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Ciências

Identification of genetic mutations in patients with familial central diabetes insipidus

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Para os meus pais
com imenso amor!

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Resumo alargado

A diabetes insípida (DI) é uma doença rara, caracterizada principalmente pela excreção de elevados volumes de urina na forma diluída podendo, entre várias causas possíveis, ter origem num defeito genético.

O desenvolvimento da doença pode dever-se a quatro causas possíveis. A mais comum deve-se a uma deficiência na secreção da hormona antidiurética arginina vasopressina (AVP), sendo referida como DI central ou neurohipofisária. Outra possível causa da doença deve-se a uma insensibilidade, por parte das células renais, aos efeitos da AVP, sendo neste caso designada como DI nefrogénica. A DI pode também dever-se a uma excessiva ingestão de líquidos, que conduz à supressão da libertação da hormona AVP, sendo referida como polidipsia primária. Por fim, um aumento do metabolismo da hormona AVP durante a gravidez pode também ser uma causa da doença, designada por DI gestacional.

A hormona AVP é sintetizada nos neurónios magnocelulares. Estes têm origem no núcleo supra-óptico e para-ventricular do hipotálamo e os seus prolongamentos terminam na neurohipófise. A destruição destes neurónios resulta numa deficiência na produção da hormona, conduzindo à DI central. Esta destruição pode ter inúmeras causas, incluindo acidentes, cirurgias, doenças autoimunes, entre outras. Contudo, a doença também apresenta uma base familiar, correspondendo a 1% de todas as causas de DI central. A DI central apresenta sintomas persistentes de poliúria, polidipsia e sede, que geralmente se começam a manifestar vários meses ou anos após o nascimento.

A DI central familiar apresenta duas características principais: está associada a mutações num único alelo do gene que codifica a hormona (gene AVP), apresentando assim uma transmissão autossómica dominante; e é causada por uma deficiência progressiva pós-natal na secreção da hormona AVP, que se pensa resultar da degeneração seletiva dos neurónios magnocelulares.

O gene AVP é composto por 3 mil pares de bases e encontra-se localizado no braço curto do cromossoma 20. Este gene contém três exões que codificam para o péptido sinalizador, para a hormona AVP, para a neurofisina II (transportador da hormona) e ainda para um glicopéptido, conhecido como copeptina. Após sintetizados, a hormona, a neurofisina II e o glicopéptido são armazenados em vesiculas secretoras, nos terminais axonais dos neurónios, e são libertados após a ocorrência de estímulos. Após a entrada na corrente sanguínea, a hormona vai atuar a nível das células renais de modo a aumentar a sua permeabilidade para as moléculas de água, favorecendo assim a absorção de água no rim.

Até à data do início deste trabalho, a doença estava associada a 70 mutações diferentes no gene AVP localizadas ao longo de todo o precursor proteico. Pensa-se que estas mutações são a causa da doença uma vez que interferem na estabilidade da cadeia de aminoácidos, alterando a sua estrutura primária. Teoricamente, mutações que afetem a conformação de

proteínas secretoras resultam no desenvolvimento de patologias devido ao seu impacto na função da proteína não conseguindo alcançar o seu destino, ficando retidas no reticulo endoplasmático. Contudo, a razão dos precursores AVP mutados serem tóxicos para os neurónios produtores de AVP está ainda por esclarecer.

Existem, até ao momento, três teorias que tentam explicar o mecanismo da doença. O mecanismo não tóxico defende que há uma expressão simultânea dos precursores “wild-type” e dos precursores mutados resultando numa associação de ambos. Assim, o precursor “wild-type” é alterado, uma vez que ambos ficam retidos no reticulo endoplasmático. Contudo, este mecanismo não explica a morte dos neurónios magnocelulares. O mecanismo tóxico defende que a constante acumulação de precursores com conformações alteradas pode interferir com a produção de proteínas essenciais à sobrevivência celular, resultando assim na morte neuronal. Recentemente, um novo mecanismo foi proposto para explicar a patogénese da doença. Observou-se a formação de vesiculas autofágicas, após acumulação de precursores mutados, que resultam na destruição dos retículos endoplasmáticos danificados, juntamente com os agregados proteicos. Durante este processo, se as células forem expostas a insultos metabólicos e ambientais, pode ocorrer apoptose dependente de autofagia, resultando na destruição dos neurónios magnocelulares.

A DI central familiar apresenta uma natureza benigna, contudo é uma doença que apresenta uma intensa pesquiza em torno dos seus mecanismos moleculares uma vez que se trata de um modelo de interesse para o estudo de doenças neuro-endócrinas e de transmissões autossómica dominante.

O presente estudo tem por objetivos fazer uma revisão das mutações descritas na literatura científica para o gene AVP, aumentar o número de mutações descritas com a análise de novos pacientes diagnosticados com DI central familiar e caracterizar as consequências funcionais das novas mutações identificadas.

Para alcançar os objetivos descritos, utilizou-se a seguinte metodologia: a revisão de todas as mutações descritas até à data, através de pesquisa bibliográfica de artigos científicos; realização de estudos genéticos, baseados na amplificação por PCR e na posterior sequenciação dos três exões do gene AVP de 9 pacientes diagnosticados com DI central familiar; inserção das novas mutações num vector de expressão contendo o cDNA do gene AVP, através de técnicas de clonagem, digestão enzimática e mutagénese dirigida; e finalmente a realização de estudos funcionais, por otimização das técnicas de transfecção e imunocitoquímica com o vector de expressão AVP “wild-type”.

Os resultados obtidos mostraram que as 3 famílias apresentam mutações no gene AVP. O paciente III-1, da família A, apresenta a alteração de uma timina para uma citosina na posição 154 do cDNA (c.154T>C) que origina a substituição de uma cisteína por arginina na posição 52 da proteína (p.C52R). O paciente II-1, da família B, apresenta uma alteração de

citosina para guanina na posição 289 do cDNA (c.289C>G) que resulta na substituição de uma arginina por glicina, na posição 97 da proteína. O paciente II-4 da família C apresenta a alteração de uma guanina para uma timina na posição 343 do cDNA (c.343G>T) que resulta na substituição de um ácido glutâmico por um codão de terminação na posição 115 da proteína. As três mutações estão em heterozigotia e as duas mutações encontradas no exão 2 correspondem a mutações novas, enquanto a mutação presente no exão 3 já se encontra descrita na literatura.

Um vector de expressão contendo o cDNA do gene AVP (pRc/RSV-AVP), foi-nos gentilmente oferecido por investigadores da área. O cDNA do gene AVP contido no vector de expressão (pRc/RSV-AVP) foi sub-clonado no vector pVAX/*lacZ* e, através de mutagénese dirigida, as mutações desejadas (c.154T>C e c.289C>G) foram introduzidas no cDNA. Assim, o cDNA com as mutações está pronto a ser inserido no plasmídeo de expressão. Os ensaios de transfecção e imunocitoquímica foram otimizados para o vector de expressão “wild-type”, uma vez que foi observada marcação para a neurofisina II nos prolongamentos dos neurónios após transfecção de uma linha celular neuronal (N2A) e marcação com anticorpos específicos.

Com este estudo, o número de mutações descritas para o gene AVP aumentou de 70 para 72 e mais três famílias fazem parte do número total de famílias estudadas com DI central familiar. É importante continuar o desenvolvimento de estudos funcionais, de modo a obter respostas sobre os mecanismos moleculares responsáveis pelo desenvolvimento da doença uma vez que estas serão importantes não só para a DI central familiar, mas também para o esclarecimento de outras doenças que apresentem mecanismos moleculares semelhantes.

Palavras chave

Diabetes insípida central, mutações AVP.

Abstract

Diabetes insipidus (DI) is associated with defects that involve the secretion and the action of hormone arginine vasopressin (AVP) resulting in the excretion of abnormally large volumes of diluted urine. The most common defect that results in disease development is the deficient secretion of the hormone AVP and the disease is referred to as central or neurohypophyseal DI. The AVP hormone is synthesized in magnocellular neurons, that originate in the supraoptic and paraventricular nuclei of the hypothalamus and are projected to neurohypophysis, and the destruction of these neurons leads to a deficiency of AVP hormone, resulting in neurohypophyseal DI. The familial form of disease represents 1% of all causes of neurohypophyseal DI and the main points of the disease are: it is associated with mutations in one allele of the AVP gene, and it is caused by postnatal development of deficient AVP secretion, proposed to result from selective degeneration of the magnocellular neurons.

The aims of this thesis are: to review AVP mutations described in the scientific literature, to expand the spectrum of mutations through the analysis of additional patients with DI and to characterize the functional consequences of identified novel AVP mutations. To achieve these aims a bibliographic research was developed; genetic studies were performed to amplify and to sequence the three exons of the AVP gene in 9 patients; an expression vector containing the desired mutations was constructed by subcloning, site-directed mutagenesis and enzymatic digestion; and the functional studies were initialized by optimization of transfection and immunocytochemistry assays for WT AVP cDNA expression vector.

Three mutations were identified: c.154T>C, c.289C>G and c.343G>T. The first two mutations are novel and the last mutation is already described in the scientific literature. The AVP cDNA from the expression vector was subcloned in the pVAX/*lacZ* vector and the mutations were inserted in the AVP cDNA by site-directed mutagenesis and enzymatic digestion. The mutated AVP cDNAs were sequenced and have been prepared to be inserted in the expression vector. The transfection and immunocytochemistry protocols have been optimized for WT AVP cDNA expression vector.

This study allowed the increase in the number of mutations from 70 to 72 different mutations, although further work is necessary in order to understand the molecular mechanisms responsible for the development of the disease and to give help and information to patients affected with this disease.

Keywords

Neurohypophyseal diabetes insipidus, AVP mutations.

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List of Acronyms

AC	Adenylyl cyclase
adFNDI	Autosomal dominant familial neurohypophyseal diabetes insipidus
AMP	Adenosine monophosphate
AQP2	Aquaporin 2
AQP3	Aquaporin 3
AQP4	Aquaporin 4
AQP2-P	Phosphorilated aquaporin 2
AVP	Arginine vasopressin
bp	Base pair
BSA	Bovine serum albumin
cAMP	cyclic
cDNA	Complementary DNA
DDAVP	Des-amino-D-arginin vasopressin
DI	Diabetes insipidus
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ddNTPs	2’,3’-dideoxyribonucleoside triphosphates
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
Fig.	Figure
Frag1	Fragment 1
Frag2	Fragment 2
Frag3	Fragment 3
FragAB	Fragment AB
FragBB	Fragment BB
FragCB	Fragment CB
GP	glycopeptide
HSP70	70 kilodalton heat shock proteins
kb	Kilo bases
MRI	Magnetic resonance image
N2A cells	Neuro 2A cells
NPII	Neurophysin II
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PKA	Protein kinase A
Opti-MEM	Opti-modified eagle’s medium

SP	Signal peptide
UV	Ultra-violet
V_2	Arginine vasopressin type 2 receptors
WT	Wild-type

1. Introduction

1.1. Definition and classification of Diabetes Insipidus

Blood osmolality in healthy individuals is maintained within restricted limits by a series of complex mechanisms. Adjustments in water balance determine the level of that osmolality. These adjustments are mediated by delicate alterations in the thirst mechanism plus the capacity of the kidney to alter urine flow rate and its osmolality in response to small changes in the plasma concentration of the hormone arginine vasopressin (AVP) [1]. Thus, through all these mechanisms, healthy humans can conserve their osmotic level despite extreme climatic conditions and, to a certain degree, when water supply is inadequate [1].

However, alterations in these mechanisms can occur and lead to one of two main states: inappropriate accumulation of water in organism, which is recognized as hypoosmolar states, and loss of renal water, which is recognized as hyperosmolar syndrome [1].

Diabetes insipidus (DI) is a rare disease and is characterized by excretion of abnormal large volumes, known as polyuria (>50mL/Kg/day) of dilute urine (<300mmol/Kg) [2, 3]. This definition allows the exclusion of osmotic diuresis, which occurs when an excess of solute is being excreted, like in the case of glucose in patients with diabetes mellitus and this is the main difference between the two disorders [3]. This disorder can be acquired as a result of various injuries or diseases, but can also be idiopathic or have a genetic origin [2].

In DI, the magnitude of the abnormality in concentration and excretion of the urine varies according to some factors like the severity of the defect which results in the disorder, the age of the patient, and the rate of solute and water intake [2].

Four basic defects are responsible for the development of DI. The first and the most common defect that occurs in this disorder is the deficient secretion of the hormone AVP, and in this case the DI is referred as neurohypophyseal, neurogenic, central or hypothalamic [3]. This form of DI can be completely controlled by administration of AVP or its analogue, des-amino-D-arginin vasopressin (DDAVP) [4]. The second type of DI is caused by renal defects, where the cells of the kidneys are insensitive to the antidiuretic effects of AVP and is referred as nephrogenic DI. In this case, the patients are unresponsive or poorly responsive to the administration of AVP or DDAVP [3, 4]. In both forms of disease, the thirst mechanism remains normal to regulate water balance [1]. Another defect that causes DI is excessive water intake (polydipsia) that leads to suppression of AVP release and consequent polyuria. This form of DI is called primary polydipsia and may be due to defects in the thirst mechanism or to cognitive impairment. Hormone supplements like AVP and DDAVP can reduce the symptoms of polyuria,

although these treatments should not be used because they can originate water intoxication, since they reduce polyuria more than polydipsia, which results in rapid retention of excess water and development of hyponatremia [1, 3, 4]. The fourth type of DI is due to increased metabolism of AVP during pregnancy resulting in gestational DI. This form of DI can be treated with DDAVP but is unresponsive to AVP. This occurs because the analogue of AVP is much less susceptible to degradation by placental vasopressinase [3, 4].

Depending on the cause that originates DI, the deficiency in vasopressin action or secretion can be partial or nearly total. Thus, the deficiency may or may not be associated with concentration of the urine after a fluid-deprivation test or in response to other strong stimuli like in the case of nausea, severe hypovolemia or severe hypotension [2].

Differentiating between the forms of DI is relatively easy if patients have severe deficiency in either the secretion or action of AVP. In both cases, the patients undergo dehydration induced by fluid deprivation, but the urine remains dilute [4]. This first result excludes the possibility of primary polydipsia since in this form of disease, a fluid deprivation results in concentration of urine because the hormonal mechanism remains normal and the problem resides in excess of water intake. The next step to differentiate nephrogenic DI from neurohypophyseal and gestational DI is the injection of AVP and DDAVP and measurement of the urinary response [4]. Patients with nephrogenic DI do not respond to treatment since their problem resides in renal insensitivity to AVP and not in hormonal deficiency and their urine remains dilute. However, patients with neurohypophyseal or gestational DI are able to concentrate their urine when AVP or DDAVP are administered because of the increased plasma levels of AVP. If fluid deprivation results in concentration of urine, other tests are necessary to differentiate between primary polydipsia and a less severe deficiency in the secretion or action of AVP [4]. The most reliable way to make this distinction is to measure plasma AVP and to relate the results to the plasma and urine osmolality during a fluid deprivation and/or hypertonic saline infusion test [4].

However, with time the diagnosis becomes more complicated and the forms of the disease can be confused. After prolonged periods of polydipsia, a decrease in maximal urine-concentration ability occurs in the kidneys, regardless of the primary cause [5]. The passage of large amounts of dilute urine through the distal nephron removes existent solutes from the renal medullary interstitium, a process known as washout phenomenon, and results in the decrease of osmotic gradient across the collecting tubular cells [1]. Since this gradient is essential for the antidiuretic action of AVP, any mechanism responsible for DI may lead to an additional defect at the renal level that complicates the interpretation of diagnostic tests based on indirect analyses of the antidiuretic action of AVP [1].

1.2. Clinical aspects of Familial Neurohypophyseal Diabetes Insipidus

AVP is synthesized in magnocellular neurons that originate in the supraoptic and paraventricular nuclei of the hypothalamus, which project down through the diaphragma sellae to form the neurohypophysis [3, 4]. In contrast to the adenohypophysis, the neurohypophysis does not synthesize hormones but functions as a reservoir for the storage and release of hormones synthesized in the hypothalamus [6].

Destruction of magnocellular neurons results in a deficiency of AVP, leading to neurohypophyseal DI. This neuronal destruction can have a variety of causes, including trauma from surgery or accident, infections, autoimmune disease, congenital brain malformations, aneurysms, and others [6]. However, neurohypophyseal DI can also occur on an inherited, or familial, basis representing 1% of all causes of neurohypophyseal DI [7]. Usually, the disease has an autosomal dominant transmission, however in 1996 an X-linked recessive form was discovered [4].

There is another type of neurons that can produce and segregate AVP, known as parvocellular neurons [4]. The projections of these neurons are located in the median eminence of the hypothalamus [4]. In some studies, it was observed that these projections are apparently unaffected in patients with neurohypophyseal DI and this fact may explain the preservation of normal circadian rhythm and pituitary-adrenal function in these patients [4] because these neurons also produce corticotrophin releasing factor, which is thought to interact with AVP in the regulation of adrenocorticotropic hormone secretion [8]. Since these neurons are not affected by neurohypophyseal DI, it is believed that the two types of AVP-producing neurons have very different susceptibilities to the cytotoxic effects of the genetic mutations that lead to development of the disease [4].

Autosomal dominant familial neurohypophyseal DI (adFNDI) is a rare disease with persistent symptoms of polyuria, polydipsia and thirst which usually manifest several months or years after birth [9]. Studies performed with mice with AVP gene mutations revealed some differences, when compared with mice with the normal AVP gene: mice with a mutated AVP gene consumed larger volumes of water, they excreted much more urine and the volume of urine excreted is worse over time, and their urine osmolality is lower [9]. Thus, like in humans with adFNDI, mice with certain mutations produce excessive amounts of dilute urine but compensate by increasing water intake and so they can avoid severe dehydration and this fact demonstrates that thirst mechanisms remain intact in the presence of the disease [9]. Despite these symptoms, adFNDI causes relatively few and well-tolerated symptoms. Nocturia (elimination of urine at night, disturbing sleep) is common and in children may present as

enuresis (urine during sleep) [4]. Physical exams and routine laboratorial analyses are usually within normal limits and hypernatremia or signs of hypertonic dehydration are minimal or absent, except in the case of patients that are comatose, have an impaired thirst mechanism or if the patients are unable to increase fluid intake [4].

The symptoms of severe polyuria and polydipsia, which segregate in an autosomal-dominant pattern and respond to exogenous DDAVP, show several intriguing features. First, the affected family members show a completely normal water balance at birth and during early infancy but develop progressive symptoms of compulsive drinking at some point in childhood [10]. Second, in some studies it was demonstrated that during repetitive fluid-deprivation tests, the secretion of AVP is normal before the onset of the disease but then starts diminishing during early childhood [11]. Finally, once fully developed, the symptoms of polyuria and polydipsia continue throughout life [10]. Occasionally, spontaneous remissions of polyuria and polydipsia during middle-age are observed, even though the patients continue to have a deficient AVP secretion as severe as in their symptomatic kin. However, this remission mechanism remains unexplained [4].

Magnetic resonance image (MRI) exams have been used to investigate neurohypophysis anatomy in patients with adFNDI [4]. Some authors found that anterior and posterior lobes of a normal pituitary gland have different signal intensities in images of magnetic resonance. The posterior lobe presents a well-defined oval or round area of hyperintensity in both normal and abnormal pituitary glands, although there are some variations in size and shape of the signal from one person to another [6]. Some patients with adFNDI lack the characteristic bright spot, or high-intensity signal [4, 12], which is common in the posterior lobe of the pituitary in 52%-100% of healthy adults [6]. Signal intensity seems to be correlated closely with posterior lobe function as it is suspected to result from neurovesicles in axon endings of AVP-producing neurons [7, 13]. If neurovesicles really are responsible for the bright spot, it is not clear why oxytocin-containing vesicles or vesicles located in the hypothalamus do not cause a high-intense signal, maybe their concentration is insufficient [7]. Thus, the absence of the bright spot in the posterior pituitary lobe could result from a neurotoxic accumulation of precursor proteins that consequently lead to cell death [7, 12, 13].

However, the significance of MRI results is uncertain because the exact cellular source of the signal is not yet known [4]. It is believed that the bright spot is absent in all patients with neurohypophyseal DI due to destructive or unidentified pathological processes [6], however to date relatively few patients with adFNDI have been studied and the results have been conflicting since in some affected individuals the bright spot has been observed [4]. A positive bright spot in a patient with adFNDI can be caused by a defect in hormone release from the posterior pituitary leading to accumulation of neurovesicles and, thus, to a normal MRI instead of defects in intra-axonal transport or processing of proteins [7]. However, at present, neither the presence nor the absence of the bright spot can be related with the presence or

absence of AVP-producing neurons in the posterior pituitary [4]. Thus, it is important that additional MRI studies including adFNDI patients are performed to clarify the importance and significance of the high-intensity signal found in some people [4].

1.3. AVP gene and AVP processing

adFNDI is caused by mutations in one allele of the gene that encodes for AVP. The gene has approximately 3 kb (Gene ID: ENSG00000101200, Esemble) and is located on the short arm of chromosome 20 (20p13) [3]. It consists of 3 exons and 2 introns and encodes AVP and neurophysin II (NPII), the carrier of AVP. The first exon encodes the signal peptide, the hormone AVP, and the NH_2-terminal region of NPII [3]. The second exon encodes the highly conserved central region of NPII and the third exon encodes the COOH-terminal region of NPII and the glycopeptide, which is known as copeptin [3, 14]. The small size of the AVP gene facilitates the mutational analysis [3] and the study of the mutations at the protein level (Fig. 1).

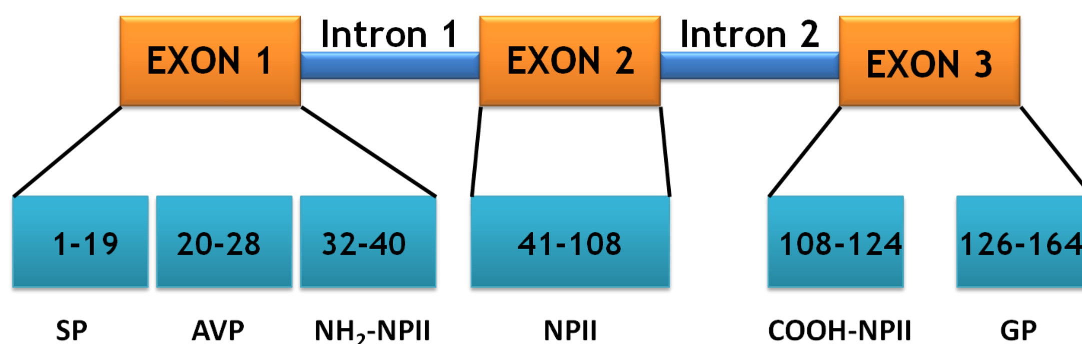

Fig. 1. Structural organization of the AVP gene and the protein vasopressin precursor. The gene is composed of 3 exons and 2 introns. The signal peptide contains 19 amino acids, AVP hormone contains 9 amino acids, NPII contains 93 amino acids and copeptin contains 39 amino acids. SP, signal peptide; AVP, arginine vasopressin; NPII, neurophysin II; GP, glycopeptide.

Several studies to analyze the expression of the AVP gene were performed using AVP transgenes derived from some animals. These results support the hypothesis that cell-specific enhancers and/or silencers that restrict expression of the AVP gene to specific neuronal cell-types in the hypothalamus are present in the regions either downstream or upstream of the AVP gene [15]. Recently, some authors demonstrated that DNA sequences in a 178 bp region immediately downstream of exon 3 of the AVP are necessary for cell-specific expression of AVP in rat hypothalamus [16].

AVP and NPII are synthesized as a single precursor, prepro-vasopressin. The prepro-hormone presumably is translated on ribosomes in the cytosol and translocated across the membrane of the rough endoplasmic reticulum [4]. Once inside the endoplasmic reticulum, it is supposed that the signal peptide remains attached noncovalently to the inner surface of the membrane via the positive charges at its N-terminal. This ligation is thought to facilitate accurate cleavage of the signal peptide by ensuring proper alignment with the signal peptidase [4]. However, the presence of certain small and neutral amino acids at the -1 and -3 positions immediately adjacent to the cleavage site of the signal peptide are required for efficient and accurate cleavage [4]. In this case, the signal peptide of AVP gene has an alanine and serine at -1 and -3 positions, respectively [4]. The pro-hormone is generated by removal of the signal peptide from the prepro-hormone and from by addition of a carbohydrate chain to the copeptin [3, 9]. There are no certainties that the glycosylation process and copeptin are important for protein proper folding, trafficking or further processing, however, it seems possible that copeptin glycosylation plays an important role by assisting refolding of misfolded AVP pro-hormone monomers through its interaction with the calnexin-calreticulin system in the endoplasmic reticulum [17]. This system monitors protein folding and interacts principally with the sugars of glycosylated proteins and places these proteins into the proximity of a glycoprotein-specific member of the protein disulfide isomerase family [17].

After removal of the signal peptide, the precursor generally must fold and dimerize correctly in the lumen of the endoplasmic reticulum [4], where the unique oxidizing environment allows the formation of disulphide bridges [10], before they can proceed through the Golgi apparatus [4]. If folding is not correct, usually precursors are retained in the endoplasmic reticulum, where they may be taken up by chaperones or heat shock proteins and degraded [4]. In vitro studies suggest that the stability of folding is dependent on binding of the N-terminal of the hormone to a specific site located in the N-terminal of NPII [4]. Also, in the case of AVP and NPII, like in others proteins, the correct folding of AVP-NPII in the endoplasmic reticulum probably also depends of the position of critically situated amino acids, like in the case of glycine or proline residues that enable the molecule either to rotate freely or to form a rigid bend. Cysteine positions are also very important because, under the action of a disulphide isomerase found in endoplasmic reticulum, they form specific disulphide bridges, which also serve to stabilize the molecule in the correctly folded conformation [4]. Binding of AVP to NPII also facilitates self-association of the folded pro-hormone into dimmers which are then transported to the Golgi apparatus [4]. Here, final glycosylation takes place and the correctly folded pro-hormones are finally packaged into dense granules which are transported along the axon to the posterior pituitary [4, 9]. The pro-hormone has different cleavage signal sequences. Whereas the hormone is followed by a sequence of three residues, glycine-lysine-arginine, NPII is followed by a monobasic cleavage site, an arginine residue (Fig. 2) [10]. During axonal transport, additional posttranslation processing occurs inside granules yielding AVP, NPII and the glycopeptides in separated forms [3]. This posttranslation process consists of two

successive cleavages; the first occurs between the hormone and the NPII by the action of a dibasic endopeptidase, and the second between NPII and copeptin by a monobasic endopeptidase [18]. Then, these molecules are stored within neurosecretory vesicles in the nerve terminals and released into the blood in response to osmotic stimuli [9]. Inside the vesicles, reversible noncovalent interactions between AVP and NPII persist until these complexes are secreted into the bloodstream and they dissociate into free hormone and NPII [4]. NPII can be seen as a chaperone-like molecule facilitating intracellular transport in magnocellular cells [3], protecting AVP from proteolytic degradation during axonal transport of the secretory granule to the posterior pituitary [13].

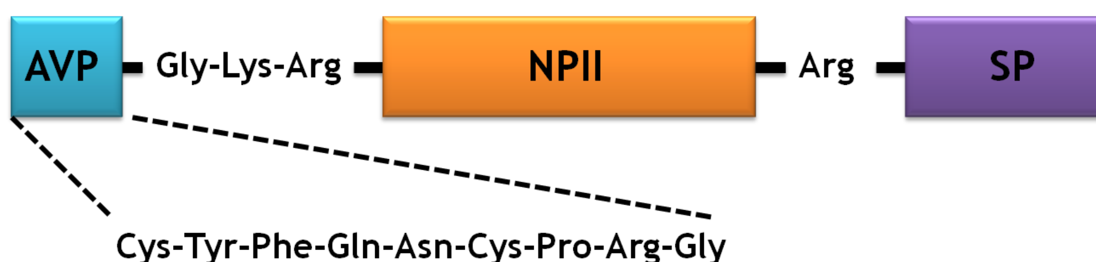

Fig. 2. Structural organization of the AVP pro-hormone. Each rectangle represents the individual domains of the pro-hormone. The amino acid sequence of the hormone and the cleavage sites are represented.

AVP controls serum osmolality by altering renal water absorption. Its release is a calcium-mediated process of exocytosis when the axon is depolarized by an appropriate stimulus [2, 19], which is determined mainly by the osmotic pressure of the plasma and extracellular fluid of the body [2]. There are specialized hypothalamic cells, called osmoregulatory neurons, which mediate the secretion of the hormone by responding to extremely small alterations in the plasma concentration of sodium and other exogenous solutes [2]. The secretion of AVP is stimulated by increases in serum osmolality, like in the case of hypernatremia, and by more pronounced decreases in extracellular fluid [3].

The antidiuretic function of AVP can be summarized in few steps. After AVP release into the systemic circulation, it binds to arginine vasopressin type 2 receptors (V_2) on the basolateral membrane of the collecting ducts cells of the kidneys, initiating a signal-transduction cascade [3, 10]. The V_2 receptor is coupled to a G_s protein and when AVP is present, the V_2 receptor activates the α subunit of the G protein which stimulates the adenylyl cyclase leading to an increase in cyclic AMP (cAMP) inside collecting ducts cells and to the consequent activation of protein kinase A (PKA) [2, 3]. The activation of the cAMP-PKA pathway originates two related mechanisms: it increases the expression of a specific water pore, known as aquaporin 2 (AQP2) [2, 20], and it leads to the phosporylation of homotetrameric AQP2, which results in the fusion of AQP2-containing vesicles with the luminal membrane of these cells [2, 21]. When these channels are incorporated into the luminal membrane, water molecules diffuse into the

cells and exit through the basolateral sides via different water channels, called aquaporin 3 and 4 (Fig. 3) [21].

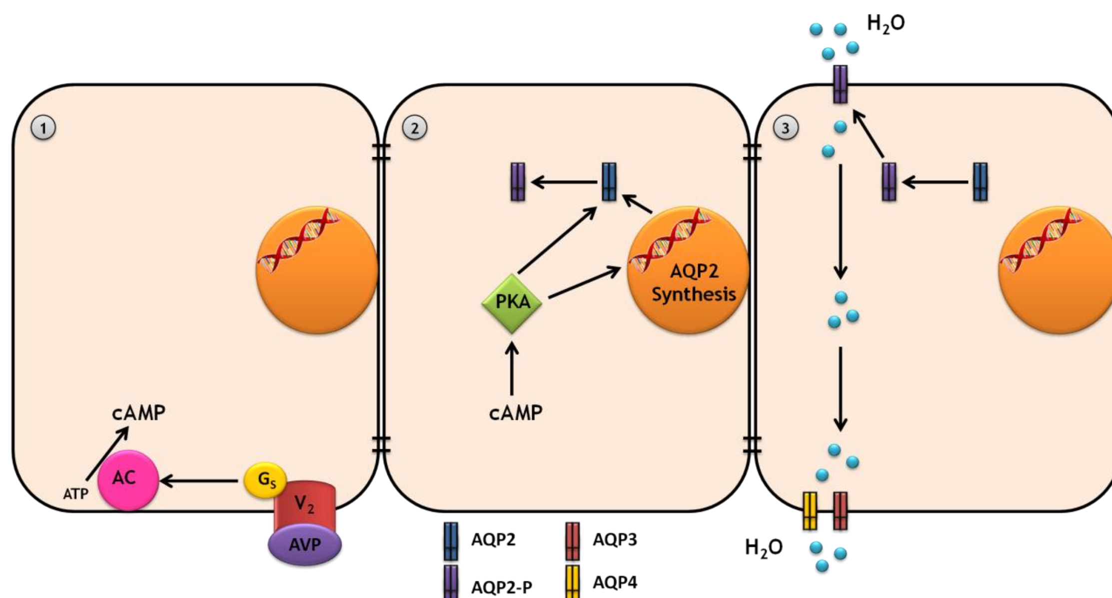

Fig. 3. Model of the regulation of water permeability in renal collecting duct cells. AVP binds to its receptor (V_2) which activates adenylyl cyclase (AC), increasing the cyclic AMP (cAMP) concentration. This intermediate activates protein kinase A (PKA) which stimulates aquaporin 2 (AQP2) synthesis and its phosporylation, leading these transporters to the apical membrane in renal cells. AQP3, aquaporin 3; AQP4, aquaporin 4; AQP2-P, phosphorilated aquaporin 2.

The described process is the molecular basis of the vasopressin-induced increase in the water permeability of the apical membrane of the collecting tubule leading to a decrease in renal water excretion [3]. In the absence of AVP stimulation, the cells of the collecting duct remain impermeable to water and the large volumes of diluted urine that enter the collecting tubules pass unmodified [2]. Thus, the excretion of urine reaches high rates and low osmolarity [2].

AVP also increases the water reabsorption capacity of the kidney by regulating the urea transporter in the collecting duct and the permeability of principal collecting duct cells to sodium [20]. Thus, in the absence of AVP stimulation, the collecting duct cells have very low permeability to water, sodium and urea, allowing the excretion of large volumes of hypotonic urine [10].

Some patients with adFNDI retain some limited capacity to secrete AVP during severe dehydration, however in most cases the deficiency of AVP secretion progresses and eventually becomes so severe that the organism can no longer concentrate urine, even during severe hypertonic dehydration [3, 4]. Symptoms of the disease usually appear after the first year of life, in contrast with nephrogenic DI, in which the defects result from mutations in V_2

receptors or in AQP2 and the symptoms are present during the first week of life [3]. In the first years of life, AVP deficiency can be partial and patients can concentrate their urine during a fluid deprivation test [4]. Thus, this result can lead to a misdiagnosis of primary polydipsia and to a delay in effective treatment [4].

1.4. Genetic basis of adFNDI

Until now, adFNDI has been associated with several different mutations in the AVP gene and all, except two, are located in the coding region [10] (Human Gene Mutation Database). Most of the mutations are single base substitutions, few are dinucleotide substitutions and the remaining are deletions of 1 or 3 nucleotides [10].

Although varied in location and nature, mutations appear to have several characteristics in common. The first similarity is that mutations appear to result in a similar clinical phenotype. Second, most of the mutations affect residues that are in hydrophilic regions of the molecule. Finally, all except one of the mutations are predicted to alter or remove one or more residues that are important for folding and self-association of the pro-hormone [4, 10, 22], changing its primary structure. Production of an abnormal precursor caused by changes in its primary structure may be due to three types of mutations: those predicted to interfere with binding of the AVP and NPII, those predicted to alter the flexibility, rigidity and disulphide bridge formation of the pro-hormone and mutations predicted to encode a truncated NPII by introducing premature stop codons [4, 10]. Mutations that interfere with binding of AVP and NPII can result from changes in the N-terminal of AVP, like mutations that impair or misdirect cleavage of the signal peptide or mutations that alter any of the first three amino acids of AVP, or can result from alterations in the shape of the NPII binding pocket [4]. The second type of mutations is the most common, and these mutations modify pro-hormone characteristics by replacing, deleting or creating de novo a glycine, proline or cysteine residue [4]. Mutations that delete glycine residues would be expected to interfere with folding of the pro-hormone due to a loss of flexibility at those sites, mutations that replace or create proline residues increase the rigidity of the molecule and mutations that replace or delete cysteine residues are likely to impair folding by eliminating or modifying one or more of the eight disulphide bridges that normally stabilize the pro-hormone into its proper conformation [4, 13].

The mutations responsible for disease development are distributed throughout the precursor protein [12]. Several mutations modify the signal peptide but the substitution of a alanine for threonine at position 19 (A19T) is the most common mutation described in adFNDI and has been found in several unrelated families around the world [10, 14]. This mutation is caused by a single base substitution (guanine to adenine) in exon 1 [14] and gives rise to an aberrant prepro-hormone that is glycosylated but retains the signal peptide as a result of inefficient

cleavage by the signal peptidase [9, 23]. In mouse studies, mice that had this mutation did not develop an apparent DI phenotype and the authors did not detect loss of AVP-producing neurons, even in homozygous mice. Thus, like in humans, this mutation originates a relatively mild phenotype in mice [9]. As for mutations in the NPII domain, a number of different mutations have been identified, including missense mutations, nonsense mutations and a single amino acid deletion [9]. There is evidence suggesting that the age of onset of the symptoms is lower in several kindreds with mutations in the NPII domain than in those with the A19T mutation in the signal peptide [4]. This fact can be explained because mutations affecting the signal peptide cleavage site would be expected to allow the formation of some normal pro-hormone from the mutant alleles, whereas the NPII mutations would not [14]. Mutations that alter the AVP hormone were also found [10]. No mutations predicting changes in the linker regions connecting the pro-hormone domains or in the copeptin domains have been identified, apart from the premature stop codons, which also truncate copeptin together with distal portions of the NPII domain [4, 10]. Recently, Hedrich et al. identified one variant in the copeptin domain which predicts a replacement of guanine by adenosine. However, individuals carrying this nucleotide substitution alone do not show disease symptoms and authors concluded that this alteration seems to be a rare polymorphism and not a disease-causing mutation [13]. To date, the intronic mutation, found by Tae and colleagues [24], is the only described mutation that does not occur in the exon regions of the AVP gene and is predicted to cause retention of intron 2 during mRNA splicing. This mutation causes a frameshift from position +1 of intron 2 and the introduction of a premature stop codon in exon 3 [24]. The aberrant protein formed consists of 167 amino acids that lack the C-terminal of NPII due to a premature codon insertion, whereas the protein translated by normal mRNA sequence of AVP gene consists of 164 amino acids that include signal peptide, the hormone AVP, NPII and copeptin [24].

All of the mutations described seem to be completely penetrant, although a few mutations might not result in appearance of adFNDI until late adolescence [2].

1.5. Pathogenesis of adFNDI

The pathogenesis of adFNDI has been studied in different model systems during the past few years [10]. The main points of the disease are: the disease is associated with mutations in one allele of the AVP gene and is caused by postnatal development of deficient AVP secretion proposed to result from selective degeneration of the magnocellular neurons that produce the hormone in normal conditions [10].

Theoretically, mutations that affect the folding of secretory proteins result in loss-of-function phenotypes due to their direct impact on protein function because these mutant proteins are prevented from reaching their final destination [3]. Thus, mutant proteins that fail to fold

correctly are retained initially in the endoplasmic reticulum [3], as this organelle has the ability to recognize, retain and degrade misfolded, incompletely folded or partially assembled copies of the proteins in a mechanism known as endoplasmic reticulum quality control [25], and subsequently the proteins are degraded either by proteasomes or by another degradation mechanism (Fig. 4) [3]. However, why AVP misfolded mutants are cytotoxic to AVP-producing neurons is a question without answer, for now [3].

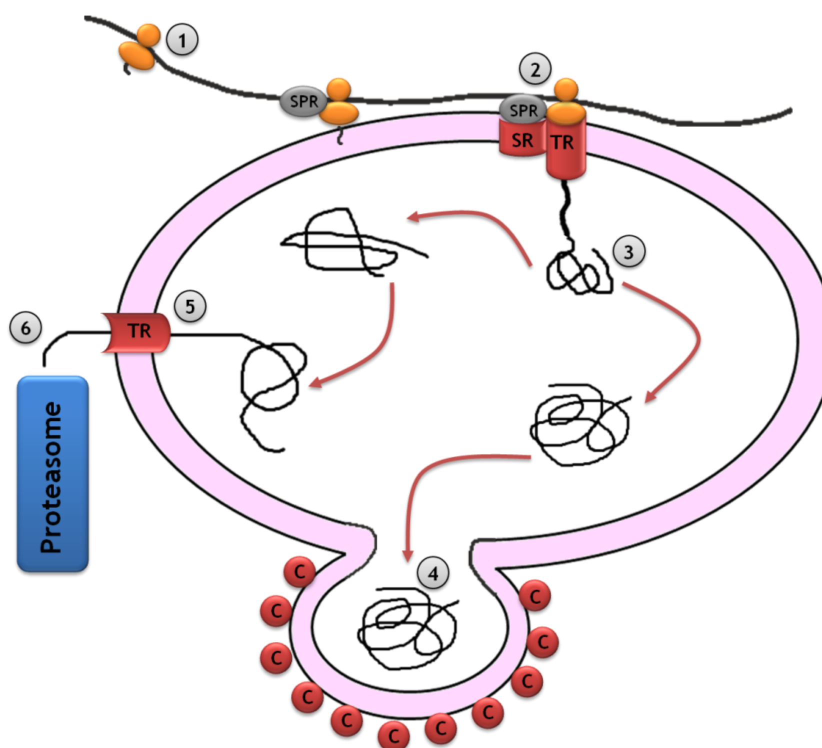

Fig. 4. Synthesis and processing of secretory proteins like AVP. mRNA and respective ribosomes migrate to endoplasmic reticulum. Then, ribosomes attach to endoplasmic reticulum by a signal recognition peptide (SRP) and the SRP receptor (SR). The growing peptide passes through the membrane via a translocon (TR). Proteins with the correct fold are stored in vesicles which will proceed to the Golgi apparatus. Misfolded proteins are initially retained in the endoplasmic reticulum, but then they are translocated to the cytosol and degraded by proteasomes. C, vesicle coat protein. Adapted from [3].

Mutant hormone precursors that do not fold and self-associate correctly probably do not move from the endoplasmic reticulum to the Golgi apparatus and to neurosecretory granules, finally, where processing mechanisms that leads to NPII, copeptin and active AVP normally occur [4]. The block in trafficking and processing of the precursor could completely eliminate AVP production from the mutant allele [4]. However, the other allele remains normal and a simple block in processing of the mutant allele would be insufficient to cause the clinical symptoms that patients with adFNDI develop, especially because the deficiency of AVP secretion is much greater than 50% [4]. This means that the mutations also interfere with the expression of the normal allele, in a mechanism known as dominant negative effect and this

mechanism can occur at any level like in transcription, translation, precursor processing and in molecule secretion [4]. Based on some studies, the processing and secretion of mutant precursors are delayed relative to processing of the wild type (WT) precursors [12] and this fact can explain the accumulation of the mutant precursor in the endoplasmic reticulum. Evidence that proves the existence of misfolded proteins in the endoplasmic reticulum is the induction of a molecular chaperone called BiP, a member of the 70 kilodalton heat shock proteins (HSP70) family [9]. This chaperone binds to misfolded proteins whose transport from the endoplasmic reticulum is blocked and BiP expression is increased as part of the unfolded protein response [9].

There are at least two mechanisms by which retention of misfolded mutant precursors in the endoplasmic reticulum could impair production of AVP from the normal allele [4]. In the first place, there is a 'nontoxic' mechanism when the mutant precursor is expressed at the same time as the WT precursor leading to the association of both precursors to form abnormal heteroligomers [4]. Thus, the mutant precursor impairs the trafficking of the WT precursor and both precursors are retained in the endoplasmic reticulum where they can be degraded or otherwise eliminated by the cell, leading to a decrease in protein activity of the WT precursors [4, 26]. With time and the high rate of mutant precursors/dimers accumulation in endoplasmic reticulum, together with the rapid degradation by the cytosolic proteasome of these heterodimers, this mechanism could easily result in the development of a severe AVP deficiency, even though the normal allele remains to be expressed at its usual rate [4, 10]. The formation of heterodimers and homodimers between mutant and WT AVP pro-hormones was already shown, such as the impairment of WT precursor trafficking by the mutant precursor during heterologous expression in cell cultures [26], resulting in formation of abnormally configured heterodimers that are retained in the endoplasmic reticulum [10].

However, the nontoxic mechanism does not explain the autopsy evidence for selective degeneration of AVP-producing magnocellular neurons [4]. Thus, it is postulated that the continuous accumulation of unfolded or misfolded mutant precursors in the endoplasmic reticulum prevents expression of the normal allele by interfering with the production of essential proteins that are important for survival of these neurons leading to a toxic mechanism [4]. However, there is little evidence of cell death caused by apoptosis, suggesting that it may occur by other pathways [9]. Some studies using immunohistochemical analyses to detect cell death of AVP-producing neurons were negative to apoptosis by using apoptosis markers [9]. But, this observation does not mean that apoptosis really does not occur in these neurons because given the small number of AVP-producing neurons and the progressive loss of cells over weeks to months, these assays may not be sensitive enough to detect apoptosis of a small number of neurons [9].

The nontoxic and the toxic theories are not mutually exclusive and together they could explain some facts like the delayed onset of the disease and its occurrence despite the

presence of a normal allele [9]. On the other hand, these two mechanisms can represent different phases of the same pathologic process (Fig. 5) [4].

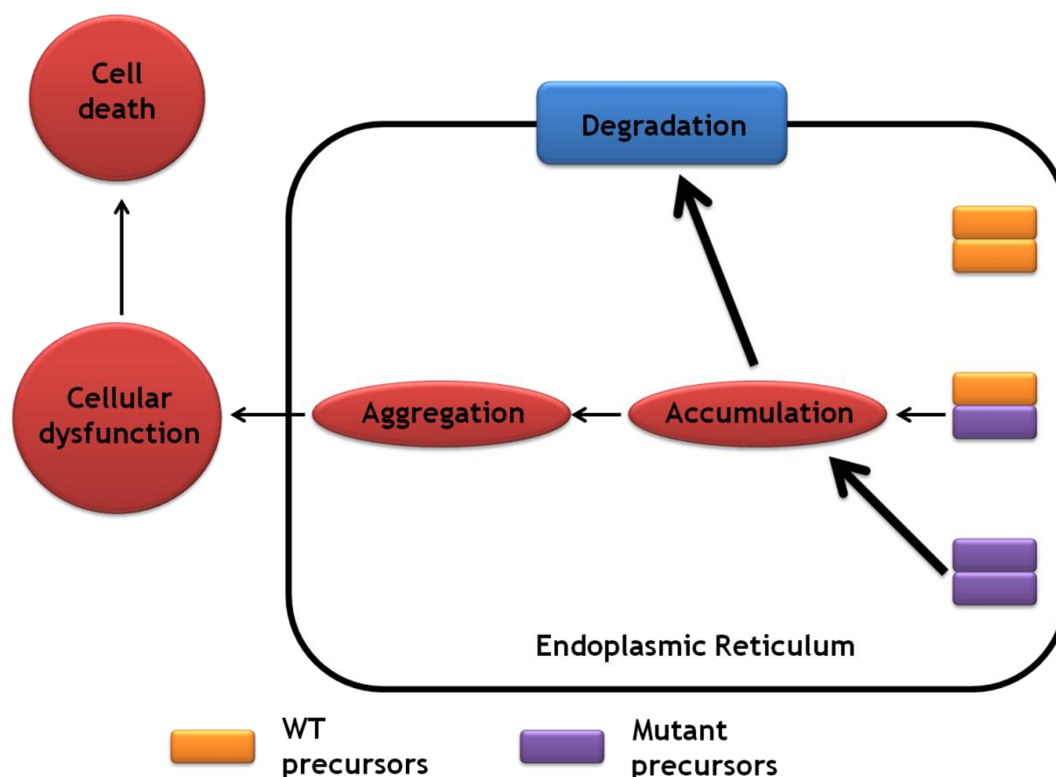

Fig. 5. A proposed model for the molecular basis of adFNDI. Functional and physical interactions occur between WT and mutated precursor that are retained in endoplasmic reticulum, impairing the transport and processing of WT. These aggregates will lead to cellular toxicity and to posterior cell death. Adapted from [26].

The hypothesis of toxicity caused by misfolded proteins (misfolding-neurotoxicity hypothesis) is consistent with all clinical, hormonal and biochemical existent data [4]. However, it is based on several factors that remain to be tested and validated and they include the following: the disease is always linked to a mutant AVP gene that originates a mutant precursor that does not fold and self-associate like the WT precursor and is not carried from the endoplasmic reticulum to the Golgi apparatus; the endoplasmic reticulum retention results in lethal accumulation and/or aggregation of mutant precursors; the decrease in AVP secretion is associated with selective degeneration of the AVP-producing magnocellular neurons; and AVP-producing parvocellular neurons are not affected by mutations that cause death in magnocellular neurons due to a much lower rate of AVP production by these cells, and/or more robust elimination mechanisms to eliminate misfolded proteins [4, 10].

Recently, new studies suggested a new mechanism that explains the pathogenesis of adFNDI. Castino et al. [27] have shown that some mutations result in accumulation of mutated

precursores in the endoplasmic reticulum, forming insoluble aggregates [27]. This accumulation results in the development of a pathology characterized by a grossly deranged endoplasmic reticulum which contains both mutated and WT protein [27]. With the aid of morphological observations, Davies et al. suggest that these structures represent autophagic vesicles [28]. Autophagy results in organelle destruction together with the WT AVP, resulting in a progressive AVP deficiency [27]. Under these circumstances, autophagy is a cell survival mechanism that removes the deranged structures [27]. However, the cells are continuously exposed to environmental and metabolic insults that can lead the cell to an autophagy-dependent apoptosis once the neurons are frail, already undergoing autophagy in order to clear mutant proteins [29]. This hypothesis does not exclude the misfolded-neurotoxicity hypothesis as, although authophagy may be responsible for the initiation of adFNDI's symptoms, it does not exclude the possibility that degeneration of the AVP-producing magnocellular neurons can be a long-term consequence [10].

The accumulation and cellular death caused by cytotoxicity of mutated precursors is a slow and prolonged process, which explains some facts like the progressive onset of the symptoms of the disease and the AVP deficiency [3, 14]. Autopsy studies performed in adFNDI patients show a selective loss of AVP-producing magnocellular neurons in the supraoptic and, to a lesser extent, in the paraventricular nucleus along with loss of their axonal extensions into the neurohypophysis [4, 30]. These studies also show atrophy of the neurohypophysis and gliosis [4, 30].

Some authors suggest that cell survival depends on its efficiency to degrade unfolded or incompletely folded proteins. Thus, degradation-resistant proteins that accumulate in the endoplasmic reticulum cause a more profound cytotoxic effect than proteins that are not resistant to the degradation process [12].

It is difficult to determine significant differences in the severity of the disease produced by the various AVP gene mutations [14]. The number of patients available for careful evaluation is very limited and there is a high degree of variability, even for patients of the same family, like the debut of symptoms, severity of polyuria and the degree of AVP deficiency [14]. Thus, these factors result in a lack of genotype-phenotype correlation which could help to determine the best treatment for the patients.

1.6. Diagnosis and treatment

The clinical diagnosis of DI can be made easily by measuring urine osmolality during a fluid-deprivation test, at least when the disease is present in its complete form, as described above [10]. However, with the development of knowledge related with the disease, some authors suggested a new diagnosis based on molecular genetic evaluation that should be

performed in all patients with familial occurrence of DI symptoms [10]. Once the molecular diagnosis is established in adFNDI kindreds, it is easier to screen other family members for the same mutations [10]. This fact has particular importance in infants at risk of inheriting the mutation as this method allows the presymptomatic diagnosis, relieving years of parental concern about the evolution of the disease in their offspring [10]. As adFNDI presents very few symptoms and allows a normal quality of life, at least when offered an appropriable treatment, and because there is little evidence for an associated risk of severe central nervous system sequelae [31] compared with nephrogenic DI, a prenatal diagnosis seems not to be indicated [10].

The treatment of adFNDI is relatively simple as the administration of the AVP analogue, DDAVP, 2 to 3 times daily eliminates symptoms [32]. Patients with adFNDI have preservation of the osmoregulation of thirst, thus only minor fluctuations in plasma osmolality are seen even during irregular pharmacological treatment and the risk of inducing hyponatremia is very small in these patients [10]. To date, no other V_2 receptor agonist has been introduced in the treatment of adFNDI but several delivery methods have been investigated and they are available in nasal sprays, in common tablets and more recently in sublingual instant melting tablet [10, 33]. However, it remains unknown if each delivery method results in better control of polyuria and polydipsia or if it is only a matter of preference [10].

In an ideal perspective, the treatment of adFNDI should be able to provide a long-lasting antidiuretic effect with the possibility to provide escape in case of higher-than-required fluid intake, like in case of social reasons. This treatment can be obtained with gene therapy which provides constantly high levels of AVP through the expression of the AVP gene contained in a viral vector [10]. Many studies have shown the efficiency of gene therapy in AVP-deficient rats using electroporation [34]. The next step in this treatment is the escape from the constant antidiuresis induced by gene therapy and this can be achieved using the recently developed V_2 receptor antagonists [34]. However, there are several diverging opinions relative to the safety of such viral approaches [10] and a further work is needed to clarify all the questions around gene therapy.

1.7. Future perspectives

adFNDI is a disease with low morbidity and an effective treatment but, despite its benign nature, the disease has been subject of intense research. This fact occurs due to its potential value as a model for studies of neuroendocrinological diseases and for studies of dominant negative mutations and due to its importance in the understanding of the effects of such mutations on the folding of hormone precursors and the role of the protein quality control machinery in the cellular handling of misfolded protein [35].

Russel et al. [9] proposed that adFNDI could be considered a neurodegenerative disorder like Alzheimer disease, Parkinson disease and others [9]. This suggestion is due to accumulation of cytotoxic precursors inside neuronal cells in adFNDI, as in the above diseases, leading to the posterior death of the cell.

A possible therapeutic approach to diseases caused by accumulation of misfolded proteins inside the endoplasmic reticulum can be the use of pharmacologic chaperones to promote the escape of proteins from this organelle [3]. Thus, the proteins can proceed their transport to its target cells. In this case, without trafficking impairment, the mutant proteins could be sufficiently functional if the problem resided in the transport of the proteins [3].

It is very important to proceed with genetic and molecular studies of adFNDI as the results can give answers not only about adFNDI, but also help to explain other diseases that have the same molecular mechanism like the case of neurodegenerative diseases or other pathologies that involve protein misfolding or aggregates. On the other hand, it is necessary that patients are informed about their state more deeply, principally in case of genetic diseases that are transmitted through several generations.

1.8. Aims of the thesis

The present study is based on three main aims. First, to review AVP mutations described in the scientific literature. Second, to expand the spectrum of mutations through the analysis of additional patients with DI. Third, and last, to characterize the functional consequences of identified novel AVP mutations.

2. Methods

2.1. Literature search of AVP gene mutations

A database of the described and published AVP gene mutations was constructed by searching the National Center Of Biotechnology Information Pubmed literature ~database for articles in English, using the keywords AVP, mutation and Neurohypophyseal Diabetes Insipidus.

A total of 61 articles that described 70 different mutations were identified and evaluated. The most relevant information was analyzed and a new nomenclature was assigned to each mutation, based on recommendations from the authors Dunnen and Antonarakis [36]. Beyond the alteration in AVP cDNA, others changes were also taken into account like the exon in which mutations occur, the alterations caused at the protein level (amino acid changes), protein domain, the population and the existence of functional studies.

2.2. Subjects and clinical procedures

A total of 9 patients diagnosed with neurohypophyseal DI, consisting of 3 familial cases and 6 sporadic cases, gave their informed consent for genetic studies of their AVP gene, in order to identify possible mutations which could be responsible for their disease. Diagnosis of patients was performed at the Endocrinology, Diabetes and Metabolism Service (University Hospital from Coimbra, Portugal) and was based on a fluid deprivation test followed by DDAVP administration.

The present study was approved by the Ethics Committee of the Faculty of Health Sciences at the University of Beira Interior.

2.3. DNA extraction

When a blood sample is collected to perform molecular analysis, like the identification of genetic mutations, the first step in laboratorial procedure is DNA extraction. The method chosen for DNA extraction from peripheral blood is very important as it is necessary to obtain a highly purified DNA without fragmentation. Some points are very important when a particular technique is chosen like technical requirements, the time required to develop the protocol, the efficiency of the method and its monetary cost [37]. Several methods are used to extract DNA, including the use of organic solvents, but the contamination with proteins is a frequent problem [38]. Miller et al. published, in 1987, a new method to extract DNA that

involves the salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution [38].

A total of 10 mL of blood was collected from each patient with Neurohypophyseal DI and the genomic DNA was extracted by the salting out method. The first stage in DNA extraction is cell lysis in order to have the DNA in solution. To perform the red blood cells (RBC) lysis, blood was transferred to 50 mL tubes and 30 mL of cold RBC lysis buffer (155 mM NH_4Cl; 20 mM $KHCO_3$; 0,1 mM Na_2EDTA; pH 7,4), was added. This buffer is a hypotonic solution which allows water intake into RBC, promoting their disruption. The mixture was incubated on ice during 15 min and was centrifuged at 2500 rpm, during 10 min at 4ºC. It is very important to remove hemoglobin, since its iron content can be a limitation to further downstream applications, so the previous step is repeated as long as the pellet remains red. During leucocyte lysis, 5mL of secondary extraction (SE) buffer (75mM NaCl; 25mM Na_2EDTA; pH 8,0), 12,5µL of proteinase K (20mg/mL) and 500µL of 10% (w/v) sodium dodecyl sulfate (SDS) was added to the mixture and it was incubated overnight at 55ºC in a thermal block (Star Lab). Each reagent has a specific function: SE buffer contains chelating agents, which bind to nuclease cofactors and prevent DNA degradation by these enzymes, SDS is a detergent so it dissolves the cell membrane and denatures proteins and proteinase K digests proteins [39]. Protein precipitation was performed by adding 3mL of saturated NaCl (6M), since this reagent decreases the solubility of proteins, followed by an incubation time of 10 min at 55ºC. The mixture was vortexed during 25 sec and finally it was centrifuged at 4000 rpm, during 30 min at 15ºC. The pellet was rejected and 100% (v/v) cold ethanol was added to the supernatant. As DNA is insoluble in ethanol, when this reagent is added, DNA molecules form aggregates that can be obtained in a pellet form upon centrifugation at 4500 rpm, during 5 min at 4ºC. The pellet was washed with 70% (v/v) cold ethanol followed by a last centrifugation at 4500 rpm, during 5 min at 4ºC. In the end, the pellet was solubilized in 1mL of Tris-EDTA (TE) buffer.

A most common method to quantify DNA samples is based on using a spectrophotometer, in a wave length (λ) of 260 nm. This method permits to estimate the quantity (Beer-Lambert law: $A_{260}=\varepsilon bc$, where A_{260} corresponds to absorbance, ε corresponds to molar absorbitivity with a value of $20cm.mg.ml^{-1}$, b corresponds to path length of the cuvette in which the sample is contained, and c corresponds to the concentration of the compound in solution) and the relative purity of DNA samples (in the case of proteins or RNA contamination), since proteins absorb light at 280 nm. A pure DNA sample will have a ratio (A_{260}/A_{280}) value of approximately 1.8 - 2.

The DNA was quantified using nanophotometer (IMPLEN).

2.4. Amplification of the AVP gene by polymerase chain reaction

The preparation of large amounts of specific DNA fragments is an indispensable tool in experiments in molecular biology. Polymerase chain reaction (PCR) is an enzymatic amplification technique that can be used, when the nucleotide sequences at the ends of a particular DNA region are known, to prepare significant quantities of a specific DNA fragment [40, 41].

The PCR procedure begins with heat-denaturation (95ºC) of a DNA sample into single strands (denaturation step) so that in the next step, two synthetic oligonucleotides, added in great excess, complementary to the 3' ends of the DNA fragment of interest can hybridize with their complementary sequences (annealing step). Annealing step occurs at lower temperatures (50-60ºC). The hybridized oligonucleotides will serve as primers for synthesis of a new DNA chain (extension step), in the presence of deoxynucleotides (dNTPs) and a thermoresistant DNA polymerase, such as that from *Thermus aquaticus* (hence, its name Taq polymerase). These three steps form a cycle, and when the extension step finishes, the whole mixture is heated again to 95º C to denature the newly formed double stranded DNA and a new annealing step occurs, since an excess of primers is present. Repeated cycles, each one with a denaturation step, an annealing step and an extension step, quickly amplify the sequence of interest [41].

The three exons of AVP gene were amplified separately by PCR, using specific primers flanking each exon (Table 1). The PCR reaction was performed in a total volume of 25 µL with 100 ng of purified genomic DNA, 0.2 mM of deoxyribonucleotides (dNTPs) (nzytech), 1U of Dream Taq DNA polymerase and complete Dream Taq buffer in a final $MgCl_2$ concentration of 1 mM (Fermentas) and 0.25 µM of each primer (AVP_1F and AVP_1R for exon 1, AVP_2Fc and AVP_2Rc for exon 2 and AVP_3Fd and AVP_2+3R for exon 3) (Stabvida). The PCR protocol was initiated with a denaturation step at 95ºC during 5 min followed by 35 cicles, each cycle with a denaturation step at 95ºC during 30 sec, an annealing step at 59ºC for exon 1, 75ºC for exon 2 and 66ºC for exon 3, during 30 sec and a extension step at 72ºC during 30 sec and finally a longer extension step at 72ºC during 10 min so that Taq polymerase can synthesize the remaining DNA chains. PCR protocols were performed in a T100 thermal cycler (Bio-Rad).

Table 1. Sequence of the primers used for amplification of AVP exons.

Primers name	Exon	Sequence
AVP_1F	1	5' CACCAAGCAGTGCTGCATAC 3'
AVP_1R		5' CTCTTTCCTAGCCCCTGACC 3'
AVP_2Fc	2	5' ACTCCCGGCTCCCCTCCTCC 3'
AVP_2Rc		5' TGCGCGGCGGGGGCGGGCCTG 3'
AVP_3Fd	3	5' AGGGCGCCCGTGCTCACACG 3'
AVP_2+3R		5' CCTCTCTCCCCTTCCCTCTTCCCGCCAGAG 3'
AVP_3R		5’ CATTGGCGGAGGTTTATTGT 3’

2.5. Gel electrophoresis

Electrophoresis in agarose gel is used to separate, identify and purify DNA fragments. The technique is rapid and simple to perform, and the DNA location within the gel is determined by staining of fluorescent intercalating dyes, such as ethidium bromide and greensafe (nzytech), allowing the detection of DNA bands by directed examination of the gel under ultra-violet (UV) light [42]. The matrix is formed by agarose, which is a linear polymer composed of D- and L-galactose [42]. When agarose is dissolved in a hot buffer and after it gelates by lowering the temperature, the chains of agarose will form helical fibers that aggregate into supercoiled structures resulting in a network of channels, for which diameters will depend on the agarose concentration [42, 43].

Electrophoresis will depend on the capacity of the charged molecules to migrate through gel pores when placed in an electric field [43]. Near neutral pH, DNA molecules have a negative charge due to phosphate backbone and therefore they migrate towards the positive electrode [41]. The rate of migration of DNA through agarose gels depends on some factors like the molecular size of DNA (larger molecules migrate more slowly than the smaller molecules), the concentration of agarose (the more concentrated the gel is, the more closed are the gel pores and, in consequence, the more difficult it is for the migration of larger DNA fragments), the conformation of DNA (superhelical circular, nicked circular and linear forms of DNA migrate at different rates in the same agarose gel and their migration depend primarily on the concentration and type of agarose used but the migration is also influenced by the strength of the applied current, the ionic strength of the buffer used and others factors) and the applied voltage (at low voltage, the rate of migration of linear DNA is proportional to the applied

voltage but at higher voltages the mobility of high-molecular-weight DNA fragments increases differentially) [43].

After the PCR reaction, the results were analyzed by electrophoresis. The agarose gel (1% (w/v)) was prepared with TAE buffer (40mM Tris-acetate, 1mM EDTA, pH=8,0). Agarose was dissolved in TAE buffer and, in order to visualize the PCR products, greensafe (nzytech) was added (1 µL per mL of gel). After gel polymerization, it was placed in a TAE buffer-containing electrophoresis tank with opposing immersed electrodes (Bio-Rad). The samples and the DNA size standard (VC 100bp Plus DNA Ladder, Vivantis) were loaded in the slots along the top of the gel and a 120 voltage was applied. At the end of the run, the gel was analyzed by UV illumination (Uvitec) and it was photographed using FireReader software (Version 15.15, Uvitec).

2.6. AVP gene sequencing

In order to identify mutations in genes, their sequence must be analyzed. The dideoxy chain-termination method was described first in 1977 by Sanger F. and colleagues. The principal aim of this method is to synthesize a set of daughter strands, from the DNA fragment of interest, that are labeled at one end and differ in length by one nucleotide. When these daughter strands are separated by gel electrophoresis, the nucleotide sequence of the fragment can be established in automated DNA sequencing machines, since a fluorescence detector that can distinguish the four fluorescent tags is located at the end of the gel. Synthesis of truncated strands is due to the use of 2',3'-dideoxyribonucleoside triphosphates (ddNTPs) that, in contrast to normal dNTPs, lack a 3'-hydroxyl group. Due to the lack of this group in ddNTPs, they can be incorporated into a growing DNA chain by the action of a DNA polymerase, but once incorporated they cannot form a phosphodiester bond with the next nucleotide leading to termination of the chain synthesis [41].

The method begins with the denaturation of a double-stranded DNA sample to generate the template strands where a primer will hybridize for the polymerization reaction. In these reactions, the ddNTPs, at lower concentrations when compared with dNTPs, are randomly incorporated at the positions of the corresponding dNTP, causing termination of synthesis at those positions in the sequence. The inclusion of different fluorochromes in each ddNTP allows the identification of each truncated fragment after their migration through the electrophoresis gel and thus, the DNA sequence can be determined by the order in which each fragment passes through the fluorescence detector [41].

Before sequencing, the PCR products were purified by spin column technique following the manufacturer's instructions (JETQUICK PCR purification Spin Kit, Genomed). After

purification, the presence of DNA was confirmed by electrophoresis in agarose gel (1% (w/v)) with DNA size standard HyperLadder II (Bioline).

DNA sequencing has three main steps: preparation of the DNA sequencing reaction, ethanol precipitation and sample preparation for loading into the sequencing equipment (GenomeLab GeXP, Beckman Coulter). In preparation of the DNA sequencing reaction, for one DNA sample, two reactions were done. Each 20 µL reaction was prepared with approximately 14 ng of DNA sample, 0.25 µM of the primer (Stabvida) and 8 µL of DTCS Quick Start Master Mix (GenomeLab, Beckman Coulter). The primers used for sequencing reaction were the same used in the PCR reaction. The reactions were placed in T100 thermal cycler with the following protocol: 30 cycles, each cycle with one denaturating step at 96ºC during 20 sec, one annealing step at 55ºC for exon 2 and 50ºC for exon 1 and 3 during 20 sec and one extension step at 60ºC during 4 min. At the end of DNA sequencing reaction, ethanol precipitation and sample preparation were performed according to manufacturer's instructions (GenomeLab, Beckman Coulter). The method used to sequence the samples comprised a capillary temperature of 50ºC, a denature temperature of 90ºC during 120 sec, an injection voltage of 2.0 kV during 15 sec and a separation voltage of 4.0 kV during 70 min..

The results were analyzed using GenomeLab Genetic Analysis System software (Version 10.2.3, Beckman Coulter).

2.7. pRc/RSV Sequencing

The expression vector pRc/RSV (Fig. 6), which contains the human AVP cDNA, was a kind gift from Dr. J. Larry Jameson (University of Pennsylvania, USA). The cDNA was sequenced by the Sanger method. For preparation of DNA sequencing reaction, 190 ng of plasmid DNA (pDNA) was initially denaturated at 95ºC during 3 min. Then, two reactions with a final volume of 20 µL were prepared. 0.25 µM of BHG-R primer (5' GGCTGGCAACTAGAAGGCACAGTCGAGG 3') was added in one reaction and 0.25 µM of AVP_1F in the other reaction. Finally, 8 µL of DTCS Quick Start Master Mix were added in both reactions. The reactions were placed in T100 thermal cycler with the following protocol: 30 cycles, each cycle with one denaturating step at 96ºC during 20 sec, one annealing step at 50ºC during 20 sec and one extension step at 60ºC during 4 min. At the end of DNA sequencing reaction, ethanol precipitation and sample preparation for loading into the instrument were performed according to manufacturer's instructions (GenomeLab, Beckman Coulter). The method used to sequence the pDNA was the same used to sequence AVP exons with the exception of separation duration which was 150 min.

The results were analyzed using GenomeLab Genetic Analysis System software (Version 10.2.3, Beckman Coulter).

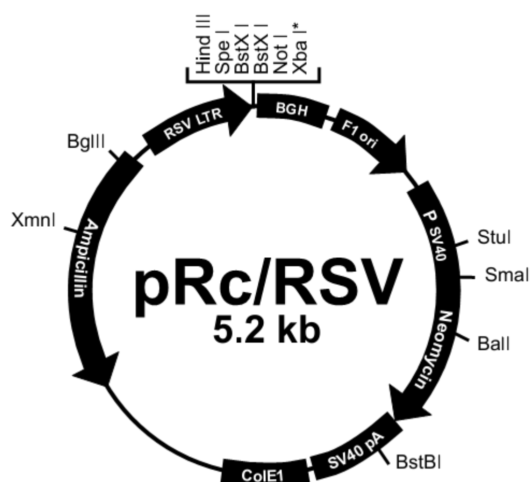

Fig. 6. Expression vector pRc/RSV (Adapted from Invitrogen).

2.8. Cloning of AVP cDNA into pVAX1/lacZ

In order to study the effects of AVP mutations on gene expression at the molecular level, it is important to have large quantities of the gene in a pure form. DNA cloning allows the preparation of large numbers of identical DNA molecules [41]. The DNA fragment of interest, AVP cDNA, is linked to a vector which allows the transport of foreign DNA and its replication into a host cell, such as the bacterium *Escherichia coli* (*E. coli*). Once inside the host cell, the inserted DNA is replicated along with the vector generating a large number of identical DNA molecules [41, 43].

The aim of this experimental step was the transfer (subcloning) of AVP cDNA, present in the expression vector pRc/RSV, to the pVAX1/*lacz* (Fig. 7) plasmid in order to insert the desired mutations in cDNA AVP. This step was crucial since the expression vector had restriction sites to the enzymes chosen for the insertion of the mutations. The *XbaI* (New England BioLabs)/*HindIII* (Takara Biotechnology) cDNA fragment was transferred from pRc/RSV to pVAX1/*lacZ* in the same sites.

A total of 2.7 µg of pRc/RSV were digested in a total volume of 50 µL with 40U of *XbaI*, 1x NEBuffer 4 (New England Biolabs) and 100 µg/mL of bovine serum albumin (BSA). A total of 3 µg of pVAX1/*lacZ* were digested in a total volume of 50 µL with 40 U of *XbaI*, 1x NEBuffer 4 and 100 µg/mL of BSA. Both reactions were incubated during 2 hours (h) at 37ºC in a thermal block. An electrophoresis in agarose gel (1% (w/v)) was undertaken to analyze the digestion results. About 40-100 U of *HindIII* were added to pRc/RSV and pVAX1/*lacZ*. The reactions were incubated at 37ºC during 2 h and 30 min in a thermal block. A final electrophoresis was done in agarose gel (1% (w/v)) in order to analyze the digested fragments.

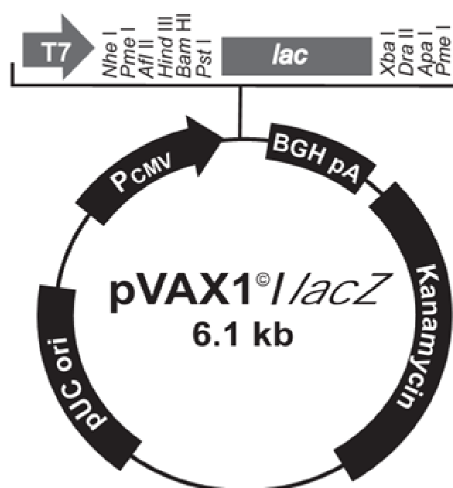

Fig. 7. Cloning vector (Adapted from Invitrogen).

To purify the AVP cDNA (*XbaI/HindIII*) released from pRc/RSV, a low-melting point agarose (2.5% (w/v)) (nzytech) was prepared. A 120 voltage was applied and at the end of migration the corresponding bands were excised. DNA was extracted from gel by spin column technique (JETQUICK Gel Extraction Spin Kit, Genomed) and the results analyzed by electrophoresis in agarose gel (1% (w/v)). Regarding digested pVAX1/*lacZ*, the purification step was performed by spin column technique (JETQUICK PCR purification Spin Kit, Genomed).

After purification, *XbaI/HindIII* AVP cDNA was ligated to *XbaI/HindIII* pVAX1/*lacZ*. Three reactions were prepared: reaction A with a final volume of 16 µL, contained 200 ng of *XbaI/HindIII* pVAX1/*lacZ*, 65 ng of *XbaI/HindIII* AVP cDNA, 3 U of T4 DNA ligase (pGEM-T Easy Vector System I, Promega) and 1x Rapid Ligation Buffer (pGEM-T Easy Vector System I, Promega); reaction B with a final volume of 20 µL, contained 200 ng of *XbaI/HindIII* pVAX1/*lacZ*, 91 ng of *XbaI/HindIII* AVP cDNA, 3 U of T4 DNA ligase and 1x Rapid Ligation Buffer; reaction C with a final volume of 14 µL, contained 100 ng of *XbaI/HindIII* pVAX1/*lacZ*, 65 ng of *XbaI/HindIII* AVP cDNA, 3 U of T4 DNA ligase and 1x Rapid Ligation Buffer. The reactions were incubated during 1h at room temperature.

2.9. Competent cells

There are two methods that allow the cells to acquire competence in order to take up the DNA from the medium: a chemical and a physical method. The chemical method is based on washing *E. coli* cells with simple salt solutions, which allow cells to achieve the desired competence state [42]. Then, DNA is added to the bacterial culture, which is subjected to a brief heat shock that stimulates the cells to take up DNA from their surrounding medium [43]. Once inside the cell, the plasmid replicates autonomously and is passed to the next generations during cell division [43].

This simple chemical procedure generates enough transformed colonies four routine tasks but the mechanisms by which these combinations of chemical agents and physical treatments induce a state of competence remains unknown, such as the mechanism by which the plasmid DNA enters and establishes itself in competent cells [42].

In order to prepare competent cells, the material and all solutions needed were sterile. The environment in which cells were prepared was sterile too. Initially, 100 µL of *E. coli* (JM109) cells were inoculated in 5 mL of LB – Broth medium at 37ºC with orbital shaking of 200 rpm overnight. The following day, 2 mL of growing culture were inoculated in 250 mL of new LB-Broth mediu at 37ºC with orbital shaking of 250 rpm until an optical density between 0.3 and 0.4 was obtained (λ = 600 nm). The absorbance was measured using a spectrophotometer (Ultraspec 3000, Pharmacia Biotech). Then, the culture was centrifuged at 5000 rpm during 10 min at 4ºC. The pellet was resuspended in 62.5 mL of $MgCl_2$ 100 mM at 4ºC, during 3 min and a new centrifugation was performed at 4000 rpm, during 10 min at 4ºC. Then, the pellet was resuspended in 12.5 mL of $CaCl_2$ 100 mM at 4ºC and a further 112.5 mL of $CaCl_2$ 100mM at 4ºC were added. The mixture was maintained on ice during 30 min and one last centrifugation was performed at 4000 rpm during 10 min at 4ºC. Finally, the cells were resuspended in 5 mL of $CaCl_2$ 85 mM at 4ºC with 15% (v/v) of glycerol and aliquots of 100 µL were immersed in liquid nitrogen and stored at -80ºC.

2.10. Transformation of competent cells with recombinant pVAX1/lacZ

After 1h of ligation between *XbaI/HindIII* pVAX1/*lacZ* and *XbaI/HindIII* AVP cDNA, performed in section 2.8, competent *E. coli* cells (JM109) were transformed with the ligation reaction product. For each ligation, the total volume of reaction was added to 50 µL of competent cells and the mixtures were incubated on ice during 30 min. Then, a heat shock at 42ºC during 1 min was performed in a thermal block for each mixture and a new incubation on ice during 2 min was performed. After this step, 200 µL of LB-Broth medium, without antibiotic, was added and the cells were incubated during 2h at 37ºC with orbital shaking of 250 rpm. A volume of 100 µL of the previous mixture was spread on LB-agar/Kanamycin plates (50 µg/mL) that were incubated at 37ºC overnight. A control was performed with 50 ng of initial pVAX1/*lacZ*.

The following day, 6 colonies of each plate (except the control) were inoculated in 20 µL of LB – Broth medium in order to performed PCR as a confirmation method of insertion of AVP cDNA in the plasmid. The PCR were performed in a total volume of 25 µL using 2 µL of inoculum, 0.2 mM of dNTPs, 1 U of Dream Taq DNA polymerase and complete Dream Taq buffer in a final $MgCl_2$ concentration of 1.5 mM and 0.25 µM of each primer (AVP_Mut1 (5’

GCCCCGGGGGCAAAGGCCGCT 3’) and AVP_Mut4 (5’ CAAGGCCCCGGCCGGCCCGT 3’)). The PCR protocol was initiated with a denaturation step at 95ºC during 5 min followed by 35 cicles, each cycle with a denaturation step at 95ºC during 30 sec, an annealing step at 60ºC during 30 sec and a extension step at 72ºC during 30 sec and finally a longer extension step at 72ºC during 10 min. PCR protocols were performed in T100 thermal cycler. An electrophoresis in agarose gel (1% (w/v)) was performed to analyze the PCR results by comparer to a DNA size standard (100bp DNA Ladder, New England Biolabs).

The positive colonies were inoculated in 4 mL of LB – Broth medium with kanamycin (50 µg/mL) and incubated overnight at 37ºC with orbital shaking of 250 rpm. After overnight growth, the cells were harvested and the recombinant plasmids were purified using Wizard Plus SV Minipreps DNA Purification System (Promega).

In order to confirm the recombinant plasmids, pDNA was subjected to digestion with *XbaI*. In a final volume of 10 µL, 5 µL of each purified pDNA, 10 U of *XbaI*, 1x of respective buffer and 100 µg/mL of BSA were added. The reactions were incubated during 2 h at 37ºC and an electrophoresis in agarose gel (1%(w/v)) was performed using a new DNA size standard (1 kb DNA Ladder, New England Biolabs). The recombinant plasmid which presented the size corresponding to recombinant pVAX1/*lacZ* (with *XbaI*/*HindIII* AVP cDNA) (pVAX/AVP) was also digested with *XbaI* and *HindIII* to confirm the AVP cDNA presence in a final volume of 25 µL, where 20 U of *XbaI*, 16-40 U of *HindIII*, 1x of *XbaI* buffer and 100 µg/mL of BSA were added. The reaction was incubated at 37ºC during 3 h and the results analyzed by an electrophoresis in agarose gel (1% (w/v)). Then, competent *E. coli* cells were transformed by the same protocol with approximately 34 ng of pVAX/AVP. The recombinant plasmids were purified using Wizard Plus SV Minipreps DNA Purification System. The pDNA (pVAX/AVP) was quantified using nanophotometer, as in section 2.3.

2.11. Site – directed mutagenesis

Site-directed mutagenesis allows researchers to make small and specific changes in a DNA sequence of interest. Alterations like substitutions of one base, deletions or insertions of a very small number of bases can be performed by this technique [43].

The PCR site-directed mutagenesis required the synthesis of four DNA oligonucleotides wherein two of them contained the desired mutation. Two primary PCR reactions produced two overlapping DNA fragments and each fragment contained the same mutation introduced by the primers, in the region of overlap. This overlap region allowed the recombination of two fragments after their mixture, denaturation and renaturation, and could be extended by DNA polymerase in order to produce a complete double-stranded DNA fragment which served as template, for a second PCR reaction using only the outermost two primers to amplify the

final sequence [40]. Finally, the modified DNA could be cloned in an expression vector and their effect analyzed by introducing the DNA into a host cell.

In order to introduce the two desired mutations in the expression vector, site-directed mutagenesis was used to create them. Seven primers were designed and synthesized (Stabvida), one of them is common to both mutations (Table 2).

Table 2. Sequence of primers used for site-directed mutagenesis.

Name	Sequence	Mutation
AVP_Mut1	5' GCCCCGGGGGCAAAGGCCGCT 3'	c.289C>G
AVP_Mut2B	5' AAGGCGGCGCAGCCGCCCCCGCT 3'	c.289C>G
AVP_Mut3B	5' AGCGGGGGCGGCTGCGCCGCCTT 3'	c.289C>G
AVP_Mut4	5' CAAGGCCCCGGCCGGCCCGT 3'	c.289C>G c.154T>C
AVP_Mut5	5'GCAAAGGCCGCCGCTTCGGGCCCA 3'	c.154T>C
AVP_Mut6	5'TGGGCCCGAAGCGGCGGCCTTTGC 3'	c.154T>C
AVP1F_Mut7	5' ATGCCTGACACCATGCTGCCCGCC 3'	c.154T>C

For the first mutation (c.154T>C) (Fig. 8), two initial PCR reactions were performed and primers AVP_Mut6 and AVP1F_Mut7 were used to create fragment 1 (Frag1) and primers AVP_Mut5 and AVP_Mut4 were used to create fragment 2 (Frag2). In a final volume of 25 µL, for both reactions, the following reagents were added: 95 ng of expression vector (pRc/RSV), 0.2 mM of dNTPs, 1 U of Dream Taq DNA polymerase and complete Dream Taq buffer, in a final $MgCl_2$ concentration of 1 mM for both fragments and 0.25 µM of each primer. The PCR protocol was the same for both reactions: a denaturation step at 95ºC during 5 min followed by 35 cycles, each cycle with a denaturation step at 95ºC during 30 sec, an annealing step at 75ºC during 30 sec and a extension step at 72ºC during 30 sec and, finally, a longer extension step at 72ºC during 10 min. The results were confirmed by an electrophoresis in agarose gel (1% (w/v)). The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and a new electrophoresis in agarose gel (1% (w/v)) was performed to analyze and quantify the purified DNA. Finally, a third PCR reaction was prepared using primers AVP1F_Mut7 and AVP_Mut4 yielding fragment 3 (Frag3). For a final volume of 25 µL, approximately 10 ng of Frag 1 and 16 ng of Frag2 were added, together with 0.2 mM of dNTPs, 1 U of Dream Taq and complete Dream Taq buffer, in a final $MgCl_2$ concentration of 1mM, and 0.25 µL of each primer. The PCR protocol was initiated with a denaturation step at

95ºC during 5 min followed by 40 cycles, each cycle with a denaturation step at 95ºC during 30 sec and an annealing/extension step at 72ºC during 1 min and finally a longer extension step at 72ºC during 10 min. A final electrophoresis in agarose gel (1% (w/v)) was prepared to analyze the PCR results.

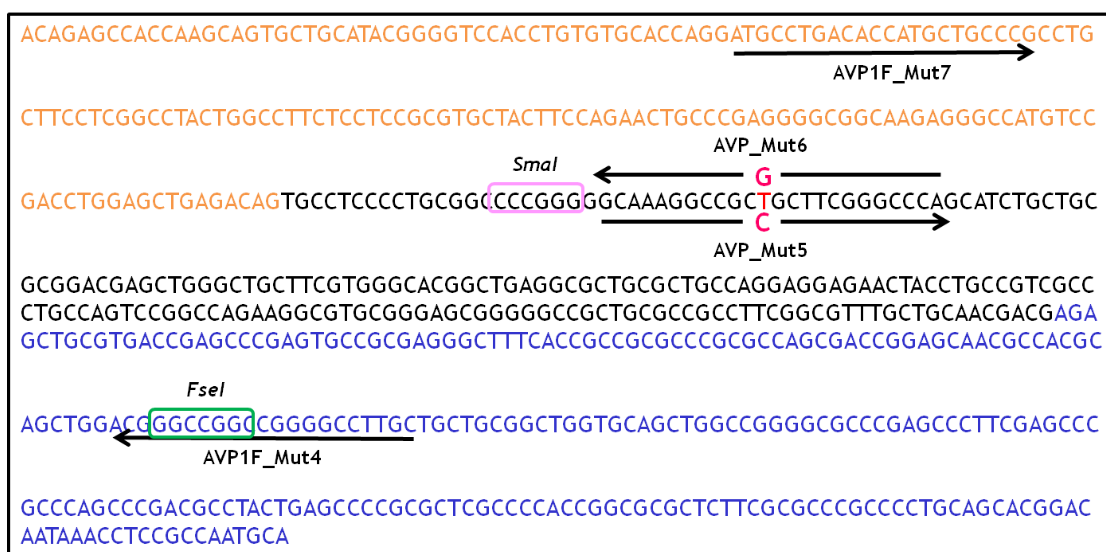

Fig. 8. AVP cDNA with primers used in site-directed mutagenesis for the first mutation (c.154T>C). Orange represents first exon, black represents second exon and blue represents third exon. Red nucleotide represents nucleotide to be substituted and pink nucleotide, in the center of the primer, represents the altered nucleotide. Surrounded by pink is the restriction site for *SmaI* and surrounded by green is the restriction site for *FseI*.

The Frag 3 was sequenced in order to confirm the insertion of the desired mutation. PCR products were purified by enzymatic digestion using *exonuclease I* (Fermentas) and *alkaline phosphatase* (Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase, Fermentas). The manufacturer's instructions for nucleotide purification were followed with an exception: 10 µL of PCR product were used instead of 5 µL. For DNA sequencing reaction, two reactions with a final volume of 20 µL were prepared. 50 ng of purified PCR product were used in each reaction and 0.25 µM of AVP_Mut4 was added in one reaction and 0.25 µM of AVP1F_Mut7 in the other reaction. Finally, 4 µL of DTCS Quick Start Master Mix were added in both reactions. The reactions were placed in the thermal cycler with the following protocol: a initial denaturation step ate 96ºC during 3 min and 30 cycles, each cycle with one denaturating step at 96ºC during 20 sec, one annealing step at 55ºC during 20 sec and one extension step at 60ºC during 4 min. At the end of the DNA sequencing reaction, ethanol precipitation and sample preparation for loading into the instrument were performed according to manufacturer's instructions (GenomeLab, Beckman Coulter). The method used to sequence the Frag3 was the same used to sequence the AVP exons with the exception of separation

duration that was 120 min. The final results were analyzed using GenomeLab Genetic Analysis System software (Version 10.2.3, Beckman Coulter).

For the second mutation (c.289C>G) (Fig. 9), primers AVP_Mut1 and AVP_Mut2B were used to create fragment AB (FragAB) in a first PCR and primers AVP_Mut3B and AVP_Mut4 were used to create fragment BB (FragBB) in a second PCR. In a final volume of 25 µL, for both reactions, the following reagents were added: 95 ng of expression vector (pRc/RSV), 0.2 mM of dNTPs, 1 U of Dream Taq DNA polymerase and complete Dream Taq buffer, in a final $MgCl_2$ concentration of 1.5 mM for FragAB and 1 mM for FragBB, and 0.25 µM of each primer. The PCR protocol used in both reactions was the same used for Frag1 and Frag2. An electrophoresis in agarose gel (1% (w/v)) was performed to confirm the PCR reaction. The products were purified using PCR & Gel Band Purification Kit (Grisp) and a new electrophoresis was performed to analyze and quantify the purified DNA fragment. Finally, a third PCR reaction was done using primers AVP1F_Mut7 and AVP_Mut4 yielding fragment CB (FragCB). For a final volume of 25 µL, 16 ng of each fragment were added, together with 0.2 mM of dNTPs, 1 U of Dream Taq and complete Dream Taq buffer, in a final $MgCl_2$ concentration of 1mM, and 0.25 µL of each primer. The PCR protocol was the same used to amplify Frag3 and a final electrophoresis in agarose gel (1% (w/v)) was performed to analyze the PCR results.

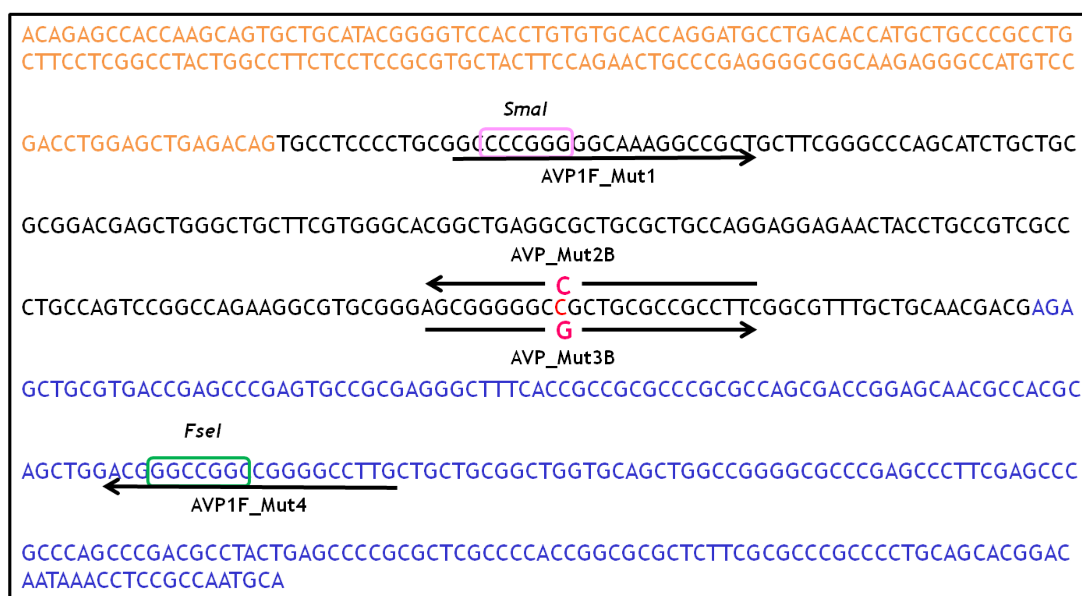

Fig. 9. AVP cDNA with primers used in site-directed mutagenesis for the second mutation (c.289C>G). Orange represents first exon, black represents second exon and blue represents third exon. Red nucleotide represents nucleotide to be substituted and pink nucleotide, in the center of the primer, represents the altered nucleotide. Surrounded by pink is the restriction site for *SmaI* and surrounded by green is the restriction site for *FseI*.

FragCB was sequenced in order to confirm the insertion of the desired mutation. PCR products were purified as previously. For preparation of DNA sequencing, two reactions with a final volume of 20 µL were prepared. 10 ng of purified PCR product were used in each reaction and 0.25 µM of AVP_Mut1 was added in one reaction and 0.25 µM of AVP_Mut4 in the other reaction. Finally, 4 µL of DTCS Quick Start Master Mix were added in both reactions. The reactions were placed in T100 thermal cycler with the same protocol used for Frag3. At the end of the DNA sequencing reaction, ethanol precipitation and sample preparation for loading into the instrument were performed according to manufacturer's instructions (GenomeLab, Beckman Coulter). The method used to sequence FragCB was the same used to sequence Frag3 and the results were analyzed using the same software.

2.12. Cloning of Frag3 and Frag CB

Fragments containing the desired mutation were digested with restricted enzymes in order to be introduced in pVAX/AVP. Both mutagenesis fragments and pVAX/AVP were digested using *SmaI* (New England Biolabs) and *FseI* (New England Biolabs). For a final volume of 50 µL, 2.5 µg of pVAX/AVP and 1 µg of PCR product (Frag3 and FragCB), 20 U of *SmaI* and 1x of respective buffer were added and the mixtures were incubated during 1 h and 30 min at 25ºC in a thermal cycler. An electrophoresis in agarose gel (1% (w/v)) was performed to analyze the results and the digested continued with addition of 10 U of *FseI* and 100 µg/mL of BSA. The reactions were incubated during 1 h and 30 min at 37ºC in a thermal block and an electrophoresis in agasore gel (1% (w/v) was performed to analyze the digest.

An electrophoresis in low melting point agarose (2.5% (w/v)) was performed in order to purify the desired fragments. The excised fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and the results analyzed by electrophoresis in agarose gel (1% (w/v)). Purified fragments were quantified.

A molar ratio of 1:3 was used to ligate *SmaI/FseI* Frag CB to *SmaI/FseI* pVAX/AVP and to ligate *SmaI/FseI* Frag CB to *SmaI/FseI* pVAX/AVP. In each reaction, 50 ng of vector and 15 ng of insert were used. T4 DNA ligase (DNA ligation kit, Takara) was used to ligate fragments. The reactions were incubated at 16ºC overnight in a T100 thermal cycler. The following day, NEB 5-alpha Competent *E. coli* (High Efficiency cells) (New England Biolabs) were transformed with ligated vectors according to manufacturer's instructions. Different colonies were incubated in LB-Broth/kanamycin medium overnight, at 37ºC with 250 rpm and pDNA were purified using Wizard Plus SV Minipreps DNA Purification System. The pDNA was quantified.

The pDNA of each colony was sequenced using the Sanger method. For preparation of the DNA sequencing reaction, 150 ng of pDNA was initially denaturated at 95ºC during 3 min. Then, two reactions for each colony were prepared to a final volume of 20 µL. 0.25 µM of

AVP1F_Mut7 primer was added in one reaction and 0.25 µM of AVP_Mut4 in the other reaction. Finally, 4 µL of DTCS Quick Start Master Mix were added in both reactions. The reactions were placed in a thermal cycler with the following protocol: a denaturing step at 95ºC during 3 min and 30 cycles, each cycle with one denaturating step at 96ºC during 20 sec, one annealing step at 50ºC during 20 sec and one extension step at 60ºC during 4 min. At the end of the DNA sequencing reaction, ethanol precipitation and sample preparation for loading into the instrument were performed according to manufacturer's instructions (GenomeLab, Beckman Coulter). The method used to sequence the pDNA was the same used to sequence the AVP exons with exception of separation duration that was 110 min. The results were analyzed using the same software.

2.13. Expression of the normal AVP gene in Neuro 2A cells

Several authors published functional studies where they show the effect of adFNDI mutations in neuronal cell lines, allowing an advancement in the understanding of consequences of the AVP gene mutations at the level of protein expression [12, 13, 44]. Several of these studies used neuroblastoma cells, known as neuro2A (N2A), as these cells are from neuronal origin and provide adequate conditions for the expression of the AVP gene [12].

N2A cells were kindly provided by Prof. Luis Pereira de Almeida (Center for Neurosciences and Cell Biology & Faculty of Pharmacy, University of Coimbra, Portugal). The cells were seeded in 12-well plates and growth in Dulbecco's modified eagle's medium (DMEM) (Biochrom AG) with 10% of fetal bovine serum (FBS) and 5% of antibiotics (penicillin and streptomycin) at 37ºC in an atmosphere of 5% of CO_2. One day before transfection, the medium was changed to DMEM with 10% of FBS and without antibiotics. To optimize the optimum ratio of DNA to lipofectamine reagent, the following day, cells were transfected with 5 µg or 10 µg of wild type AVP cDNA construct (pRc/RSV-AVP) and 4 µL or 8 µL of Lipofectamine 2000 (Invitrogen) per well (table 3). Two transfection controls were performed, one to analyze endogenous protein production, where no pRc/RSV-AVP neither lipofectamine were added to cells, and another to monitor cytotoxicity from the transfection reagent, where 8 µL of lipofectamine were added to cell culture. Another two controls for immunocytochemistry assays were performed where 5 µg of pRc/RSV-AVP and 4 µL of lipofectamine were added at each assay. All assays were duplicated and were summarized in table 3. Lipofectamine was diluted in 100 µL of Opti-modified eagle's medium (Opti-MEM) (Gibco) and incubated during 5 min at room temperature. The expression vector was diluted in 100 µL of Opti-MEM. The diluted DNA and lipofectamine were combined, mixed and incubated during 20 min at room temperature. Finally, this combined mixture was added to cell culture and incubated at 37ºC in an atmosphere of 5% of CO_2. 24 h latter, the medium was changed to DMEM with 5% of antibiotic without FBS and remained at 37ºC during 72 h.

Table 3. Summary of transfection assay conditions.

Wells	A	B	C	D
1	5 µg pRc/RSV-AVP 8 µL lipofectamine	5 µg pRc/RSV-AVP 8 µL lipofectamine	Without lipofectamine Without pRc/RSV-AVP	Without lipofectamine Without pRc/RSV-AVP
2	10 µg pRc/RSV-AVP 8 µL lipofectamine	10 µg pRc/RSV-AVP 8 µL lipofectamine	2 µL lipofectamine Without pRc/RSV-AVP	2 µL lipofectamine Without pRc/RSV-AVP
3	5 µg pRc/RSV-AVP 4 µL lipofectamine	5 µg pRc/RSV-AVP 4 µL lipofectamine	5 µg pRc/RSV-AVP 4 µL lipofectamine	5 µg pRc/RSV-AVP 4 µL lipofectamine

2.14. Immunocytochemistry

Immunocytochemistry is a technique that uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. Bound antibodies can be detected using several different methods. Earlier, the technique was based on labeling the specific antibody with a fluorophore which was applied to the cells to identify the antigen sites. This method is known as the direct method [45]. Later, some alterations were performed and the specific antibody, bound to the antigen, was detected with a secondary antibody that was tagged with either a fluorophore or an enzyme. This method is the indirected method widely used in immunocytochemistry [45]. Different fluorophores with different emission spectra made it possible to detect two or more antigens in the same cells but fluorescent labeling also has several disadvantages like the requirement of special instrumentation and the interpretation of background details, like autofluorescence [45]. Concerning antibodies, the most desirable display high specificity and affinity for the antigen, in order to avoid false-positive reactions, and are produced in high titer so that they can be used at high dilution [45].

Three important steps in immunocytochemistry are: cell fixation, membrane permeabilization and the block of unspecific reactions. Most studied antigens are soluble in aqueous solutions and they need to be fixed in place in cells before antibody addition. On the other hand, insoluble antigens also need to be structurally preserved [45]. All chemical fixatives cause chemical and conformational changes in the protein structure and usually, they disturb the secondary and tertiary structure of proteins that are mostly responsible for eliciting antigenicity [45]. Thus, it is important to choose a fixation method that minimally interferes with cellular structure and chemical composition [45]. Regarding permeabilization, it is important to note that antibodies are larger molecules that cannot diffuse into and out of cells, so, specific reagents must be used in order to permeabilize the cell and organelle

membranes allowing antibody access to intracellular and intraorganellar antigens [45]. With respect to blocking of unspecific reactions, secondary antibodies can react with charged groups like unbound aldehydes from the fixative reagents or cell components such as histones. Thus, in order to minimize these reactions, it is important to block the charged groups with proteins containing no important antigens [46].

After transfection, the medium was aspirated and the cells were washed with 1x phosphate-buffered saline (PBS) buffer. The fixation and permeabilization were performed using a 1:1 ratio of methanol:acetone with both solutions at -20ºC. Then, a 12-well plate was incubated at -20ºC during 10 min. Solvents such as alcohols and acetone are strong coagulant fixatives and they act by displacing water, which causes cellular shrinkage and the destruction of most organelles, breaking hydrogen bonds and thus disrupting the tertiary structure of proteins [45]. On the other hand, these solvents also dissolve membrane lipids leading to their permeabilization [45]. Cells were washed again with 1x PBS buffer and blocked with blocking buffer containing: 1x PBS, 0.1% Tween and 1% BSA for 1 h at room temperature. Then, cells were washed with 1x PBS and 0.1% Tween (PBS_T) and incubated with respective antibodies according to tables 4 and 5. The antibodies dilutions were performed with dilution solution containing PBS_T and 1% FBS. After incubation with each antibody, cells were washed with PBS_T during 15 min. Post-stained cell cultures on cover-slips were mounted using Dako fluorescent mounting medium (Sigma). According to table 3 (3C and 3D), two more controls for immunocytochemistry were performed: the first was used as labeling control (to analyze the contribution of endogenous fluorescence) and the second was used as secondary antibody control (to eliminate the nonspecific binding hypothesis).

The results were observed by Confocal laser scanning microscopy and the images were obtained using the software Zen 2011 (Zeiss).

Table 4. Antibodies used for immunocytochemistry assays with dilutions and incubation times.

Antibody type	Antibody	Species	Dilution	Incubation time
1º	Anti-NPII (Neurophysin II, goat polyclonal IgG, Santa Cruz Biotechnology)	Goat	1/50	2 h
1º	Anti-endoplasmic reticulum (anti-protein disulfide isomerase, mouse IgG2b monoclonal, Invitrogen)	Mouse	1/1000	1h30
2º	Anti-goat (Alexa Fluor 350 donkey anti-goat IgG, Invitrogen)	Donkey	1/1000	1 h
2º	Anti-mouse (Alexa Fluor 488 goat anti-mouse IgG, Invitrogen)	Goat	1/1000	30 min

Table 5. Immunocytochemistry assay.

Experiments	A	B	C	D
1	NPII staining	NPII staining + Endoplasmic reticulum staining	NPII staining	NPII staining + Endoplasmic reticulum staining
2	NPII staining	NPII staining + Endoplasmic reticulum staining	NPII staining	NPII staining + Endoplasmic reticulum staining
3	NPII staining	NPII staining + Endoplasmic reticulum staining	Without primary antibodies + with secundary antibodies	Without primary and secondary antibodies

3. Results

3.1. Reported mutations in the AVP gene

After a literature search, a table was drawn with all the mutations so far described in the AVP gene (Table 6). The first mutation was described in 1991 and since then, numerous families with a FNDI diagnosis have been studied and found to have mutations in the AVP gene.

So far, there are 70 different mutations described in the scientific literature and 104 different families reported with one of these mutations (families and mutations reported in this study were not taken into account). In these 70 mutations, 60 correspond to the substitution of a single nucleotide, 3 correspond to the substitution of 2 nucleotides, 2 correspond to the deletion of a single nucleotide, 3 correspond to the deletion of 3 nucleotides and 1 mutation corresponds to an almost complete deletion of the AVP gene (with the exception of exon 1). For one mutation it was not possible to determine the exact nucleotide change due to insufficient data. According to these variants, 54 mutations cause missense changes, 2 mutations cause deletion of a single amino acid residue, 3 mutations cause deletion of 4 amino acids residues, 7 mutations lead to the insertion of a premature stop codon (nonsense), 1 mutation causes a frameshift, 1 mutation causes an indel (insertion/deletion) and two mutations have an unknown effect at protein (p.?). Several families shared the same mutations; the most relevant was the c.55G>A mutation, corresponding to 8% of studied families. Regarding the type of alteration at the protein level, Fig. 10 presents their distribution between all reported families (families reported in this study were not taken into account).

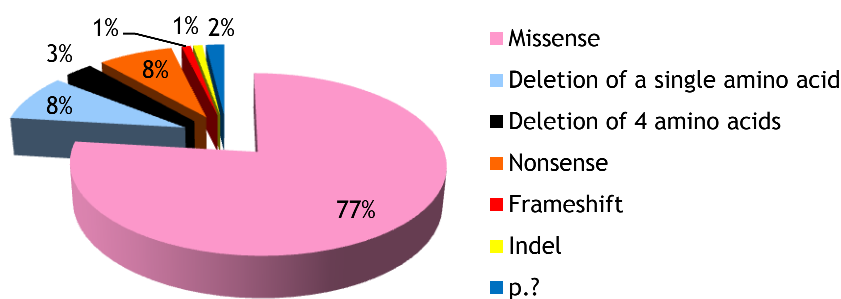

Fig. 10. Percentage of each type of protein change caused by the mutations in 104 reported families.

The mutations are distributed throughout the gene (Fig. 11)

Table 6. Reported mutations in the AVP gene causing FNDI.

Mutation (cDNA)	Exon	Predicted effect on protein[a]	Affected protein domain[b]	Population	Reference	Original designation	Confirmation by functional studies[c]
c.1A>G	1	p.M1_T4del	SP-19-16	Belgian	[49]	g.225A>G	N
c.3delG	1	p.M1_T4del	SP-19-16	Swiss	[7]	g.227delG	Y[47]
c.3G>A	1	p.M1_T4del	SP-19-16	Czech	[49]	g.227G>A	N
c.50C>T	1	p.S17F	SP-3	Danish	[22]	g.274C>T	N
c.55G>A	1	p.A19T	SP-1	Japanese	[23]	g.279G>A	Y
c.55G>A	1	p.A19T	SP-1	North American	[50]	Ala19Thr	N
c.55G>A	1	p.A19T	SP-1	Danish	[22]	g.279G>A	Y[14]
c.55G>A	1	p.A19T	SP-1	North American	[22]	g.279G>A	N
c.55G>A	1	p.A19T	SP-1	North American	[4]	g.279G>A	N
c.55G>A	1	p.A19T	SP-1	Spanish	[51]	g.279G>A	N
c.55G>A	1	p.A19T	SP-1	Brazilian	[52]	g.279G>A	N
c.55G>A	1	p.A19T	SP-1	German	[49]	g.279G>A	N
c.55G>A	1	p.A19T	SP-1	Australian	[53]	A19T	N
c.56C>T	1	p.A19V	SP-1	North American	[22]	g.280C>T	N
c.56C>T	1	p.A19V	SP-1	North American	[54]	g.280C>T	N
c.56C>T	1	p.A19V	SP-1	Lebanese	[54]	g.280C>T	N
c.56C>T	1	p.A19V	SP-1	German	[55]	g.280C>T	N
c.56C>T	1	p.A19V	SP-1	New Zealand	[49]	g.280C>T	N
c.56C>T	1	p.A19V	SP-1	Australian	[53]	A19V	N
c.61T>C	1	p.Y21H	AVP2	Turkish	[56]	g.285T>C	Y[48]
c.62A>C	1	p.Y21S	AVP2	Japanese	[57]	g.286A>C	N
c.64_66delTTC	1	p.F22del	AVP3	North American	[44]	Phe3 del	Y
c.77C>T[d]	1	p.P26L	AVP7	Palestinian Arab	[58]	g.301C>T	Y
c.123C>G	2	p.C41W	NPII10	Belgian	[59]	g.1720C>G	N
c.132C>G	2	p.C44W	NPII13	Australian	[53]	C44W	N
c.133G>C	2	p.G45R	NPII14	Israeli	[22]	g.1730G>C	N

Table 6. Reported mutations in the AVP gene causing FNDI (continuation).

Mutation (cDNA)	Exon	Predicted effect on protein[(a)]	Affected protein domain[(b)]	Population	Reference	Original designation	Confirmation by functional studies[(c)]
c.143G>T	2	p.G48V	NPII17	Dutch	[63]	NPII Gly17Val	Y[60]
c.151C>T	2	p.R51C	NPII20	Austrian	[22]	g.1748C>T	N
c.154T>C	2	p.C52R	NPII21	Portuguese	Present study	c.154T>C	N
c.160G>C	2	p.G54R	NPII23	German	[55]	g.1757G>C	N
c.160G>C	2	p.G54R	NPII23	North American	[64]	c.160G>C	N
c.160G>C	2	p.G54R	NPII23	German	[49]	g.1757G>C	N
c.160G>A	2	p.G54R	NPII23	Spanish	[65]	g.1757G>A	N
c.161G>T	2	p.G54V	NPII23	Italian	[66]	g.1758G>T	N
c.161G>A	2	p.G54E	NPII23	North American	[67]	g.1537G>A	N
c.164C>T	2	p.P55L	NPII24	North American	[68]	g.1761C>T	N
c.173G>T	2	p.C58F	NPII27	German	[69]	g.1770G>T	N
c.175T>C	2	p.C59R	NPII28	North American	[4]	g.1772T>C	N
c.176G>A	2	p.C59Y	NPII28	Cypriot	[70]	g.1773G>A	Y[61]
c.177_179delCGC	2	p.C59_A60delinsW	NPII28_NPII29	Swiss	[71]	C59del/A60W	N
c.188T>C	2	p.L63P	NPII32	Italian	[72]	g.1785T>C	N
c.192_193CT>AA	2	p.C65S	NPII34	Chinese	[73]	c.192C>A + c.193T>A	N
c.194G>T	2	p.C65F	NPII34	Dutch	[74]	Cys65Phe	N
c.200T>C	2	p.V67A	NPII36	North American	[49]	g.1797T>C	Y[62]
c.232_234delGAG	2	p.E78del	NPII47	Japanese	[75]	g.1824 -1829delAGG	Y[12]
c.232_234delGAG	2	p.E78del	NPII47	North American	[7]	g.1824 -1829delAGG	N
c.232_234delGAG	2	p.E78del	NPII47	North American	[11]	g.1824 -1829delAGG	N
c.232_234delGAG	2	p.E78del	NPII47	North American	[49]	g.1827 - 29delAGG	N
c.232_234delGAG	2	p.E78del	NPII47	North American	[49]	g.1827 - 29delAGG	N
c.232_234delGAG	2	p.E78del	NPII47	Austrian	[49]	g.1827 - 29delAGG	N
c.232_234delGAG	2	p.E78del	NPII47	Korean	[76]	c.232 - 234delGAG	N
c.232_234delGAG	2	p.E78del	NPII47	Australian	[53]	g.1824-1829del	N

Table 6. Reported mutations in the causing FNDI (continuation).

Mutation (cDNA)	Exon	Predicted effect on protein[(a)]	Affected protein domain[(b)]	Population	Reference	Original designation	Confirmation by functional studies[(c)]
c.232G>A	2	p.E78K	NPII47	Japanese	[78]	g.1829G>A	N
c.232G>A	2	p.E78K	NPII47	Moroccan	[59]	g.1829G>A	N
c.233A>G	2	p.E78G	NPII47	English	[22]	g.1830A>G	N
c.233A>G	2	p.E78G	NPII47	Norwegian	[49]	g.1830A>G	N
c.242T>C	2	p.L81P	NPII50	Danish	[22]	g.1839T>C	Y[77]
c.251C>T	2	p.P84L	NPII53	Swedish-Norwegian	[79]	g.1848C>T	N
c.260C>T	2	p.S87F	NPII56	North American	[80]	g.1857C>T	N
c.260C>A	2	p.S87Y	NPII56	Dutch	[81]	Ser87Tyr	N
c.262G>A	2	p.G88S	NPII57	Japanese	[82]	g.1859G>A	Y[12]
c.262G>A	2	p.G88S	NPII57	North American	[22]	g.1859G>A	N
c.262G>C	2	p.G88R	NPII57	North American	[22]	g.1859G>A	N
c.263G>T	2	p.G88V	NPII57	Brazilian	[83]	G88V (GGC>GTC)	N
c.274T>A	2	p.C92S	NPII61	French	[84]	Cys92Ser	N
c.275G>C	2	p.C92S	NPII61	North American	[22]	g.1872G>C	N
c.275G>C	2	p.C92S	NPII61	German	[85]	g.1872G>C	N
c.275G>A	2	p.C92Y	NPII61	North American	[80]	g.1873G>A	N
c.275G>A	2	p.C92Y	NPII61	North American	[64]	c.275G>A	N
c.276C>G	2	p.C92W	NPII61	Belgian	[59]	g.1873C>G	Y
c.276C>A	2	p.C92X	NPII61	Norwegian	[22]	g.1873C>A	N
c.276C>A	2	p.C92X	NPII61	Czech	[49]	g.1873C>A	N
c.277G>T	2	p.G93W	NPII62	Japanese	[86]	g.1874G>T	N
c.286G>T	2	p.G96C	NPII65	North American	[22]	g.1883G>T	N
c.286G>T	2	p.G96C	NPII65	Austrian	[49]	g.1883G>T	N
c.287G>T	2	p.G96V	NPII65	Japanese	[87]	g.1884G>T	N
c.287G>T	2	p.G96V	NPII65	Japanese	[88]	g.1884G>T	N
c.287G>T	2	p.G96V	NPII65	German	[89]	g.1884G>T	N

Table 6. Reported mutations in the AVP gene causing FNDI (continuation).

Mutation (cDNA)	Exon	Predicted effect on protein[(a)]	Affected protein domain[(b)]	Population	Reference	Original designation	Confirmation by functional studies[(c)]
c.287G>A	2	p.G96D	NPII65	Swedish	[49]	g.1884G>A	N
c.287G>A	2	p.G96D	NPII65	Polish	[13]	g.1884G>A	Y
c.289C>T	2	p.R97C	NPII66	North American	[91]	c.289C>T	N
c.289C>G	2	p.R97G	NPII66	Portuguese	Present study	c.289C>G	N
c.290G>C	2	p.R97P	NPII66	German	[92]	g.1887G>C	N
c.292T>G	2	p.C98G	NPII67	North American	[93]	g.1665T>G	N
c.292T>G	2	p.C98G	NPII67	Polish	[13]	g.1889T>G	Y
c.292T>A	2	p.C98S	NPII67	Italian	[94]	g.1665T>A	N
c.293_294GC>CT	2	p.C98S	NPII67	Welsh	[95]	Cys98Ser	N
c.294C>A	2	p.C98X	NPII67	Japanese	[86]	g.1891C>A	Y[12]
c.295G>C	2	p.A99P	NPII68	Brazilian	[96]	g.1892G>C	N
c.310T>G	2	p.C104G	NPII73	Czech	[49]	g.1907T>G	N
c.311G>T	2	p.C104F	NPII73	North American	[97]	g.1684G>T	N
c.311G>A	2	p.C104Y	NPII73	Dutch	[98]	Cys104Tyr	N
n/a	2	p.C105S	NPII74	Australian	[53]	C105S	N
c.313T>C	2	p.C105R	NPII74	North American	[64]	c.313T>C	N
c.314G>A	2	p.C105Y	NPII74	Japanese	[99]	g.1911G>A	N
c.322G>T	2	p.E108X/p.?	NPII74	Dutch	[100]	c.322G>T	N
c.322+1delG	Intron 2	p.E108fsX167	NPII77	Korean	[24]	IVS2+1delG	Y
c.330C>A	3	p.C110X	NPII79	Danish	[22]	g.2094C>A	Y[77]
c.337G>T	3	p.E113X	NPII82	Spanish	[51]	g.2101G>T	N
c.342_343CG>GT	3	p.E115X	NPII84	French	[22]	g.2106-07 CG>GT	N
c.343G>T	3	p.E115X	NPII84	German	[85]	g.2107G>T	N
c.343G>T	3	p.E115X	NPII84	Portuguese	Present study	c.343G>T	N
c.346T>G	3	p.C116G	NPII85	Dutch	[101]	Cys116Gly	Y[90]
c.346T>C	3	p.C116R	NPII85	Dutch	[101]	Cys116Arg	N

Table 6. Reported mutations in the AVP gene causing FNDI (continuation).

Mutation (cDNA)	Exon	Predicted effect on protein[(a)]	Affected protein domain[(b)]	Population	Reference	Original designation	Confirmation by functional studies[(c)]
c.348C>G	3	p.C116W	NPII85	Italian	[49]	g.2112C>G	N
c.352G>T	3	p.E118X	NPII87	North American	[22]	g.2116G>T	N
Gross gene deletion[(d)]		p.?		British	[102]	Deletion 10.396 bp	N

(a) Numbers represent the position in the AVP precursor. Letters correspond to universal abbreviations for amino acids.

(b) Numbers refer to the position in individual peptides originating from the prepro-vasopressin. SP, signal peptide; AVP, arginine vasopressin; NPII, neurophysin II.

(c) Y, yes; N, no. Cited references references represent later studies where the functional studies were performed.

(d) Mutation associated with autosomal recessive inheritance of FNDI.

n/a, exact nucleotide change not available in the original report.

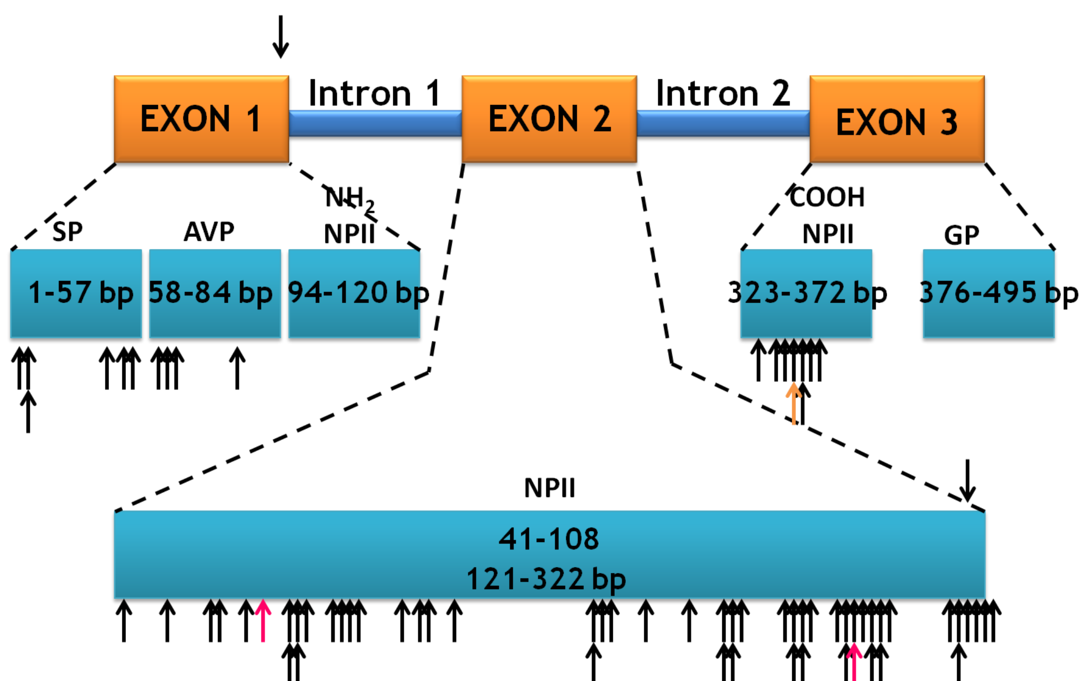

Fig. 11. Unique mutations described in the human AVP gene. Black arrows represent described mutations in scientific literature. Pink arrows represent the novel mutations studied in this thesis and the orange arrow represents the third analyzed mutation, which as previously been described.

3.2. Identification of kindreds with mutations

Nine patients were diagnosed with neurohypophyseal DI. A blood sample of each patient arrived to the Health Sciences Research Centre and the DNA of each individual was extracted from leucocytes, in order to analyze the AVP gene and detect possible mutations.

In all these patients, only the three familial cases presented mutations in the AVP gene. Although several individuals, in each family, were reported to have disease symptoms, only patients indicated by arrows (Fig. 12) were available for genetic studies, with the exception of family B (Fig. 12) in which both the index cass and his mother were studed and found to have an AVP mutation. None of the 6 sporadic cases were found to have mutations in the AVP gene.

3.3. Identification of mutations in the AVP gene

All three exons of the patients were amplified by PCR. Exon 1 amplification resulted in a 240 bp fragment, exon 2 amplification resulted in a 268 bp fragment and exon 3 amplification resulted in a 322 bp fragment (Fig 13).

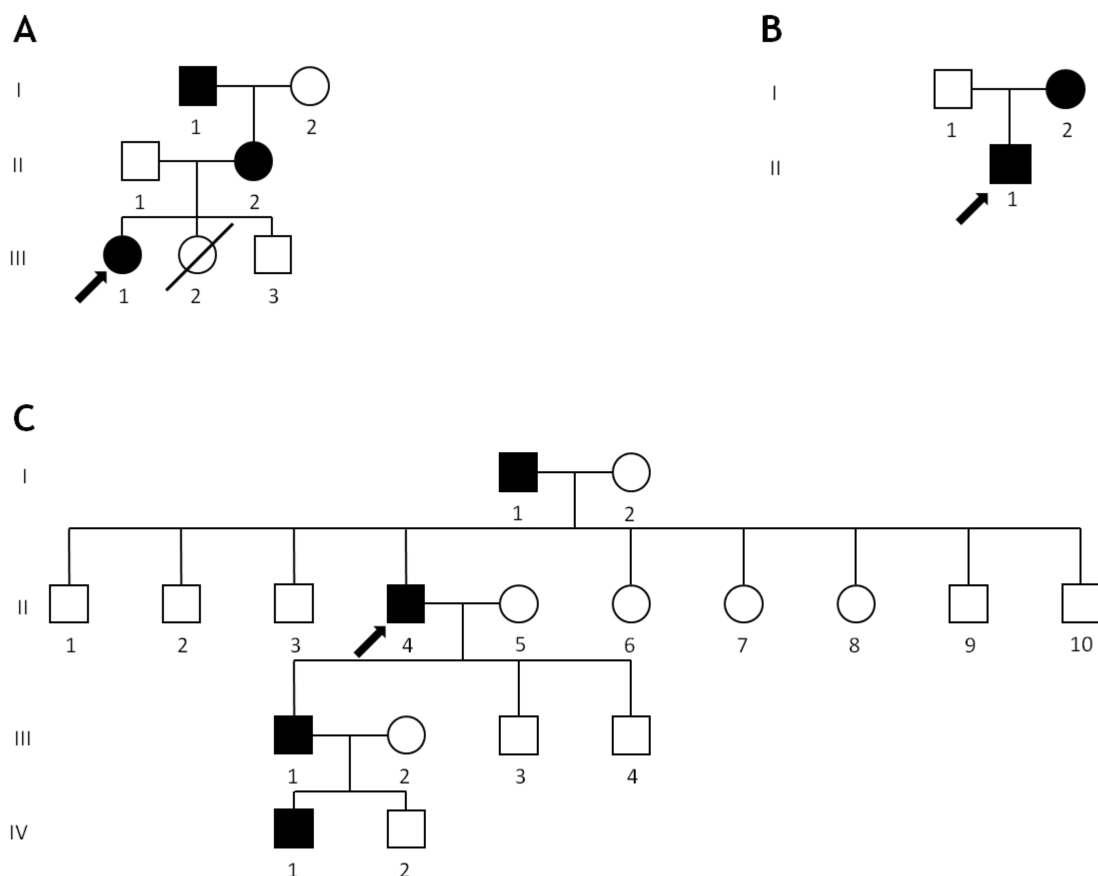

Fig. 12. Pedigrees of three families with adFNDI. Index cases are marked by an arrow. Closed symbols represent individuals with historical or laboratory evidence of DI and open symbols represent healthy individuals.

The subsequent sequencing revealed three different mutations of which two of them are novel mutations. Patient III-1 from family A showed a heterozygous T to C transition at position 154 of AVP cDNA (c.154T>C) (Fig. 14 A). This alteration occurs in the second exon and results in an amino acid substitution of cysteine to arginine at position 52 in the protein (p.C52R), affecting NPII in amino acid position 21. Patient II-1 from family B showed a heterozygous C to G transition at position 289 of AVP cDNA (c.289C>G) (Fig. 14 B). The mutation also occurs in the second exon and results in an amino acid substitution of Arg to glycine (Gly/G) at position 97 in protein (p.R97G) which corresponds to amino acid 66 of NPII. The AVP gene of the patient's mother (I-2, family B) was found to have the same mutation. Patient II-4 from family C showed a heterozygous G to T transition at position 343 of AVP cDNA (c.343G>T) (Fig. 14 C). The transition occurs in the third exon and results in a nonsense mutation as the amino acid glutamic acid is substituted by a termination codon at position 115 in the protein (p.E115X). This mutation results in a truncated protein which ends at amino acid 84 in NPII.

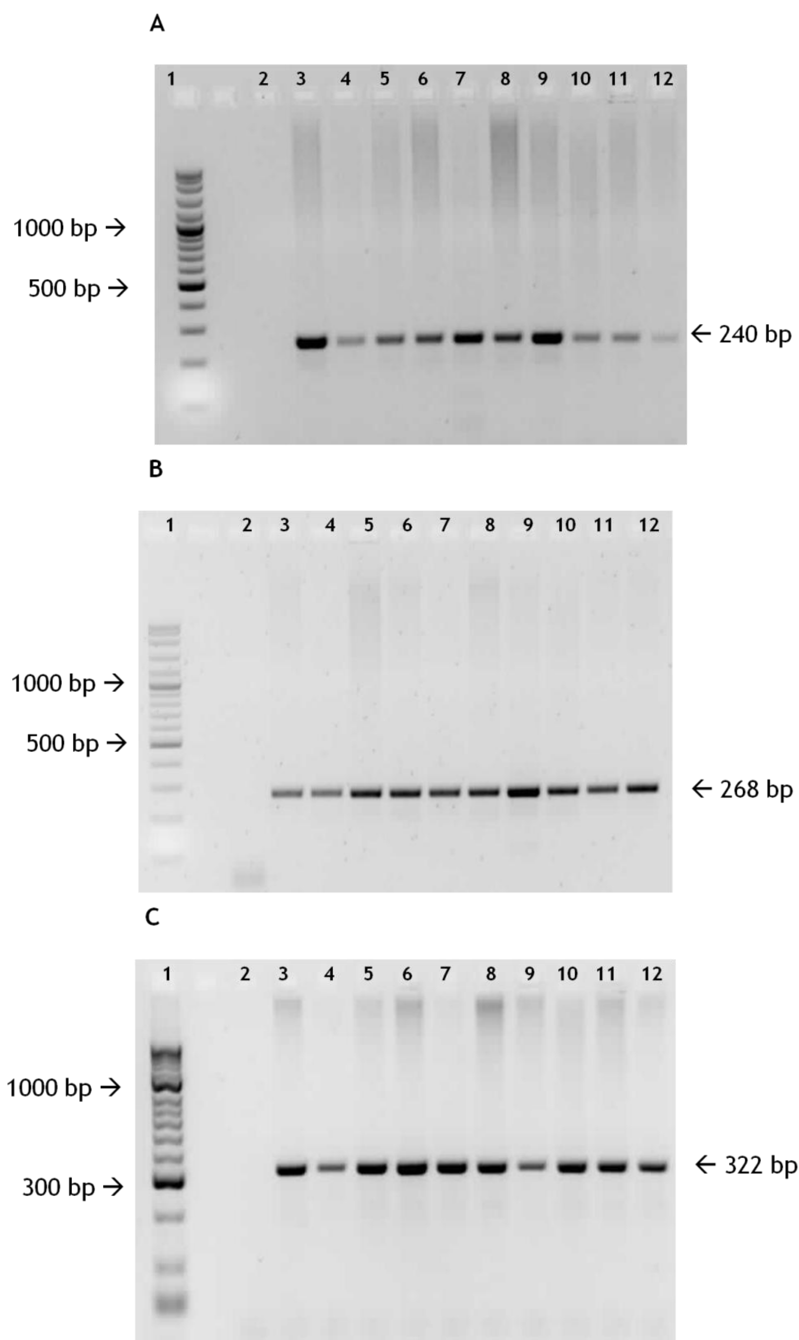

Fig. 13. Electrophoresis of PCR products for each AVP gene exon. A, B and C correspond to first, second and third exon, respectively. Lane 1 corresponds to DNA size standard, lane 2 corresponds to the negative control of PCR reaction (without DNA), lane 3 corresponds to the positive control of PCR reaction, which contains DNA of a healthy individual and the remaining lanes correspond to nine analyzed patients.

The mutations found in the patients from family A and B are novel mutations, whereas the mutation found in patient from family C has been already described [85].

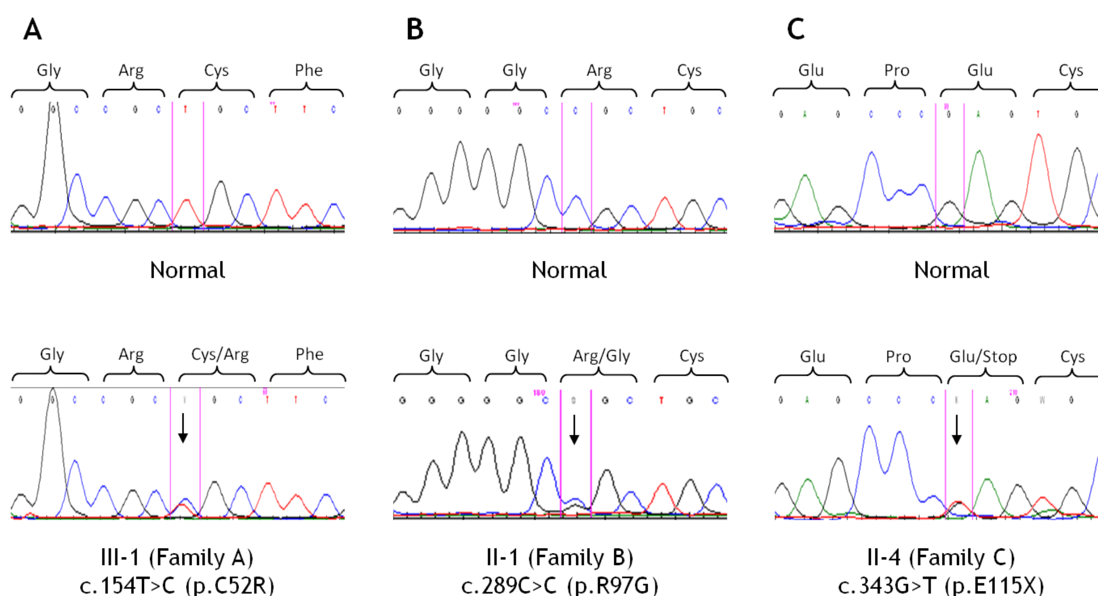

Fig. 14. Electropherograms from fragments of the AVP gene of one healthy individual and three clinically affected subjects with novel identified mutations. A, missense mutation founded in patient III-1 (family A) compared to a healthy individual in exon 2. B, missense mutation found in patients II-1 (family B) compared to a healthy individual in exon 2. C, nonsense mutation found in patient II-4 (family C) compared to a healthy individual in exon 3.The heterozygous mutations are indicated by black arrows.

3.4. Construction of the pVAX/AVP vector

pRc/RSV-AVP was sequenced to confirm the AVP cDNA sequence. There were no divergences compared with the Ensembl (Gene ID: ENSG00000101200) sequence. Therefore, the pRc/RSV-AVP was appropriate to be used as the wild-type form of AVP gene for further functional studies.

The transfer of AVP cDNA from pRc/RSV-AVP to pVAX1/*lacz* was not an easy process and required optimization. The transformation protocol of *E. coli* cells with pVAX/*lacZ* was only achieved with a stabilization step of 2 h with agitation, after transformation. The best yields of the three ligation ratios (of *XbaI*/*HindIII* AVP cDNA and *XbaI*/*HindIII* pVAX/*lacZ*) were obtained with reactions A and B, refered in section 2.8.

PCR reaction is a convenient method to confirm the presence of an insert in bacteria's pDNA. However, in this study, we observed the existence of several false positives when the pVAX/AVP was analyzed by PCR reaction, from LB-agar/ Kanamycin clones. When pVAX1/*lacZ*, with 6100 bp, was digested with *XbaI* and *HindIII*, two fragments were obtained: a larger fragment of 3125 bp, which represents the polylinker zone, and a smaller one with 2975 bp where the AVP cDNA will be inserted, leading to a 3594 bp plasmid. Thus, both plasmids

would be present in transformed colonies and in order to select the recombinants of interest, a screening of pDNA from some colonies was performed with *XbaI*, revealing some pVAX/AVP with 3594 bp length (Fig. 15, lanes 5,6,7,8,12,14,16 and 19).

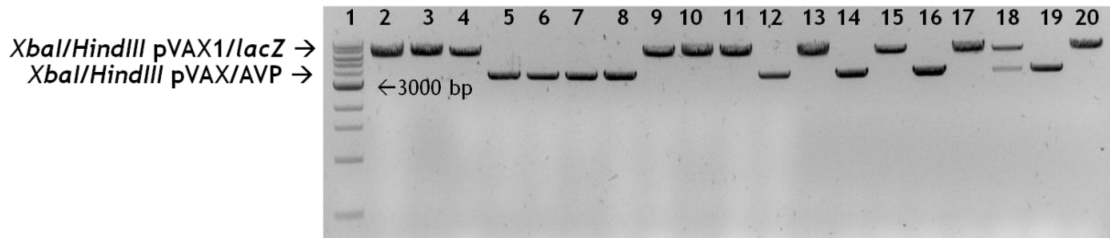

Fig. 15. Analysis of purified pDNA after enzymatic digestion with *XbaI*. Lane 1 corresponds to 1 kb DNA ladder and the remaining lanes correspond to several purified and digested pDNA. The bands with greater molecular size correspond to digested pVAX/*lacZ* and the bands with lower molecular size correspond to digested pVAX/AVP. The enzymatic digestion represented an efficient method to analyze the presence of different pDNA.

Some of the purified pDNAs were also analyzed by XbaI and *HindIII* digestion, to confirm the cDNA presence, and the correct fragments were obtained: *XbaI/HindIII* pVAX/AVP with 2975 bp and *XbaI/HindIII* AVP cDNA with 619 bp (Fig. 16).

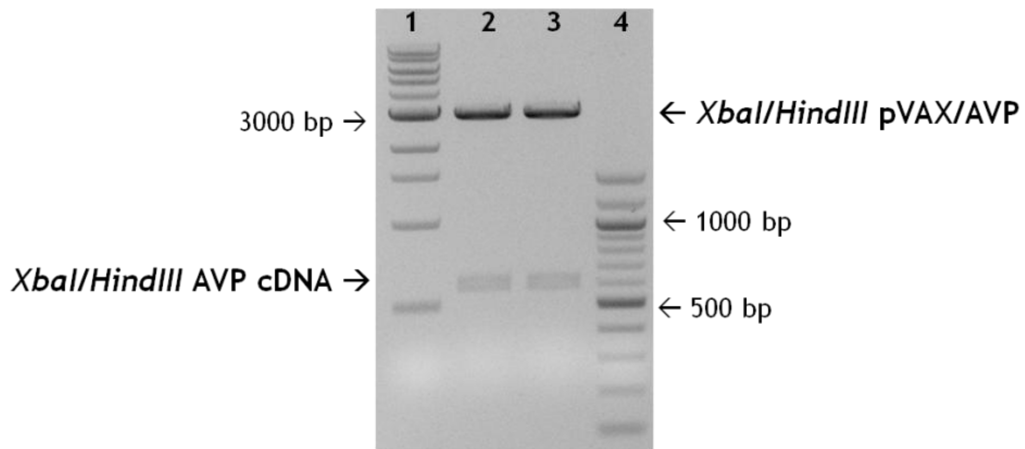

Fig. 16. Confirmation of AVP cDNA in the recombinant pVAX/AVP. Lane 1 corresponds to 1 kb DNA ladder, lanes 2 and 3 correspond to two different purified pDNAs, *XbaI* and *HindIII* digested, and lane number 4 corresponds to 100 bp DNA ladder.

3.5. Site-directed mutagenesis as a method to introduce desired mutations in AVP cDNA

Site-directed mutagenesis proves to be a very efficient technique to introduce substitutions in a DNA chain. However, it is important to consider an important factor: the position of the mutated nucleotide in the primer sequence. Initially, the mutated nucleotide was in the 3' extremity of the primer and all attempts, to insert the desired mutation in the PCR product, failed. Thus, the mutated primers were altered so that the position of the mutated nucleotide was in the middle of the primer.

The technique of site directed mutagenesis was the same for both mutations, the mutation from family A (c.154T>C) and the mutation from family B (c.289C>G). For the first mutation (c.154T>C), the PCR reaction using WT AVP cDNA as template, with primers AVP1F_Mut7 and AVP_Mut6 originated Frag1 (166 bp), and the PCR reaction with primers AVP_Mut5 and AVP_Mut4 originated Frag2 (284 bp) (Fig. 17 A). For the second mutation (c.289C>G), the PCR reaction with primers AVP_Mut1 and AVP_Mut2B originated FragAB (169 bp), and the PCR reaction with primers AVP_Mut3B and AVP_Mut4 originated FragBB (147 bp) (Fig. 17 B).

After purification of the amplified PCR products, the pairs were used as DNA template in a new PCR reaction to obtain the complete fragments (Fig. 8 and 9). For the first mutation, primers AVP1F_Mut7 and AVP_Mut4 originated Frag3, with 430 bp (Fig. 18 A), and for the second mutation, primers AVP_Mut1 and AVP_Mut4 originated FragCB, with 293 bp (Fig. 18 B).

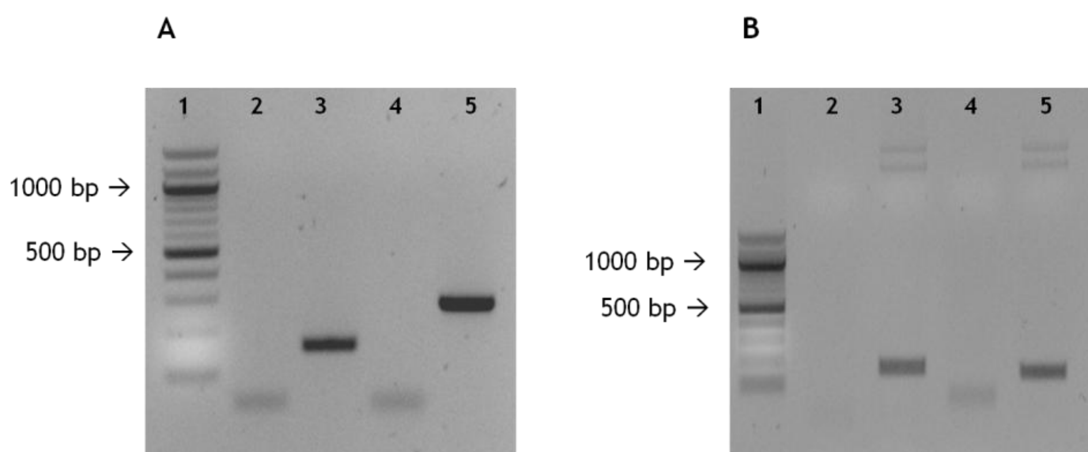

Fig. 17. First PCR reactions for site directed mutagenesis for both mutations. A corresponds to amplification of Frag1 (lane 3) and Frag2 (lane 5) whereas B corresponds to amplification of FragAB (lane 3) and FragBB (lane 5). In both images, lanes 1 correspond to 100 bp DNA ladder, lanes 2 and 4 corresponds to the negative control of the PCR reaction.

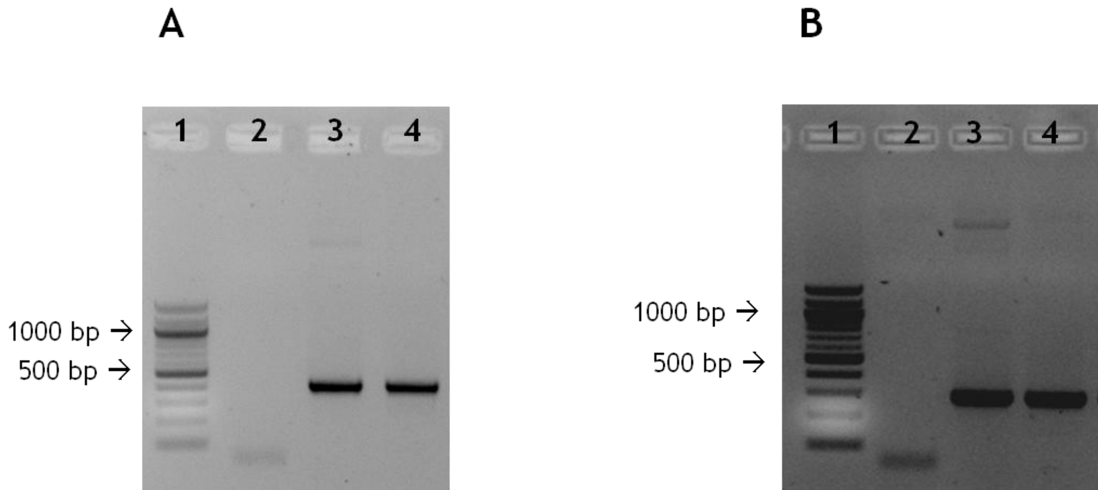

Fig. 18. Subsequent PCR reactions for both mutations. A corresponds to amplification of Frag3 (lane 4) whereas B corresponds to amplification of FragCB (lane 4) and FragBB (lane 4). In both images, lanes 1 correspond to 100 bp DNA ladder, lanes 2 to the negative control of the PCR reaction and lanes 3 to positive control (pRc/RSV-AVP).

The products of 430 and 293 bp were sequenced in order to confirm the correct insertion of mutations, and the results were consistent with the expected. Through electropherogram analysis the presence of the mutated nucleotides was observed (the cytosine in Frag3 and the guanine in FragCB) together with the WT nucleotide (thymine in Frag3 and cytosine in FragCB) (Fig. 19).

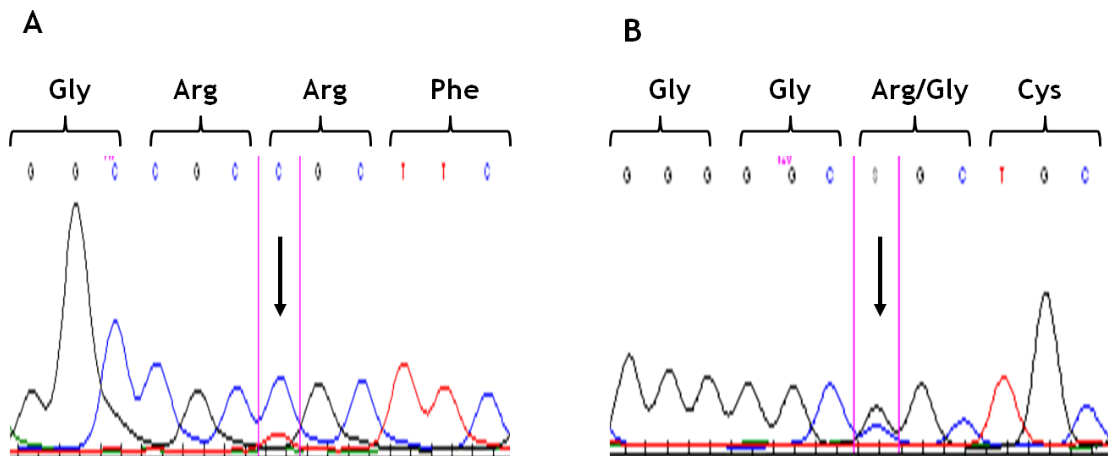

Fig. 19. Electropherograms from site-directed mutagenesis fragments. A represents a portion of Frag3 where the inserted mutation is observed and indicated by the black arrow. B represents a portion of FragCB where the inserted mutation is observed and indicated by the black arrow.

3.6. Cloning of mutations in pVAX/AVP

The process of introducing the mutations in AVP cDNA, previously inserted in pVAX/AVP, proved to be difficult. Initially, the *BbvcI* enzyme was used, in both mutations, and the fragments were correctly digested but, after ligation no transformation products were obtained. After analysis of *BbvcI* characteristics it was concluded that the *BbvcI* enzyme was not the most appropriate enzyme for re-ligation since the ligation efficacy of the digested fragments is less than 10% (New England Biolabs).

Thus, the strategy of inserting Frag3 and FragCB in pVAX/AVP was modified by the use of the *FseI restriction* enzyme, whose digestion products present a ligation percentage greater than 95% (New England Biolabs). Frag3 was digested with *SmaI* and *FseI,* originating three fragments with 138 bp, 275 bp and 13 bp. The 275 bp's fragment (*SmaI*/*FseI* Frag3) was inserted in pVAX/AVP which, when digested with the same enzymes, releases the same fragment length of 275 bp (Fig. 20). FragCB when digested with *SmaI* and *FseI* originates three fragments with, 275 bp, 13 bp and 5 bp. The fragment with 275 bp (*SmaI*/*FseI* FragCB) was inserted in *SmaI*/*FseI* pVAX/AVP (Fig. 20).

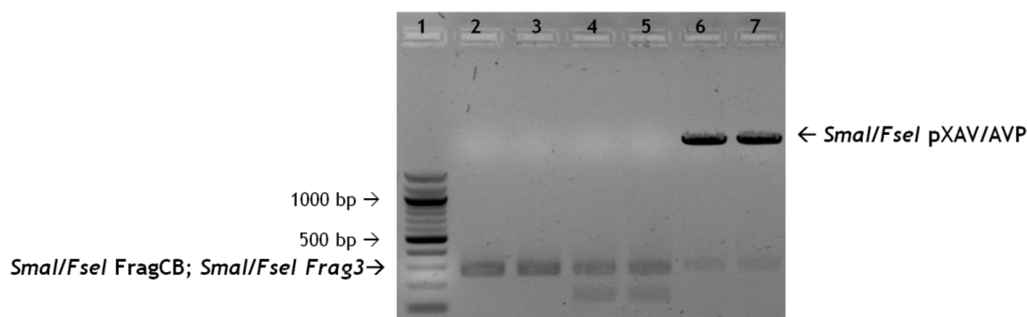

Fig. 20. *SmaI*/*FseI* digestion of FragCB, Frag3 and pVAX/AVP. Lane 1 corresponds to 100 bp DNA ladder, lanes 2 and 3 correspond to FragCB digested with *SmaI* and *FseI*, lanes 4 and 5 correspond to Frag3 digested with *SmaI* and *FseI* and lanes 6 and 7 correspond to pVAX/AVP digested with *SmaI* and *FseI*.

Competent *E. coli* cells (Promega) were transformed with ligation products, resulting from various molar ratios (1:1, 1:3, 1:5, 3:1 (vector:insert)) of *SmaI*/*FseI* Frag3 or *SmaI*/*FseI* FragCB and *SmaI*/*FseI* pVAX/AVP but no transformation products were obtained. Thus, high efficiency competent cells (New England Biolabs) were used. A molar ratio of 1:3 (vector:insert) was used to transform the cells and several transformed colonies were obtained. There were colonies with the WT nucleotide and colonies with alternative nucleotide (Fig. 21).

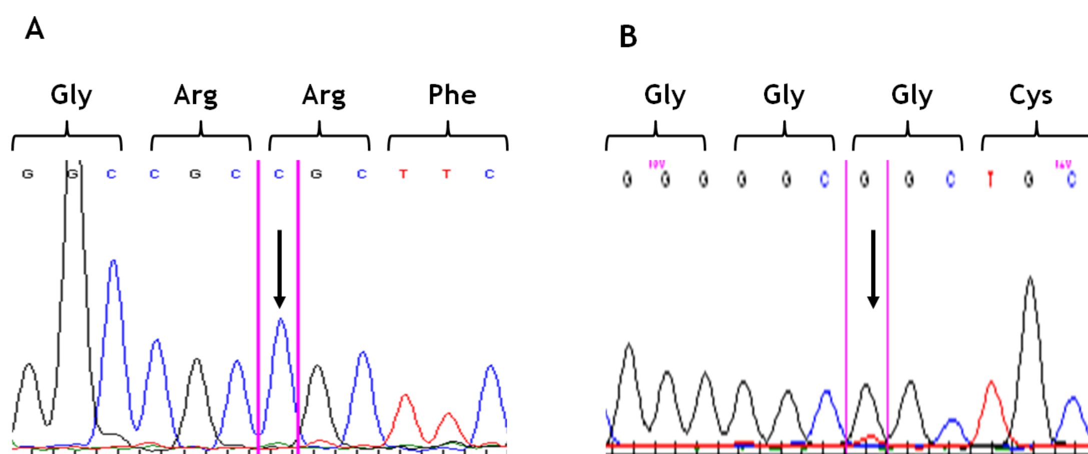

Fig. 21. Electropherograms from the sequences of the mutated pVAX/AVP vector. A represents pVAX/AVP with mutation c.154T>C and the mutated nucleotide is indicated by the black arrow. B represents pVAX/AVP with mutation c.289C>G and the mutated nucleotide is indicated by the black arrow.

3.7. Analysis of AVP WT gene expression in N2A cells

Immunocytochemistry was the chosen method to analyze the expression of the WT AVP gene in the transfected N2A cells. The transfection protocol was optimized. Initially, after transfection, the cells remained only 6 hours in medium with DNA and lipofectamine and the incubation step with DMEM with 5% of antibiotics and without FBS lasted 48 h. However, after the immunocytochemistry assay, several aggregates of poorly differentiated cells were observed and no staining for NPII was obtained. Thus, we increased the time in medium with DNA and lipofectamine to 24 h, and the incubation time with DMEM with 5% of antibiotics and without FBS to 72 h. The resulting cells were much more differentiated and some NPII staining was observed, mostly in transfection assays containing 5 μg of DNA and 8 μL of lipofectamine (Fig. 22).

The immunocytochemistry protocol was also optimized. After the transfection protocol, cells were fixed with paraformaldehyde and permeabilized with a solution containing 1x PBS and 1% Triton and were blocked with blocking buffer containing 1x PBS, 0.1% Tween and 20% FBS. The results obtained revealed a high green fluorescence, even without staining (Fig. 22). This fluorescence indicated that N2A cells presented autofluorescence when the immunocytochemistry was performed according to the previously protocol. Thus, the immunocytochemistry protocol was altered to that described in section 2.13 but some autofluorescence remained.

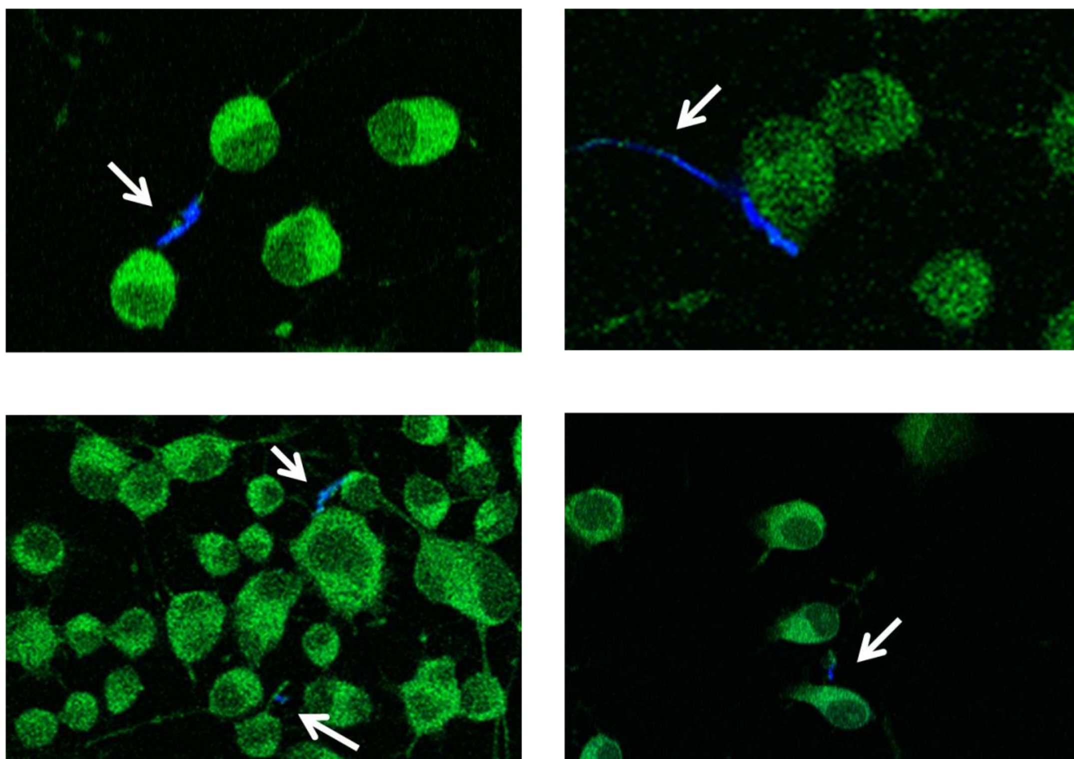

Fig. 22. Cellular localization of NPII protein in transiently transfected N2A cells as visualized by confocal laser microscopy. NPII was detected by incubation with a goat anti-NPII and an Alexa Fluor 350 donkey anti-goat (blue staining). NPII is localized in cellular axons and it is indicated by the white arrows. The green staining represents cells' autofluorescence.

After transfection and immunocytochemistry optimization, the WT AVP gene was expressed in N2A cells, as the NPII protein was observed in cells' axons using confocal laser scanning microscopy. However, a double staining of NPII and endoplasmic reticulum was not possible with the use of antibodies referred in table 4. This was due to a possible cross-reactivity between antibodies since there are two goat antibodies (anti-NPII and anti-mouse) and one anti-goat antibody which can react with both goat antibodies and, thus, eliminate the staining for NPII. To confirm this hypothesis, several double stainings were performed, for NPII and endoplasmic reticulum, and no staining for NPII was observed.

4. Discussion

Until now, adFNDI linked to mutations in the gene encoding the AVP precursor has been reported in 104 families. A total of 70 mutations have been already described, and with the novel mutations discovered and presented in this study, the total number of mutations is extended to 72.

Most mutations are located in the coding region of AVP gene, with two exceptions in mutation c.322+1delG [24], which is localized in intron 1, and a mutation recently described [102], which deletes the majority of the AVP gene and its regulatory sequences, in the intergenic region between the AVP and the oxytocin gene [102]. The mutations described in our study are also localized in the coding region of AVP gene, and, as most mutations, have an autosomal dominant transmission.

Within a total number of 72 described mutations, only two are autosomal recessive. The most recent case is reported by Christensen and colleagues [102], in which, a deletion of approximately 10 kb was described [102]. The authors suggest that, a recessive pattern is observed due to a complete abolishment of the transcription of the AVP gene [102]. Family members that are heterozygous for this deletion showed no symptoms of adFNDI, as the normal allele of AVP appears to be enough to produce sufficient AVP secretion. The second case of an autosomal recessive mutation was described by Willcutts and colleagues [58], in 1999. Their study described a mutation in the AVP domain, which results in a progressive loss of antidiuretic activity despite continued secretion of the anomalous hormone [58].

As described in the introdution, there are three types of mutations capable of producing an abnormal precursor, by causing changes in its primary structure: mutations predicted to interfere in the interaction between AVP and NPII, mutations predicted to alter the flexibility, rigidity and disulphide bridge formation of the pro-hormone, and mutations predicted to encode a truncated NPII by introducing premature stop codons [4, 10]. The three mutations identified in this study can be introduced in one, or more, groups.

Mutation c.154T>C, localized in exon 2, results in an amino acid substitution of cysteine to arginine at position 52 in the protein domain. Several mutations altering cysteine residues have been described. All these mutations are responsible for the adFNDI phenotype, since they disrupt the protein structure by eliminating a disulfide bridge that normally stabilizes the pro-hormone into its proper conformation [4, 10, 13]. This novel mutation will eliminate the disulfide bridge formed between cysteine at position 21 and cysteine at position 44 in NPII peptide. In addition to eliminate a disulfide bridge that is important to protein structure, this disulfide bridge forms the outer edge of the peptide-binding site, thus interfering with the binding of AVP hormone to NPII [103].

Mutation c.289C>G, present also in exon 2, results in an amino acid substitution of arginine to glycine at position 97 in protein domain. This mutation is similar to other described mutations that also introduce glycine residues, leading to an increase in protein flexibity and, thus impairing its correct folding [4, 13].

Mutation c.343G>T, located in exon 3, results in a substitution of glutamic acid to a stop codon, resulting in a truncated protein without copeptin and some residues in the COOH-terminal of NPII. The role of copeptin is uncertain, howerever, it is possible that copeptin glycosylation plays an important role by assisting refolding of misfolded AVP pro-hormone monomers [17], as described in the introduction. Functional studies have not yet been performed, however, protein expression can be expected for the same reason pointed for the recessive pattern of the deletion described by Christensen and colleagues [102]. Thus, this mutation can cause adFNDI phenotype by eliminating important residues that help in the folding of the precursor.

Although the novel mutations identified in this study are likely to be pathogenic, further studies are needed to demonstrate the functional effects of these mutations. For this purpose, these mutations are already been cloned into an expression vector (pRc/RSV) and the cells transfection protocol as already been optimized for the WT AVP gene. This WT AVP gene is a crucial control for immunocytochemistry assays measurement of AVP levels, western blot and apoptosis assays. It will be used as a control in all future functional studies in order to clarify the cellular mechanisms that are altered by the mutations.

The immunocytochemistry assay has also been completely optimized for the WT AVP cDNA. It will allow the analysis of NPII protein localization in N2A cells, since an accumulation of NPII protein arround the cell nucleous, and in the endoplasmic reticulum is expected to be observed. This may occur due to the aggregation of mutated precursors in the endoplasmic reticulum, since they cannot continue to the Golgi apparatus, in contrast with the WT AVP protein, which is transported along the cell axons [14].

5. Conclusion

adFNDI is a rare disease, however, with time, more studies about this disease are being reported, more mutations are being found and more cases are being studied. But, the understanding of the molecular basis underlying the disease faces a lack of answers around some questions. Why is the number of magnocellular neurons decreased in autopsies of adFNDI patients? Why do so many mutations originate so similar clinical phenotypes?

Further work will be necessary to explain the points that remain without clarification and functional studies are a useful tool in order to create the most similar environment to neuronal cells, in this case, magnocellular neurons. Thus, it may be possible to identify the mechanisms responsible for the progression of the disease and, once the cause of cellular death is identified, a therapeutic approach can be developed in order to avoid this progressive neuronal death.

Although functional studies are relevant, genetic studies also deserve a greater attention since they can be used as a preventing tool. When a family presents an adFNDI history and a genetic alteration is confirmed, the AVP gene of the younger members, despite the absence of symptoms, can be analyzed for mutations. Thus, a child without symptoms can be a potential patient, and face a dangerous situation, which can be avoided.

The understanding of the disease molecular mechanisms can be used not only in adFNDI, but in other diseases with similar mechanisms like the case of neurodegenerative diseases and other diseases that develop due to protein aggregates. Thus, it is important to continue the research work, not only to enrich scientific knowledge, but also with the intention of providing help and information to patients.

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