from above or under the focus appear to be inside the neuron. Most likely, though, is that the antibodies clogged and get stuck inside the neurons. We can see this pattern in the wild-type as well as in the knock-out samples which indicate that it is a consequence of unspecific binding of the primary or the secondary antibodies. Our P4 antibody might bind to other targets than P4. That could also explain the high background signals. Highly charged or hydrophobic structures in the sample can for example trap antibodies.
We also had problems with the tissue samples, some were partially degraded before use or were mechanically destroyed during the process which can give misleading results.
The method, Duolink PLA, can be valuable when the technique is fully optimized and results are reliable compared to the controls and IHC results.

IHC experiments show that P4 is present in the medulla of the adrenal glands as well as in the neuromuscular junctions, which confirm our hypothesis that P4 have a function in those structures.

5 Conclusions and future perspectives

We performed the PLA experiments to investigate if VAChT and P4 are co-located and if they are in proximity. If P4 and VAChT are located to the same vesicles we would also, in the future, like to know if P4 is a cotransporter to VAChT and/or VMAT2 and the details of that process, alternatively what kind of process P4 participates in. There is also a possibility that P4 and VAChT or P4 and VMAT2 are located to the same vesicles but have independent functions. Transport experiments with P4 have previously been done by J. Geyer *et al.*. In these experiments, Slc10a4(P4) transfected HEK293 cells were used with radiolabeled substrates. The substrates of the closely related transporter Slc10a1 were also investigated with taurocholat and estrone-3-sulfate, but none of them were found to be transported by P4. Acetylcholine and the two monoamines dopamine and serotonin are interesting target for transport experiments to find what P4 is eventually transporting. Since P4 has been located to cholinergic and monoaminergic neurons and the brain areas where serotonin and dopamine are produced, acetylcholine, serotonin and dopamine are possible substrates of P4. Purified vesicles can be analysed using mass spectrometry to try to find the possible substrate of P4.

To further optimize the PLA technique one could play with the following parameters; dilutions of primary and secondary antibodies, incubation time and incubation temperature.
We have tried to dilute both the primary and the secondary antibodies, but the right dilution of the primary antibody was not found in these experiments. As mentioned earlier, the inconsistent results can be a consequence of unspecific binding of the primary antibody. One could also make negative controls with no primary antibody to evaluate the binding of the secondary antibody and compare the background signals. The washing and blocking steps are also critical points in the process. Olink has a blocking enhancer that perhaps could be useful to decrease the background signals. One could also fix the tissue only with PFA, other compartments can destroy the epitope. To avoid that the tissue is destroyed during the process there are special ceilings that can be used to protect the tissue.
We might need a monoclonal antibody against the N-terminal of P4 because the N-terminal is facing the outside of the vesicle, where the antibodies more easily can bind to their epitope.

Further, in the attempt to find the function of P4, finding the structures where P4 is expressed can give guidance. Since the adrenal gland had expression of P4, other structures affected by the autonomic nervous system where acetylcholine is a major transmitter are interesting, for example heart, liver and pancreas. The enteric nervous system is also affected by the autonomic nervous system. Acetylcholine, dopamine

and serotonin play important roles in the function of the enteric nervous system, which makes it an interesting field to investigate to see if P4 participates the function of the autonomic system. If P4 is involved in the transport of acetylcholine and monoamines, it is involved in many important functions of our body.

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