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Physiological and pathological role of serine 96 phosphorylation in the regulation of androgen receptor

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Declaration of original authorship

I, Diana Piol, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Spinal and bulbar muscular atrophy (SBMA) is an X-linked neuromuscular disorder characterized by the progressive dysfunction and loss of lower motor neurons. SBMA is caused by the expansion of a CAG tandem repeat encoding a polyglutamine (polyQ) tract in the androgen receptor (*AR*) gene. SBMA belongs to the family of polyQ diseases, which includes eight other neurological diseases caused by the same mutation in unrelated genes. PolyQ diseases share common features, such as that polyQ proteins are typically expressed throughout the body, yet they cause specific neuronal loss. It remains to be clarified why specific sub-populations of neurons degenerate in each polyQ disease. The well-known structure and function of AR make SBMA a good model to investigate polyQ disease pathogenesis. Androgen binding to AR results in its nuclear translocation and binding to androgen-responsive elements (AREs) to regulate gene expression. Moreover, AR is highly phosphorylated. Recently, we obtained evidence that phosphorylation of polyQ-AR by cyclin-dependent kinase 2 (CDK2) at serine 96 increases toxicity. This post-translational modification was enriched in neurons. Therefore, we hypothesized that phosphorylation of polyQ-AR at serine 96 modulates its function in response to activation of neuronal activity, a level of regulation altered in SBMA. We carried out a microarray analysis in resting and stimulated neurons in which AR was activated by androgens. Our preliminary results suggest that AR activation drives a differential gene expression program in stimulated neurons. In order to analyze the role of CDK2 and serine 96 phosphorylation *in vivo*, we deleted one or both CDK2 alleles in SBMA mice. Modulation of CDK2 expression reduced polyQ-AR phosphorylation at serine 96, decreased polyQ-AR accumulation in neurons, and attenuated disease manifestations in SBMA mice. Finally, we carried out an unbiased high-throughput screening of phosphatase and kinase inhibitors. As read-out, we analyzed polyQ-AR nuclear translocation induced by testosterone, in order to identify compounds to lower polyQ-AR toxicity. We isolated 6 phosphatase and 17 kinase inhibitors as modifiers of polyQ-AR nuclear shuttling. Among them, we found two compounds targeting Cdc25, a known activator of CDK2. Cdc25 modulation altered serine 96 phosphorylation, toxicity and transcriptional activity of polyQ-AR in cells. Our results support the idea that Cdc25 represents a potential candidate to develop new therapeutic strategies for SBMA. In summary, our findings show that serine 96 phosphorylation modifies AR physiological functions in neurons and polyQ-AR toxicity in SBMA.

1. INTRODUCTION

1.1. The motor neuron

The motor neuron is the basic unit of the motor system. A motor neuron is composed of cell body, dendrites, axons and presynaptic terminals. Each part of the neuron has a specific role in the generation and propagation of nervous signal. The cell body, called also soma, is the metabolic center of the cell, and contains the nucleus and the endoplasmic reticulum. Dendrites are short tree-shaped processes that originate from the soma. They receive numerous incoming signals from other neurons, which are integrated and processed to exert the specific neuronal function. The motor neuron axon is a long tubular projection that originates from the soma and carry electrical signals to its target tissue, the muscle. The electrical signal, named action potential, starts from the initial segment of the axon and propagates along its extension, due to the presence of a myelin sheath. This envelopment serves as insulating material for the immediate propagation of electric signal. Myelin is a lipid substance, which enwraps the axons with regular interruption, called nodes of Ranvier, that permit saltatory conduction to speed up the electric signal propagation.

The classification of motor neurons is based on their anatomical origin or their neuronal targets. The anatomical classification distinguishes between two classes of motor neurons, called upper motor neurons and lower motor neurons. Upper motor neurons (UMNs) are responsible for carrying the signal for voluntary movements through descending motor tracts. UMNs project to the lower motor neurons (LMNs) of the cranial and spinal nerves. LMNs directly innervate skeletal muscles. UMNs localize in the primary motor cortex and in the premotor areas and are responsible for the initiation and control of skeletal muscle contraction. The LMNs are located in the cranial nerve nuclei of the brainstem and the anterior nerve roots of the spinal cord.

As mentioned above, motor neurons can also be classified based on their neuronal targets, in somatic and visceral motor neurons. Somatic motor neurons have cell bodies in the central nervous system (CNS) and project their axons to skeletal muscles. The signal that triggers

skeletal muscle contraction is monosynaptic, as it involves only one somatic motor neuron that synapses the muscle. Visceral motor neurons synapse with other neurons located in the ganglia of the sympathetic and parasympathetic nervous system (or autonomic nervous system) located in the peripheral nervous system (PNS). They indirectly innervate involuntary muscles, such as cardiac muscle and smooth muscles of the arteries. The function of involuntary muscles results from non-conscious brain activity. In this case, the signal is disynaptic, as the visceral motor neuron that originate from the CNS synapses onto a ganglionic neuron located in the parasympathetic or sympathetic component of PNS, which projects to the muscle. Somatic motor neurons are further subdivided into three types: alpha, beta and gamma efferent motor neurons and information come out from the CNS to end in the periphery. The cell bodies of the alpha motor neurons are located in the ventral horn of the spinal cord (SC) and innervate extrafusal muscle fibers. On the contrary the cell body of the gamma MNs innervate intrafusal muscle fibers within the muscle spindle.

1.1.1. The motor unit

In 1925, Dr. Charles Sherrington proposed that movement is controlled by the basic functional unit composed of a motor neuron and the innervated muscle fibers. This unit is known as the "motor unit". This specific unit consists of a large anterior horn cell, its axons and the skeletal muscle fibers innervated by the axon (***Fig. 1***). In particular, the cell body of a motor neuron located in the central nervous system (CNS) projects its axon outside the CNS to directly control muscle fibers, carrying the signal from the spinal cord to the muscles to produce movement. Motor neurons form synapses with one or more fibers that are typically distributed over a relatively wide area within the muscle. There are three categories of motor units: slow (S), fast fatiguing (FF) and fast fatigue-resistant (FR) motor units.

The slow motor units are used to sustain muscular contraction and are important for keeping upright posture. They are composed of small, "red" muscle fibers that contract slowly to generate a small amount of energy, and, because they have high content of myoglobin, mitochondria and rich capillary beds, these fibers are resistant to fatigue.

The fast fatiguing motor units are important to stimulate larger muscle groups, applying large amounts of force, but they fatigue quickly. They are required for tasks that need brief burst of energy (e.g. jumping or running).

The third type of motor units, the fast fatigue-resistant types, has properties that lie between the other two types of motor units: they stimulate moderate sized muscle groups similar to the fast fatiguing type, but they are able to sustain much longer effort and provide more energy than the S motor units.

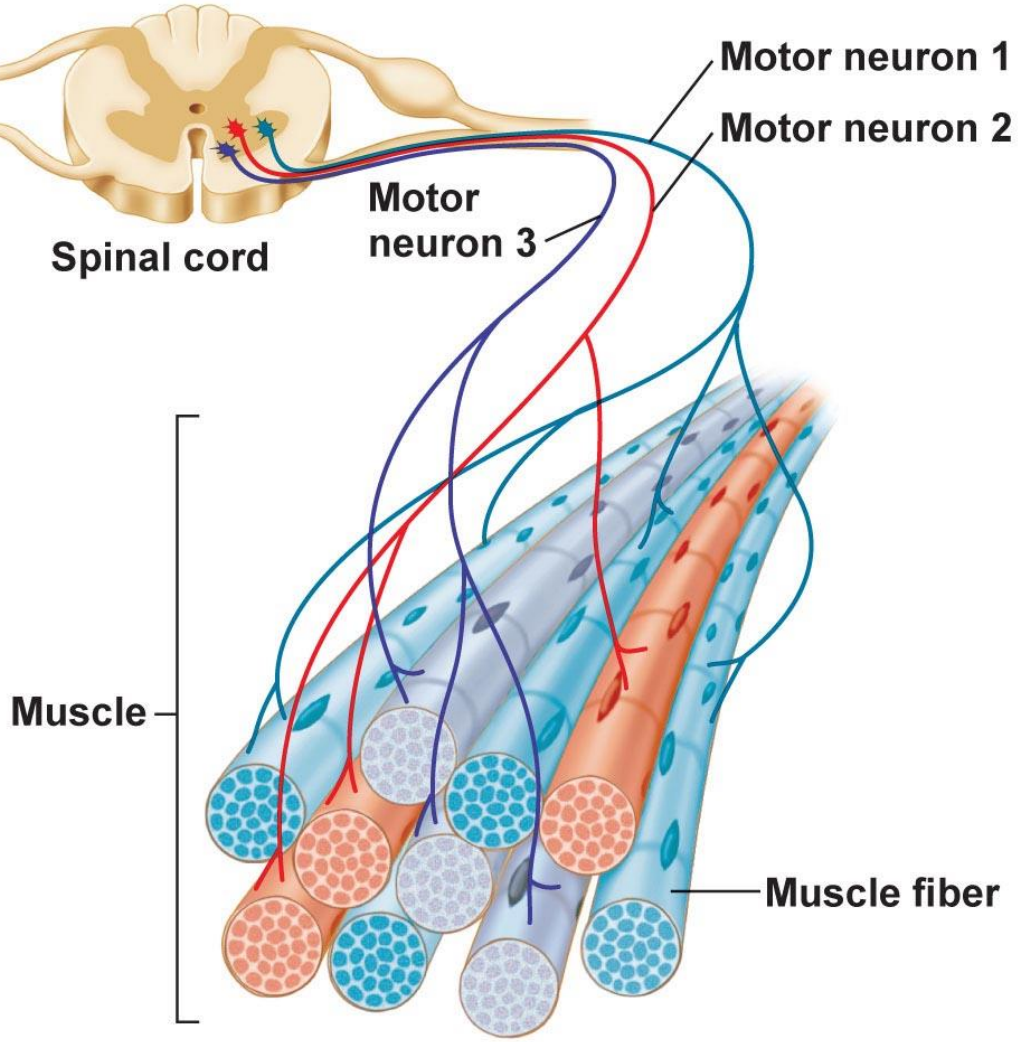


***Figure 1*. Motor unit.** A motor unit is composed by the motor neuron and the skeletal muscle fibers innervate directly by it. Image from ©Pearson education, Ink, 2011.

In most muscles, slow motor units have lower thresholds for activation than the larger units (fast fatiguing or fast-fatigue resistant) and are tonically active during motor actions that require sustained effort (standing, for instance). The threshold for the fast motor units is reached only when movements that require high energy are made, such as fast jumping. This functional distinction between the different categories of motor units is also important because it explains some structural differences among muscle groups. For instance, a motor

unit in the soleus (a muscle important for posture that comprises mostly small units) has an average innervation ratio of 180 muscle fibers for each motor neuron. In contrast, gastrocnemius, a muscle that comprises both small and large units, has an innervation ratio of 1000–2000 muscle fibers per motor neuron and can generate the force needed for sudden changes in body position.

1.1.2. Motor neuron recruitment

The progressive activation of additional motor units with increasing strength of voluntary muscle contraction is known as motor neuron recruitment. The central nervous system can increase the strength of muscle contraction by increasing the number of active motor units (i. e., spatial recruitment) or increasing the firing rate (firing frequency) at which individual motor units fire to optimize the summated tension generated (i. e., temporal recruitment). Both mechanisms occur concurrently. The primary mechanism at lower levels of muscle contraction strength is the addition of more motor units, but the firing rate of the initially recruited motor units also increases. When nearly all motor units are recruited, increase in firing frequency becomes the predominant mechanism to increase motor strength.

The activation of one motor neuron leads to the activation of the innervated muscle fibers. This process results in a weak, but distributed muscle contraction. On the other hand, the activation of more motor neurons results in more muscle fibers being activated, and therefore a stronger muscle contraction. Motor unit recruitment is a measure of how many motor neurons are activated in a particular muscle, and therefore it is a measure of how many muscle fibers of that muscle are activated. The higher the recruitment, the stronger the muscle contraction will be.

"A particular voluntary movement appears to begin with discharge of the same motor unit. More intense contraction is secured by the addition of more and more units added in a particular sequence. This ‘recruitment’ of motor units into willed contraction is identical to that occurring in certain reflexes. The early motor units in normal gradual voluntary contraction are always in our experience small ones. The larger and more powerful units, each controlling many more muscle fibers, enter contraction late”. This principle was enunciated by Denny-Brown and Pennybacker in 1934 and establishes that the orderly recruitment of motoneurons is based on size, with the smaller neurons activated first (Denny-Brown and Pennybacker 1934). It is known, commonly, as Henneman’s Size Principle, from

the name of the first scientist who elaborate these observations in a principle. A single motor unit produces a force that results not only from the number of the innervated fibers in the unit, but also from the frequency with which the innervating axon stimulates the muscle fibers. The motor unit firing rate is the percentage at which the nerve impulses arrive, and it can change from low frequencies able to produce a series of single twitch contractions (or fiber contraction), to frequencies high enough to realize a fused titanic contraction. In general, the motor unit firing rate of each individual motor unit increases with increasing muscular effort until a maximum rate is reached. Once a motor unit is activated and stimulates muscles fibers, muscle receives a signal for contraction.

1.1.3. Neuromuscular junction

The electrical information that the axon carries and transmits to muscle is converted to a chemical stimulus inside the nerve-muscle synapse, which is the junction between the motor neuron and the skeletal muscle fiber. This type of synapse is known as the neuromuscular junction. The endplate is the region of the muscle where the motor neuron loses its myelin sheath to become branched, forming expansions named synaptic boutons. Motor neurons release the neurotransmitter, acetylcholine, from the synaptic boutons in the synaptic cleft, which is a tight region between the presynaptic and postsynaptic membranes. Acetylcholine is released over a specialized region of the muscle membrane that contains depressions (junctional folds), in which a specific class of neurotransmitter receptor, the nicotinic type of acetylcholine receptors, are located. Synaptic boutons contain highly specialized structures, known as active zones, which are the sites of acetylcholine release (***Fig. 2***). At the active zones, there is high accumulation of the synaptic vesicles, which contain the neurotransmitter and voltage-gated Ca^{2+} channels that allow calcium to enter the terminal at the arrival of the action potential. Calcium influx leads to fusion of synaptic vesicles with the plasma membrane, thus releasing the transmitter in the synaptic cleft through a process known as regulated exocytotic. Exocytosis is the process by which a neuron releases neurotransmitters, contained in secretory vesicles, out of the cell membrane into the extracellular space.

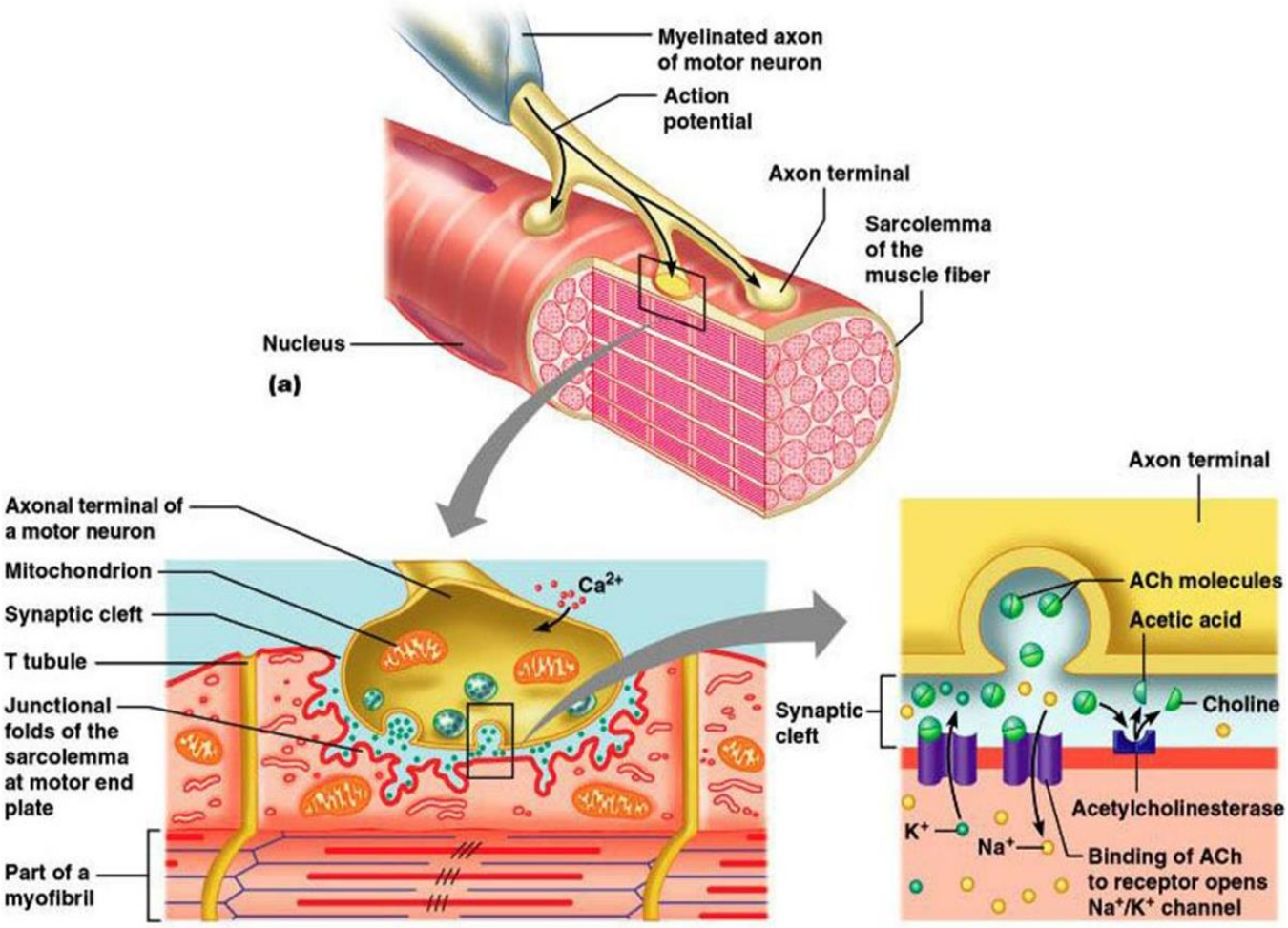


Figure 2. **Neuromuscular junction organization.** The neuromuscular junction is a chemical synapse composed by the motor neuron and a muscle fiber. The signal is transmitted through the motor neuron axon and cause the release of the neurotransmitter acetylcholine into the synaptic cleft. ©Pearson education, Ink, 2011.

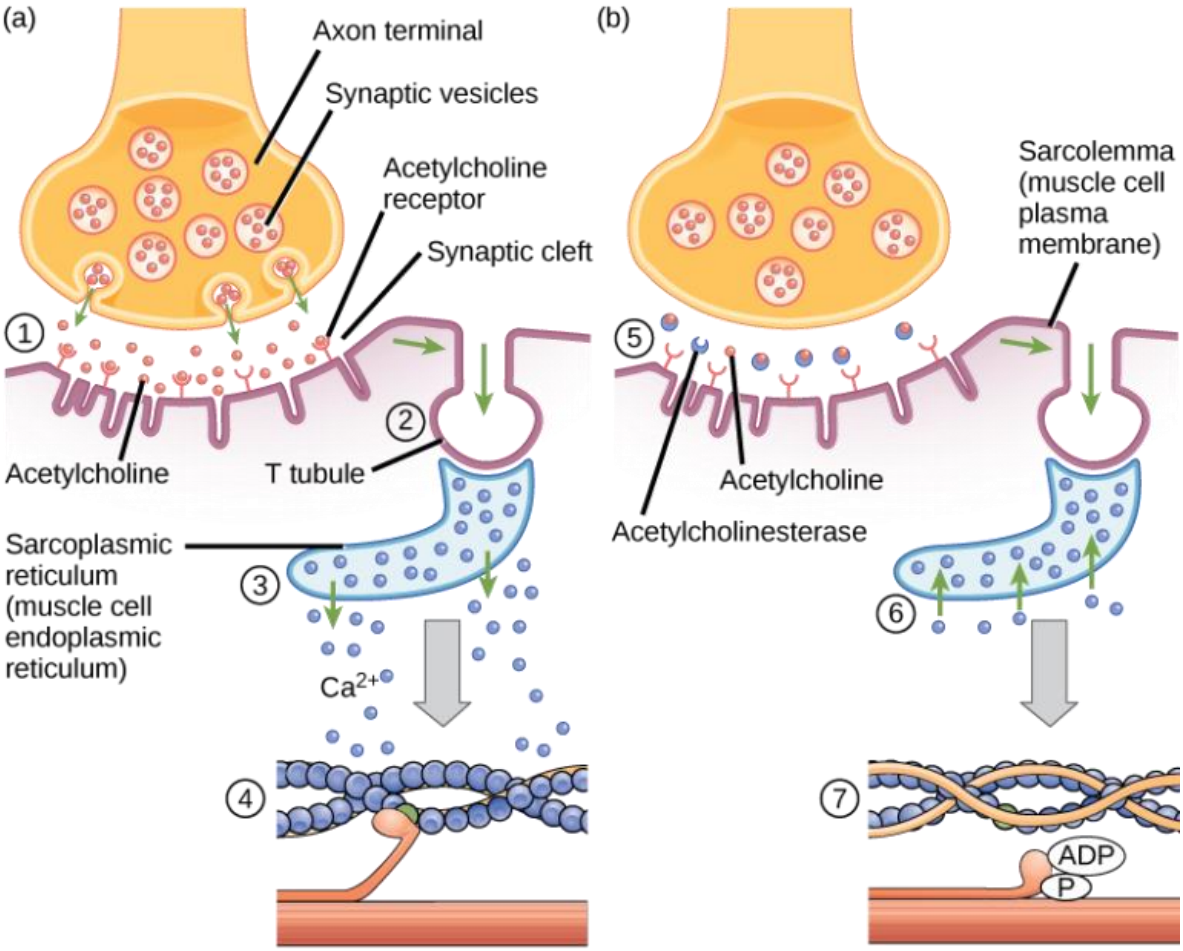
The basal lamina is the basement membrane within the cleft which is composed of collagen and other extracellular matrix components. Acetylcholine, which is anchored to the collagen fibrils of the basal laminae, is rapidly hydrolyzed by acetylcholinesterase upon arrival of the neuronal stimulus. The membrane of the junctional fold on the muscle is enriched in voltage-gated Na^+ channels. The nicotinic acetylcholine receptor is a ligand-dependent channel: once the neurotransmitter binds the receptor, an inward current is generated due to the influx of voltage-gated Na^+ channels triggering the depolarization of the membrane and initiating the action potential in the muscle necessary for fiber contraction.

1.1.4. Signal transmission and muscle contraction

Contraction of voluntary muscles (or skeletal muscles) is controlled by efferent signals that come from the brain in the form of action potentials through the motor unit. The sliding filament model describe skeletal muscle contraction and, in particular, how the action potential generated in the CNS reaches the muscle fibers, originating the tension necessary to change the well-organized pattern of A band and I band, that give to skeletal and cardiac muscle the striated aspect. An action potential originating in the CNS reaches an alpha motor neuron, which then transmits an action potential down its own axon (***Fig. 3***). The action potential propagation leads to the activation of voltage-gated sodium channels along the axon toward the neuromuscular junction. The presynaptic tract of the motor axons is demyelinated and stops 30 nanometers from the cell membrane of a muscle cell (sarcolemma), at the level of the synaptic cleft where neurotransmitters are released.

When the action potential reaches the junction, it results in a calcium ion influx through voltage-gated calcium channels. The calcium influx causes acetylcholine release into the extracellular space between the motor neuron terminal and the neuromuscular junction of the muscle fiber. The surface of the sarcolemma has invaginations called post-junctional folds that increase the area of the membrane exposed to the synaptic cleft and increase the density of acetylcholine receptors present in the synaptic cleft. Once the neurotransmitter diffuses across the synapse, it binds the nicotinic receptors on the neuromuscular junction leading to the activation of intrinsic sodium/potassium channel and generating the influx of sodium and the efflux of potassium. Due to a different membrane permeability of the two ions, the charge between internal and external surfaces of the membrane becomes less negative, triggering an action potential.

Skeletal muscles are composed of clusters of muscle cells which are long and spindle shaped. A muscle consists of packages of muscle cells called fascicles. A muscle cell contains many nuclei, the cytoplasm (sarcoplasm) and the plasma membrane, named sarcolemma. Each muscle cell is organized in myofibrils, which are aligned in parallel arrangements and extend full length of the cell.



1. Acetylcholine released from the axon terminal binds to receptors on the sarcolemma.
2. An action potential is generated and travels down the T tubule.
3. Ca^{2+} is released from the sarcoplasmic reticulum in response to the change in voltage.
4. Ca^{2+} binds troponin; Cross-bridges form between actin and myosin.
5. Acetylcholinesterase removes acetylcholine from the synaptic cleft.
6. Ca^{2+} is transported back into the sarcoplasmic reticulum.
7. Tropomyosin binds active sites on actin causing the cross-bridge to detach.

***Figure 3*. Signal transmission.** Acetylcholine released in the synaptic cleft binds to acetylcholine receptors on muscle fibers. Inside the muscle, calcium is released from sarcoplasmic reticulum triggering the cross-bridge binding between actin and myosin, initiating the contraction process. Image from ©Lumen Learning, 2017.

Myofibrils consist of protein chains called myofilaments, showing a symmetrical, alternating pattern of thick and thin elements. The thick myofilament consists of a large number of bundled myosin molecules aligned in overlapping arrays (***Fig. 4***). Myosin is a hexameric protein with two identical heavy chains and two pairs of different light chains: the regulatory chain (RLC) and the essential light chain (ELC). The thin myofilament is made of two

helically intertwined chains of G-actin (globular actin) units. Actin is associated with other proteins, such as tropomyosin and the troponin complex. The thick and thin myofilaments, with their associated proteins, are responsible for muscle contraction. Together, these fibers form the basic unit of muscle, the sarcomere.

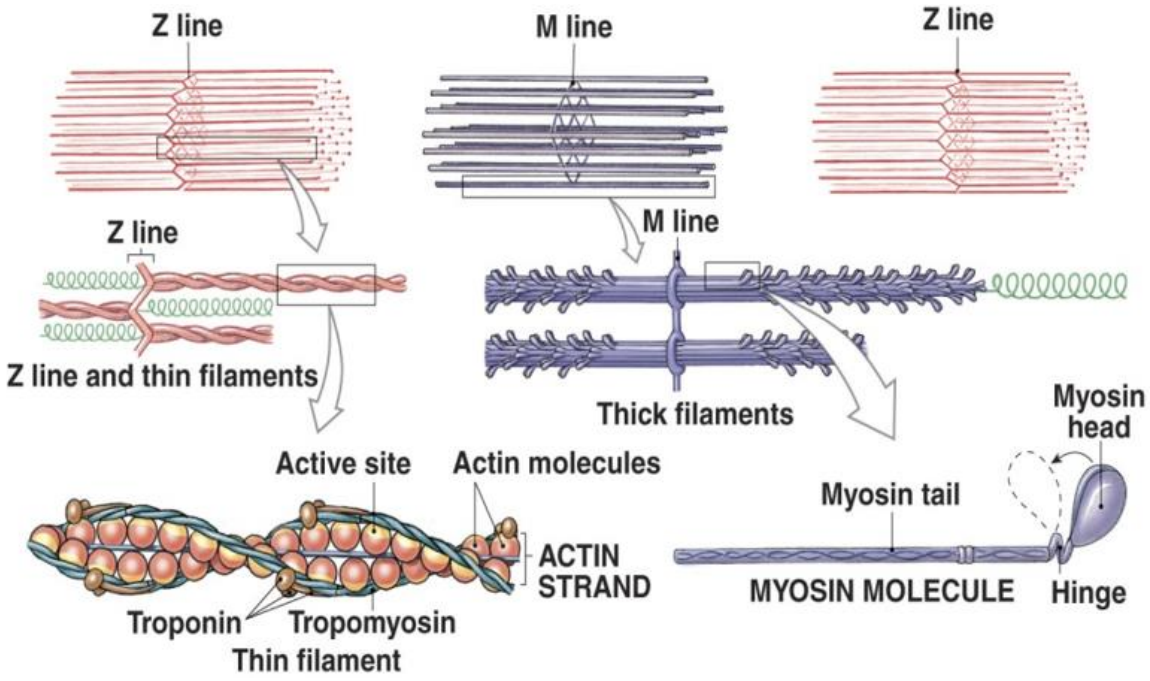


***Figure 4*. Actin and myosin filaments.** During contraction, the myosin thick filaments grab on the actin thin filaments, forming crossbridges. The thick filament pulls the thin filament past them, making the sarcomere shorter. Image from ©Pearson education, Ink, 2011.

A sarcomere is defined as the segment between two neighboring Z-lines, which is the region where actin molecules are bound (***Fig. 5***). Surrounding the Z-line is the region of the I-band which is the zone of thin filaments that is not superimposed by thick filaments. Following the I-band, there is the A-band which contains the entire length of a single thick filament. Within the A-band is a paler region called the H-zone, which is the zone of the thick filaments that is not superimposed by the thin filaments. Finally, inside the H-zone is a thin M-line formed of cross-connecting elements of the cytoskeleton.

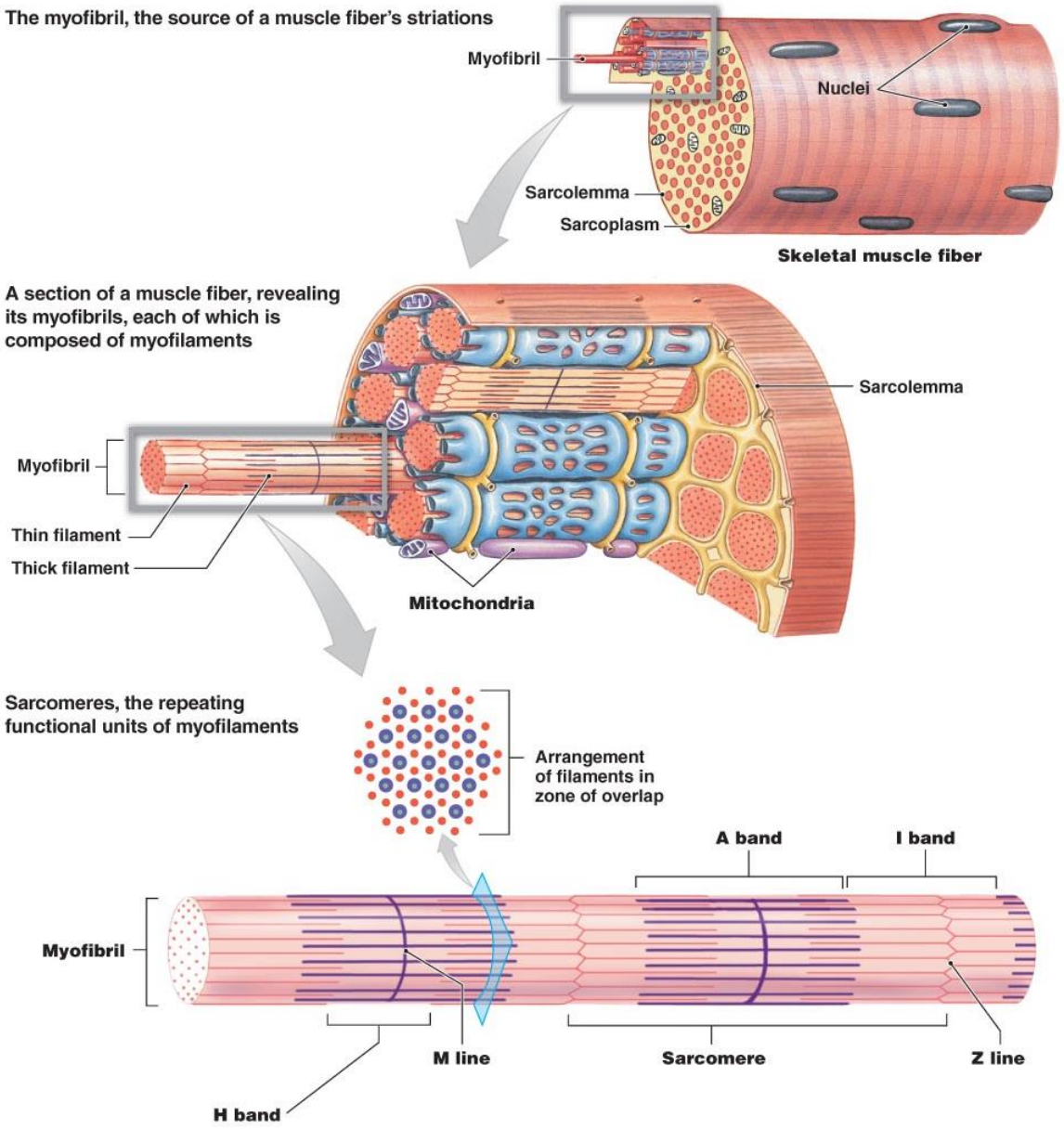


***Figure 5*. Sarcomere organization.** The thick myosin filaments account for the darker A-bands, the thin actin filaments for the light I-bands. The I-band is divided by a dark Z-line, where the actin filaments are anchored. The sarcomere region is between two Z-lines, therefore it consists of two I-band halves and an A-band. ©Pearson education, Ink, 2011.

The action potential diffuses depolarizing the inner portion of the muscle fiber by the activation of the L-type voltage-dependent calcium channels (dihydropyridine receptors) in the T tubule membrane. Consequently, the sarcoplasmic reticulum starts to release calcium from ryanodine receptors that physically interact with the L-type voltage-dependent calcium channel of the T-tubules.

Normally, tropomyosin blocks the binding region for myosin on the thin filament of muscles. When calcium is released from the sarcoplasmic reticulum, it binds to the troponin C, which belongs to the troponin complex and contains four calcium-binding hands. Troponin C is present on the actin-containing thin filaments of the myofibrils and calcium binding leads to an allosteric change. Upon calcium binding, troponin protein changes to troponin T, and this allows tropomyosin to move, triggering to open up the myosin binding sites. Myosin is in a ready state due to the presence of ADP and inorganic phosphate bound to its nucleotide binding pocket. Consequently, myosin is able to cover the unblocked binding sites on the thin filament and actin is able to act as a co-factor and sustain the release of inorganic phosphate from myosin (***Fig. 6***).

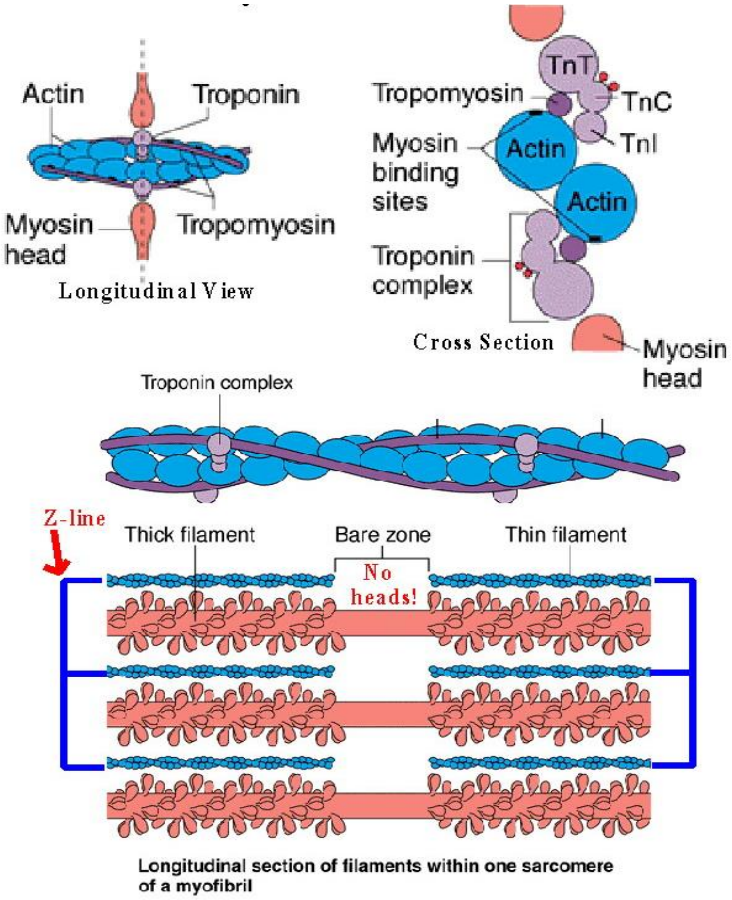


Figure 6. **Actin and myosin interaction.** The contraction of skeletal muscle is possible to the action of two accessory proteins bound to the actin filaments: tropomyosin and troponin. In striated muscles, each tropomyosin molecule is bond to troponin, which is composed by three polypeptides: troponin C (Ca^{2+} binding), troponin I (inhibitory) and troponin T (tropomyosin binding). Calcium binding to troponin C shifts the position of the complex, relieving inhibition and allowing contraction. ©Pearson education, Ink, 2011.

Once they are bound, the Z-bands are pulled towards each other and the sarcomere and the I-band start to shorten. Once ATP binds again to myosin, it dissociates from actin and returns to a weak binding state. ATP is then hydrolyzed from myosin and the energy of this process is necessary for myosin to assume the “cocked back” conformation. When calcium is no longer present on the thin filament, the tropomyosin changes conformation back to its previous state and it blocks again the binding sites. The myosin stops binding to the thin filament, and the contractions cease.

1.2. Spinal and Bulbar Muscular Atrophy (SBMA)

Motor neuron diseases (MNDs) are a particular class of disorders characterized by the selective dysfunction and death of the upper and/or lower motor neurons innervating the voluntary muscles of bulbar, respiratory, and limbs regions. This results in muscle weakness and progressive atrophy.

Spinal and bulbar muscular atrophy (SBMA, also known as Kennedy's disease) is an example of MND. SBMA was first documented by Hiroshi Kawahara in the 19th century, who described the clinical outcome and hereditary characteristics of two Japanese brothers with progressive bulbar palsy. Later, Kennedy and colleagues in 1968 described a disorder with progressive weakness and atrophy of muscle (Kennedy et al. 1968). In 1991, the cause of spinal and bulbar muscular atrophy was identified as the expansion of a trinucleotide CAG repeat in the *AR* gene (La Spada et al. 1991). SBMA is a rare X-linked MND characterized by the selective loss of lower motor neurons in the brainstem and spinal cord, leading to atrophy affecting the face, bulbar musculature and proximal limbs. SBMA patients show a well-defined muscle pathology with weakness, fasciculation and atrophy. Moreover, they show difficulty to swallow, speak and severe cramps. In addition, SBMA progression is associated with endocrine abnormalities, such as diabetes mellitus, and signs of mild androgen insensitivity, including gynecomastia, testicular atrophy and reduced fertility (Parodi and Pennuto 2011; Rocchi and Pennuto 2013). The age of onset usually is between the third and fifth decade of life, with some reported cases of juvenile forms (Parodi and Pennuto 2011; Sambataro and Pennuto 2012). SBMA is a sex specific disease, with manifestations of symptoms occurring only in males. Females present only subclinical manifestations, even if homozygous for the mutation (Schmidt et al., 2002).

SBMA is caused by the expansion of CAG trinucleotide repeat in the first exon of *AR* gene, which encodes a polyglutamine (polyQ) tract leading to polyQ expanded AR protein (polyQ-AR). PolyQ expansion is the cause of at least eight other neurodegenerative diseases, including Huntington's disease (HD), six types of spinocerebellar ataxia (SCA1, SCA2, SCA3, SCA6, SCA7, SCA17), and dentatorubral-pallidoluysian atrophy. The proteins involved are huntingtin, ataxin-1, ataxin-2, ataxin-3, CACNA1A, ataxin-7, TATA binding

protein (TBP) and atrophin-1, respectively (David et al. 1997; Imbert et al. 1996; Koide et al. 1994; MacDonald et al. 1993; Orr et al. 1993) (***Fig. 7***).

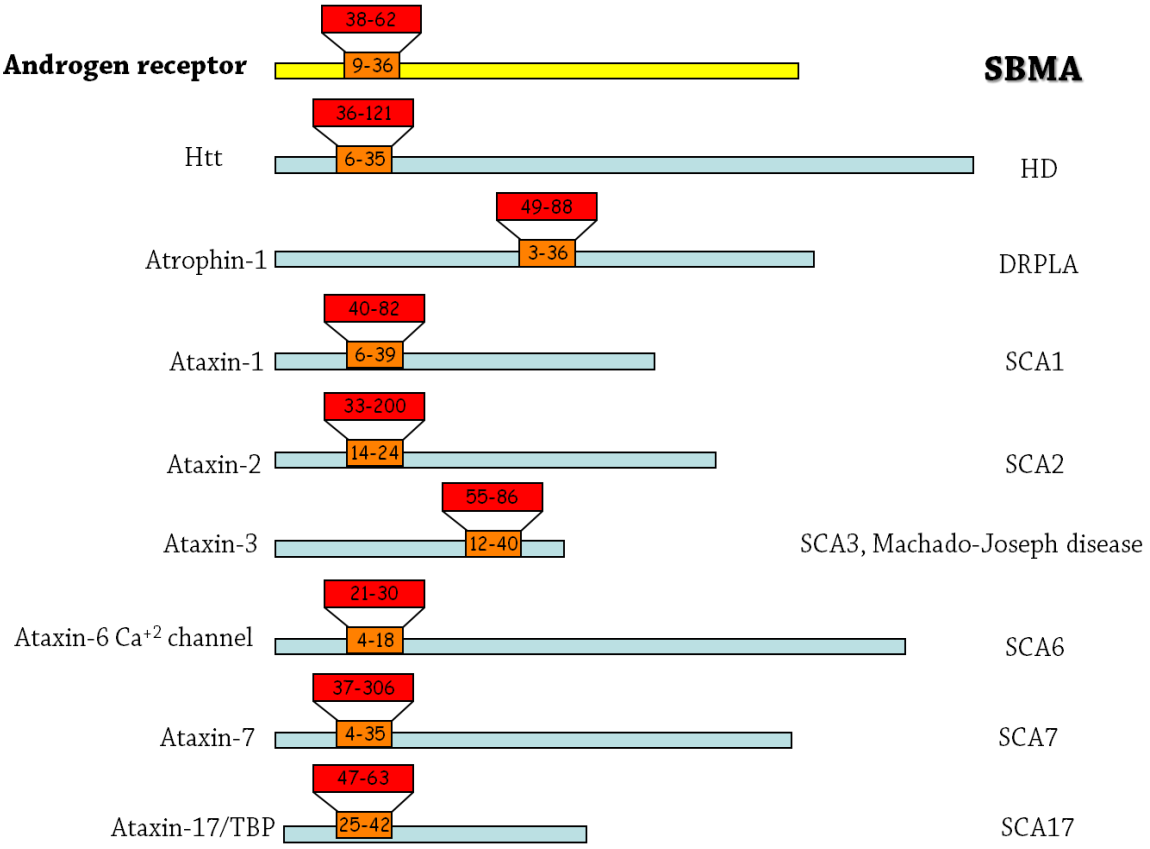


Figure 7. **Polyglutamine disease family.** CAG trinucleotide repeats expansion in at least nine proteins is the cause of polyglutamine diseases. This family is composed by SBMA, in which *AR* gene possesses a CAG expanded tract, Huntington's disease due to expansion in huntingtin (Htt), six types of spinocerebellar ataxia (SCA1, SCA2, SCA3, SCA6, SCA7, SCA17), in which the mutated genes are ataxin-1, ataxin-2, ataxin-3, CACNA1A, ataxin-7, TATA-binding protein (TBP), respectively, and dentatorubral-pallidoluysian atrophy (DRPLA) due to atrophin-1 CAG expansion.

PolyQ diseases share several features, such as late-onset manifestations of symptoms and the degeneration of specific neuronal subpopulations in each disease, thereby leading to different clinical manifestations. Moreover, there is an inverse relationship between the length of CAG expansion and the age of disease onset. This phenomenon is known as "*genetic anticipation*". There is another common pathological sign among these disorders, which is the presence of insoluble material accumulation in forms of micro-aggregates/oligomers and inclusions.

Indeed, *in vitro* studies revealed that the misfolded protein conformation is due to formations of hydrogen bonds between the main chain of one strand and the side chain of the adjacent strand, thus forming anti-parallel beta strands (Perutz et al. 1994). The polyQ proteins acquire a non-native beta-sheet conformation, leading to aggregation, which triggers cellular stress response and finally neuronal death (Perutz et al. 1994; Poletti 2004; La Spada et al. 1991; Williams et al. 2009; Williams and Paulson 2008).

AR is a nuclear transcription factor and a member of the steroid hormone receptor superfamily, which includes the estrogen receptor, glucocorticoid receptor, progesterone receptor, and mineralocorticoid receptor. AR activation is ligand-dependent, and it is due to the presence of androgens such as testosterone and its more potent derivative dihydrotestosterone (DHT). The *AR* gene is located on the X chromosome (Xq11-12) and is composed of eight exons. Exon 1 encodes the intrinsically disordered amino-terminal domain of the protein, which contains the polyQ tract. Normally, the expansion ranges between 9 and 36 residues, and expansion over 37 residues causes disease. Exon 2 and exon 3 encode for the DNA binding domain (DBD) and the hinge region of the protein, in which is contained a specific nuclear localization signal (NLS) necessary for protein translocation to the nucleus. The remaining exons codify for the carboxy-terminal region, formed by the ligand binding domain (LBD) (***Fig.8***).

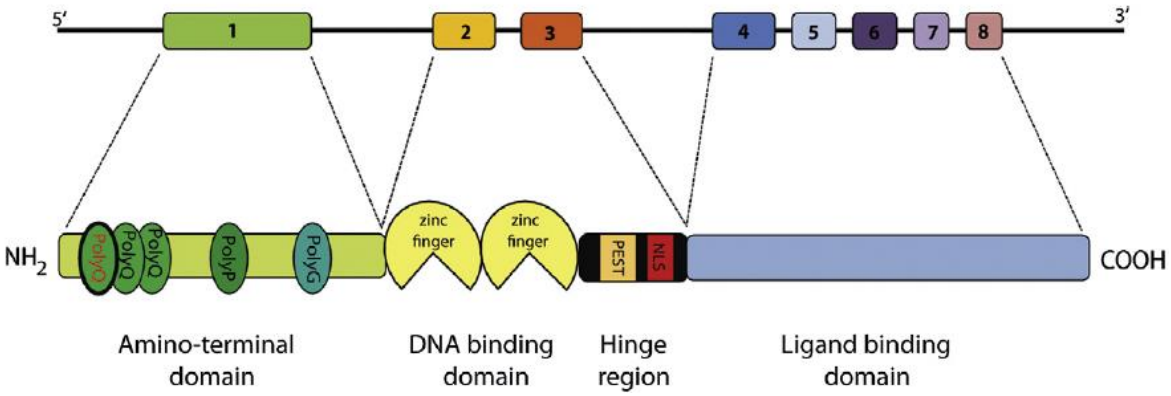


Figure 8. **Scheme of *AR* gene and protein.** The *AR* gene is composed of eight exons. The first exon encodes the amino-terminal domain, which contains three polyQ tracts (polyQ), a poly-proline tract (polyP) and a poly-glycine tract (poly-G). The first polyQ tract (red) is expanded in SBMA. Exons 2 and 3 encode the DNA-binding domain, which is formed by two zinc fingers, and the hinge region, which contains the PEST sequence and the nuclear localization signal (NLS). Exon 4 through 8 encode the ligand-binding domain (Parodi and Pennuto 2011).

1.2.1. Molecular mechanism of SBMA pathogenesis

In normal conditions, AR is sequestered in the cytosol by heat shock proteins (Hsps). These proteins contribute to maintain normal protein level, targeting AR for degradation through the help of ubiquitin-proteasome system. Upon binding to its natural ligands, testosterone and its more potent derivative dihydrotestosterone (DHT), AR dissociates from Hsps and translocates to the nucleus (***Fig.9***). It has been shown that the overexpression of Hsps in SBMA animal models reduce toxicity of expanded polyQ-AR, reducing nuclear-localized mutant AR and enhancing its degradation (Ishihara et al. 2003; M. Katsuno et al. 2003).

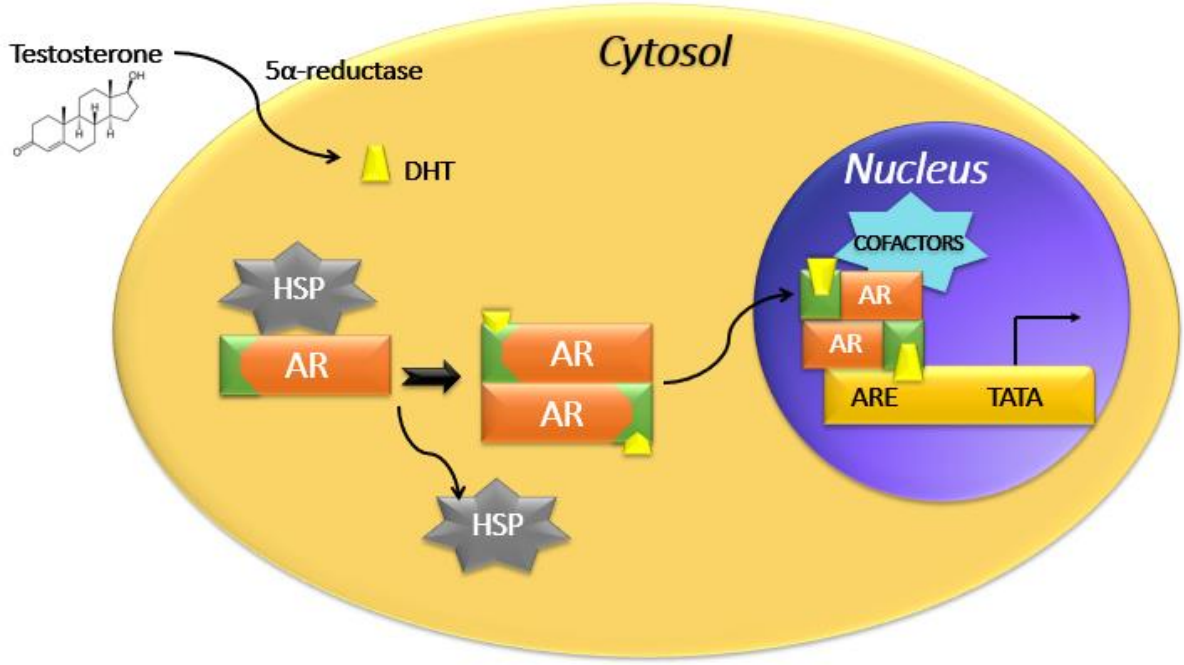


***Figure 9*. AR biology.** Testosterone may be reduced by 5α-reductase to dihydrotestosterone (DHT). When in the cytosol, DHT binds to inactive AR. Ligand binding triggers a series of sequential events: dissociation from Hsps, dimerization and translocation into the nucleus. Nuclear AR binds androgen-responsive elements (ARE) in the DNA, interacting with transcriptional cofactors to activate or repress specific genes.

Once testosterone or the reduced molecule DHT enter the cell and binds AR, the receptor is activated, Hsps dissociate and AR translocate to the nucleus, where it works like a transcription factor. The ability to move into the nucleus is due to its nuclear localization signal (NLS), in the hinge region. This event is necessary but not sufficient to establish the toxicity, as it has been reported in different studies. Indeed, mutation or deletion of this signal blocks protein translocation without abolishing ligand binding and preventing neurotoxicity, whereas polyQ-NLS fusion results in its nuclear localization but absence of toxicity because

of lack of ligand binding (Montie et al. 2009; Nedelsky et al. 2010). Inside the hinge region is situated a putative PEST sequence, which probably targets AR for proteasomal degradation.

After the binding to the ligand, the LBD undergoes to a conformational change inducing a N/C-terminal interaction which could be intramolecular (in the cytosol) or intermolecular (upon nuclear transport), and result in the exposure of two coregulator interaction surfaces, named activation function-1 (AF-1) and 2 (AF-2) (***Fig. 10***). This interaction occurs between the FxxLF motif in the N-terminal of the protein and the AF-2, located in LBD. Inside AF-2, two opposing charged residues are present (K720 and E897), which flank the hydrophobic surface of the domain, forming a binding cleft for co-factors. N/C interaction allows protein stabilization and the disruption leads to attenuation of mutant protein toxicity, as demonstrated in cell and fly models of SBMA (Zboray et al. 2015, Orr et al. 2010, He et al. 2000).

Once in the nucleus, AR binds specific regions of the genome, called androgen-responsive element (ARE), leading to the transcription activation or repression of specific androgen responsive genes. It was published that in a fly model of SBMA in which AR binding to DNA is impeded by the substitution of alanine 574 with an aspartate, the toxicity of polyQ-AR is importantly decreased (Nedelsky et al. 2010). AR transcriptional activity is modulated by subsequent recruitment of several transcriptional co-regulators, which has been demonstrated both *in vivo* and *in vitro* to be a necessary and sufficient event for toxicity (Nedelsky et al. 2010).

Figure 10. **AR functional domains.** AR protein is composed by four domains which differ for structure and function: an intrinsically disordered N-terminal domain (NTD) in which resides the polyQ, a DNA binding domain (DBD), a small hinge region (H) in which there is the nuclear localization signal and a C-terminal domain for ligand binding (LBD).

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1.2.2. Post-translational modifications of AR

Commonly, AR can be post-translationally modified through the covalent enzymatic addition of acetyl, methyl and phosphate groups, or SUMO and ubiquitin protein (***Fig. 11***). These modifications lead to the regulation of AR transcriptional activity, subcellular localization, protein stability and cell growth, altering the binding with corepressors, coactivators, other regulatory factors and ligand.

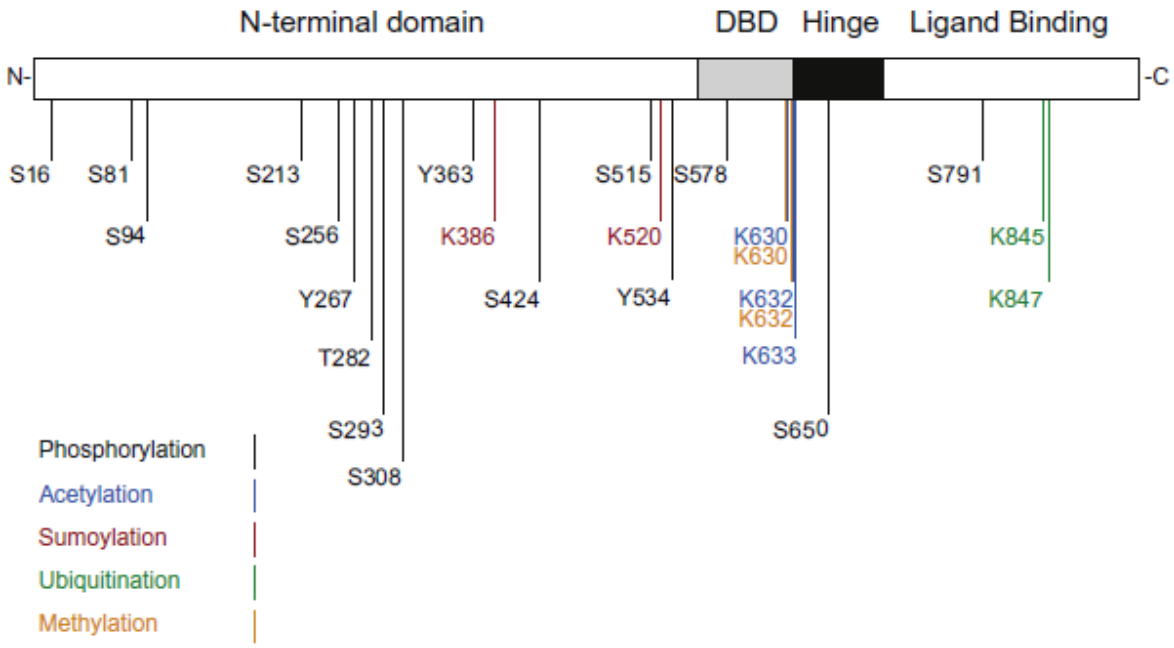


Figure 11. **Post-translational modifications of AR.** The major known site of phosphorylation (black), acetylation (blue), Sumoylation (red), Ubiquitination (green) and methylation (orange) of AR are indicated (Gioeli and Paschal 2012).

AR is acetylated at lysine (K) 630, 632 and 633, inside the hinge region, which are targeted by p300, p300/CBP associated factor (PCAF) and Tip60 (Fu et al. 2000; Gaughan et al. 2002). AR acetylation is induced by androgen stimulation (Fu et al. 2000). The deacetylation of these lysines is exerted by HDAC1 and SIRT1. AR deacetylation leads to decreased transcriptional activity (Fu et al. 2006). Mutations at acetyl acceptor sites provoke an increase binding to co-repressors, such as NCoR and SMAD3 (Fu et al. 2003). Conversely, a mutation that mimic acetylation at K630 increases AR activity on reporter promoters of PSA and MMTV (Zhou et al. 1995). Another report showed that differentially acetylated lysines on AR could have different effects on its transactivation. The mutants K630A, K632/633A and K630/632/633A increased AR activity in PSA and MMTV promoters, but the Pem promoter

was unaffected (Faus and Haendler 2008). Another stimulus that enhance AR transactivation is bombesin, a neuroactive peptide. This compound is reported to activate p300 in a Src and PKC-dependent manner, inducing AR activity (Gong et al. 2006). It was shown that the overexpression of SIRT1 was protective for motor neurons, due to a decrease in polyQ-AR aggregation (Montie et al. 2011). This protection was abrogated by lysine to glutamine substitutions at K630/632/633, which mimic acetylated sites, demonstrating that SIRT1 protection is dependent on these intact lysines (Montie et al. 2011). In addition, it was shown that polyQ-AR is hyperacetylated in a SBMA cell model and a pharmacologic intervention aimed to reduce this aberrant post-translational modification was effective in decreasing DHT-induced cell death (Montie et al. 2011). These observations suggest that the modulation of polyQ-AR acetylation is a potential approach for SBMA therapy and reveal a novel protective role of SIRT1-mediated deacetylation (Montie et al. 2011).

An AR methylation site is in the hinge region between DNA binding domain and the ligand binding domain. SET9 was found to methylate AR, but the site of modification is controversial. Ko and colleagues reported AR methylation at K630 with experiments of immunoprecipitations with mutant protein fragments (Ko et al. 2011). Another group identified the methylation site at K632, using mass spectrometry and immunoprecipitation with short peptides (Gaughan et al. 2011). Anyway, the SET9-mediated AR methylation induces N-C interdomain interactions, which are known to be transactivation enhancer (Langley et al. 1998). Recently, it was characterized AR methylation by a protein arginine methyltransferase, PRMT6, through mass spectrometry, which reveals that AR is methylated at arginine (R) 210, 212, 629, 787, and 789 (Scaramuzzino et al. 2015). R210-R212 and R787-R789 reside in the Akt consensus site of AR, RXRXXS (where X is any amino acid) (Scaramuzzino et al. 2015). Importantly PRMT6 was identified as an AR coactivator and enhancer of polyQ-AR toxity. The phosphorylation at the two serines in the Akt consensus sites, S215 and S792, induce AR repression impeding testosterone binding and preventing AR nuclear translocation, leading to neuroprotection (Palazzolo et al. 2007, 2009).

1.2.2.1. Phosphorylation

The most studied post-translational modification of AR is phosphorylation. AR is a phosphoprotein modified at serines (S), threonines(T) and tyrosines (Y), according to the activated intracellular transduction pathways. Phosphorylation modifies specific domains, affecting AR function. The principal phosphorylation sites of AR are reported in ***Table 1***. AR phosphorylation occurs 15 minutes after synthesis to allow ligand binding and protein folding and other phosphorylation events occur upon ligand binding (Blok et al. 1998; Van Laar et al. 1991). The ligand-induced phosphorylation events are S16, S81, S256, S308, S424 and S650 (nomenclature taken from NCB accession number AAA51729) (Gioeli and Paschal 2012). Other sites, such as S94 (hereby indicated as S96, according to NCBI Reference Sequence: NP_000035.2), are known to be constitutively phosphorylated (Gioeli and Paschal 2012).

Phospho-site	Function				Kinases
	Transcription	Stability	Localization	Growth	
S81	✔	✔	✔	✔	CDK1, CDK5, CDK9
S213	✔	✔	✔	✔	Akt, PIM1 (L, S)
Y223	✔				Fer
Y267	✔			✔	Ack
T282	✔			✔	Aurora-A
S293	✔			✔	Aurora-A
S308	✔			✔	CDK11^{p58}
Y363	✔				Ack
S515	✔	✔			CDK7, MAPK
Y534	✔	✔		✔	Src, Etk
S578	✔				PKC, PAK6
S650	✔		✔		JNK, p38
T850	✔			✔	PIM1L

Table 1. List of the known kinases that phosphorylates AR at the indicated sites with the indicated outcome on AR biology (Koryakina et al. 2014).

In the inactive state AR is predominantly cytosolic, while nuclear in the presence of androgen. Without hormone, AR protein can be found both in the cytosol and nucleus of transfected cell lines (Palazzolo et al. 2010, Palazzolo et al. 2007). Nuclear localization

causes AR phosphorylation at S81, S256 and S308, instead cytoplasmic localization increases S96 phosphorylation. This implies that the kinases responsible for the described phosphorylations are localized in the nucleus and cytosol, respectively (Gioeli et al. 2006).

1.2.2.1.1. AR phosphorylation by cyclin-dependent kinases (CDKs)

AR possesses 9 minimal S/TP (where P is proline) consensus sites for cyclin-dependent kinases (***Fig. 12***). S81 can be phosphorylated by CDK1, CDK5 and CDK9, and it is most abundant phosphorylation that occurs after androgen treatment. Analysis of phosphorylation during cell cycle progression revealed that AR is highly phosphorylation during mitosis, when CDK1 activity is increased. S81 is linked to AR subcellular localization. When CDK5 is overexpressed AR localized more in the nucleus, on the contrary when it is downregulated AR stays more in the cytoplasm (Hsu et al. 2011). S81 is involved also in the regulation of AR transcriptional activity, indeed S81A was observed to change the transcription of several gene reporter constructs (Gordon et al. 2010). CDK11 phosphorylates AR on S308. This phosphorylation modifies the growth rate of LNCaP and AR transactivation. CDK11 silencing leads to the decrease of AR transcriptional activity, while its overexpression results in increased AR response. CDK7, protein of the TFIIF complex, can phosphorylate AR on S515. S515A AR mutant was associated with decreased transcriptional activation in reporter assays. This phosphorylation is linked also to decreased protein turnover, which is associated with decreased recruitment of the ubiquitin-proteasome machinery (Chymkowitch et al. 2011).

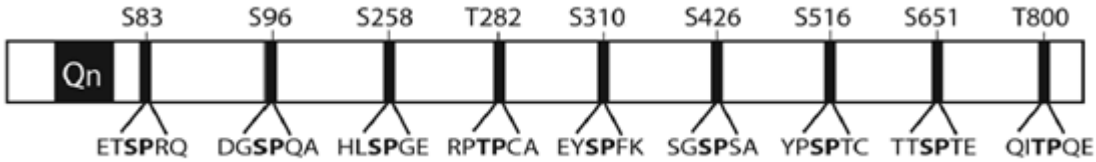


Figure 12. **AR protein has 9 S/TP consensus sites for phosphorylation by cyclin-dependent kinases along its sequence.** The pathological polyQ tract of AR (in the figure reported as Qn) is very close to S83 and S96. The majority of the phosphorylation sites reside in the N-terminal domain. S651 is inside the hinge region and T800 in the ligand binding domain (adapted from Polanco et al. 2016).

1.2.2.1.2. AR phosphorylation of serine 96 by CDK2 (Polanco et al. 2016)

Recently, we discovered that CDK2 is the kinase responsible for the phosphorylation of AR at S96 (Polanco et al. 2016). In this paragraph, I summarize the experimental workflow we adopted to discover CDK2 as the kinase of S96, and its pathological relevance.

AR protein is resolved in a 7% SDS-PAGE as a doublet of about 110 and 112 kDa (***Fig. 13A***, lane 1). The incubation of cell lysate with lambda phosphatase for 24 hours revealed that the upper isoform is composed essentially by phosphorylated androgen receptor. Indeed, the 112 kDa isoform completely disappeared after the treatment. The only remaining isoform after lamba phosphatase treatment was the one at 110 kDa, meaning that it is composed mainly by unphosphorylated AR (***Fig. 13A***, lane 2). The analysis of the expression in HEK293T cells of different phosphorylation mutants of AR shows that only the mutation at serine 96 abrogates the formation of the upper isoform of AR. The phosphodefective alanine mutant (S96A) runs as the lower 110 kDa band, instead the phosphomimetic aspartic acid mutant (S96D) runs as the upper 112 kDa band of both normal and polyQ-AR (***Fig. 13B***). Various mutants of phosphorylation sites were created in order to analyze whether the formation of the doublet was impaired. In particular, serine 16, 83, 258, 282, 310, 426, 516, 651, 800 were mutated to alanine, either individually or in combination (nomenclature according to NCBI Reference Sequence: NP_000035.2). None of the tested mutations were found to change the migration of AR (***Fig. 13C-D***, -PACAP/Forskolin treatment lines). This means that phosphorylation at serine 96 of AR specifically forms the upper isoform of about 112 kDa detectable by SDS-Page electrophoresis. An important characteristic of AR doublet is that the accumulation of the upper phosphorylated isoform was decreased by the activation of PKA/AC pathway through pituitary adenylyl cyclase activating polypeptide (PACAP) and forskolin treatment (***Fig. 13C-D***, +PACAP/Forskolin treatment lines).

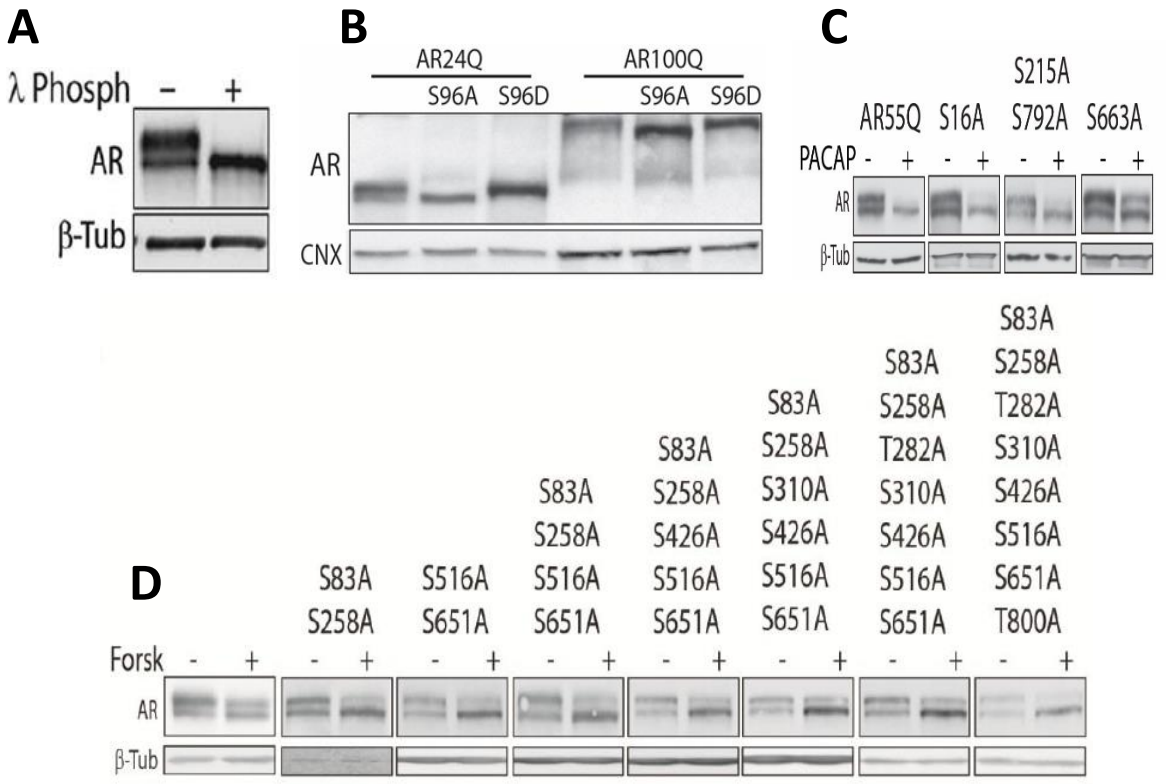


***Figure 13*. Phosphorylation state at serine 96 is influenced by PACAP/Forskolin treatment and changes the migration of normal and polyQ-AR in SDS-PAGE.** A) Western blotting analysis of HEK293T cells transfected with expanded polyQ-AR, whose cell lysate was treated with or without λ phosphatase (λ phosph) for 24 hours. N=3 independent experiments. B) Western blotting analysis in Motor neuron-derived cells-1 (MN1) stably expressing AR24Q and AR100Q, normal and polyQ AR respectively, with and without S96A and S96D substitutions. N=3 independent experiments. C) and D) Western blotting analysis of the indicated serine-to-alanine phospho-resistant polyQ-AR variants in HEK293T cells treated with vehicle, forskolin (10 μM), and PACAP (100 nM) for 5h. N = 3 independent experiments (Figure adapted from Polanco et al. 2016).

We used an unbiased approach, screening kinase and phosphatase inhibitors and looking for variation in the phosphorylation of S96. We found that a nonspecific inhibitor of CDK1/2 decreases S96 phosphorylation. We manipulate CDK1 and 2 expression levels with overexpressing constructs with WT or dominant negative mutants (DN) of the proteins. We found that CDK2 WT overexpression increases S96 phosphorylation, instead CDK2 DN abolishes the specific post-translational modification. Then, we performed an *in vitro* phosphorylation assay in which CDK2 coupled with cyclin E succeeded to phosphorylates S96, but not S96A AR mutant (***Fig. 14***). Importantly, we mutated all the consensus sites for

CDKs in AR amino acid sequence, except S96, and CDK2 was still able to phosphorylate the AR mutant isoform. These observations prove that serine 96 is phosphorylated by CDK2.

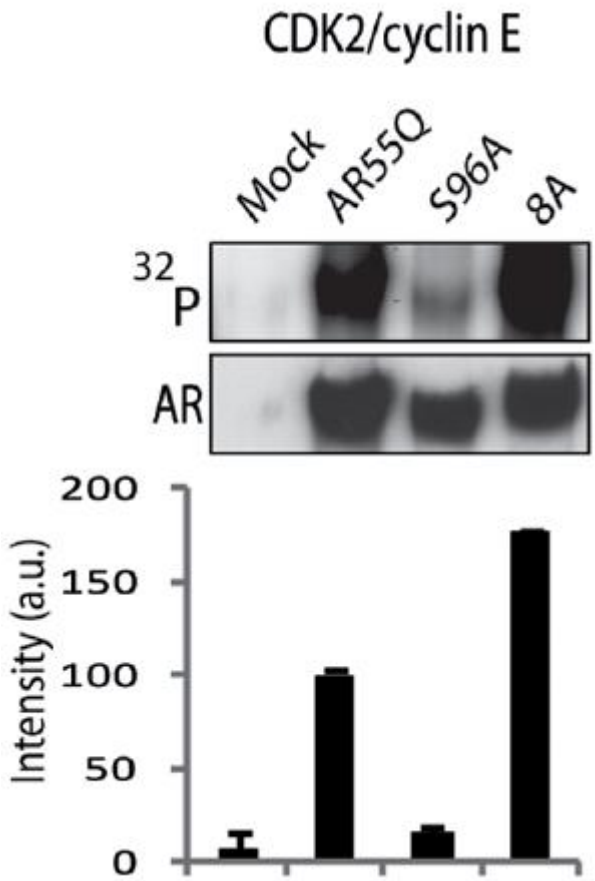


***Figure 14*. CDK2 is the kinase of serine 96 AR.** *In vitro* phosphorylation assay of AR55Q, AR55Q-S96A, and AR55Q-8A with all the (S/T)P sites substituted with alanine except for S96. They were immunopurified from HEK293T cells and incubated with recombinant CDK2 and cyclin E. Top: Autoradiography. Bottom: Western blotting analysis of AR levels. Graph,means ± SD. n = 2 independent experiments. a.u., arbitrary units (Figure adapted from Polanco et al. 2016).

Phosphorylation of S96 by CDK2 was found to be toxic in SBMA cell and mouse models (Polanco et al. 2016). We found that CDK2 was inhibited by PKA/AC pathway activation through forskolin or pituitary adenylate cyclase-activating peptide (PACAP) administration. The phosphorylation of S96 was reduced by forskolin/PACAP treatment *in vitro*, leading to the possibility to use this strategy also *in vivo*. Indeed, when we treated knock-in mice with a PACAP analog, peptide 7, we found that S96 phosphorylation and AR aggregation were reduced, leading to a rescue of the motor phenotype and survival in this mouse model (Polanco et al. 2016). The proposed model for the modulation of S96 phosphorylation is reported in ***figure 15***.

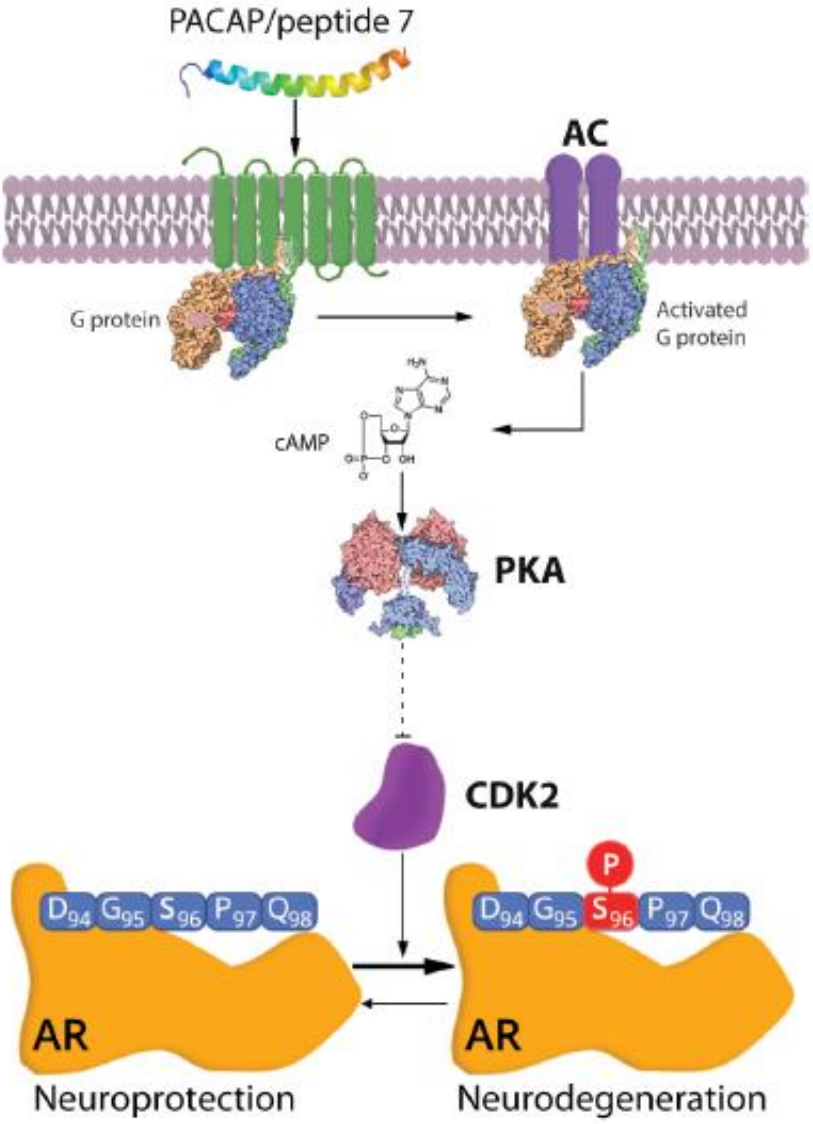


***Figure 15*. Working model of modulation of serine 96 phosphorylation through PKA pathway.** PACAP/peptide 7/forskolin treatment causes the activation of the PKA pathway which lead to inhibition of CDK2. The inhibition of CDK2 kinase activity impedes serine 96 phosphorylation, causing neuroprotection (figure adapted from Polanco et al. 2016).

1.2.2.2. AR dephosphorylation

Protein function is regulated through phosphorylation/dephosphorylation cycles which are exerted by the integrating actions of kinases and phosphatases. Phosphatases are hydrolases that remove the phosphate group from amino acid residues previously phosphorylated by kinases. Phosphatases have been categorized as promiscuous enzymes in the past, in the sense that they have poor intrinsic substrate specificity *in vitro*. Instead, *in vivo* studies show that they preferentially target specific substrates (Sacco et al. 2012).

Protein phosphatase 1 and 2A (PP1 and PP2A) bind AR (Chen et al. 2009; Yang et al. 2007). Tautomycin, a selective inhibitor of PP1, increases S650 phosphorylation levels and increases AR degradation (Chen et al. 2009). In a report of Yang and colleagues, PP2A was shown to be loaded to AR protein by simian virus 40 small t antigen, suggesting that this mechanism occurs only in transformed cells. PP2A inhibitors increases AR transcriptional activity and protein levels, proving that AR is regulated by direct or indirect PP2A action even in absence of small t antigen (Yang et al. 2007).

An extensive work to identify AR phosphatases at specific sites needs to be done, in order to regulate mutant AR toxicity in SBMA, but also in prostate cancer fields.

1.2.3. Therapeutic Approaches for SBMA

SBMA is characterized by motor neuron death partially due to a toxic gain of function of the polyQ-AR. In the last years, cell and animal models were created to dissect important features of the pathogenic mechanism of SBMA. Starting from these models, a number of different therapeutic strategies have been developed.

1.2.3.1. Disruption of the ligand-AR interaction

As previously described, ligand binding to the AR induces dissociation from heat shock proteins and nuclear translocation. In male mice, the SBMA phenotype is rescued by castration, while testosterone administration triggers the disease in females (Chevalier-Larsen 2004; Katsuno et al. 2002). The link between androgen binding and toxicity in SBMA indicates that this step of mutant AR toxicity could be a target for therapeutic intervention. Leuprorelin, a lutenizing hormone-releasing hormone agonist that reduces testosterone release from the testis, was successful in treating SBMA mice, leading to beneficial effects and preventing disease onset (M Katsuno et al. 2003b). Several randomized, placebo-controlled clinical trials have been developed in SBMA patients to reduce androgen levels. The agents used in these studies are leuprorelin and dutasteride, a 5-alpha-reductase inhibitor, which blocks the conversion of testosterone into the more potent androgen dihydrotestosterone (DHT). Even if no one of the two androgen-reducing agents significantly improved primary clinical outcome (Fernández-Rhodes et al. 2011; Katsuno et al. 2010), leuprorelin improves swallowing parameters and decreases nuclear accumulation of mutant AR in scrotal skin cells. These results suggest that androgen reduction therapy reduces

mutant AR accumulation and may modify the progression of SBMA specially at early disease stages.

1.2.3.2. PolyQ-AR silencing

Toxic gain of function contribution to neuronal dysfunction in SBMA and other polyQ diseases prompted the begin of clinical trials to reduce the levels of expression of the disease-causing proteins. Conditional mouse models of Huntington's disease and SCA1 have shown that abrogation of the expression of the polyQ-expanded proteins correlates with reduced disease progression and even reverses the pathology (Yamamoto et al. 2000; Zu 2004). These findings suggest that gene silencing approaches using RNA interference (RNAi) and antisense oligonucleotide (ASO) technology in polyQ diseases may be a successful treatment. A recently study described that the peripheral administration of AR-specific ASOs suppress mutant AR expression both *in vitro* and *in vivo*, attenuating the disease phenotype in two different mouse models of SBMA (Lieberman et al. 2014). Moreover, it has been described that a microRNA, miR-196a, enhances AR mRNA decay by silencing CELF2, an RNA-binding protein known to bind and stabilize CUG-rich 3'-UTR of mRNAs. miR-196a viral delivery reduces mutant AR accumulation and ameliorates the disease phenotype in SBMA mice (Miyazaki et al. 2012). These studies show that the decreased AR expression through antisense approaches can modify SBMA manifestations in animal models.

1.2.3.3. Degradation of polyQ-AR protein

An alternative strategy for reducing the levels of mutant AR is to accelerate the clearance of the protein. Ubiquitin-proteasome system (UPS) is the main degradation pathway of AR protein (Lieberman et al. 2002). In addition, there are several evidences in both cell and animal models, suggesting that enhancing UPS-mediated clearance of mutant AR has a protective effect on SBMA pathogenesis (Palazzolo et al. 2009; Tokui et al. 2009; Waza et al. 2005). Current pharmacological approaches for enhancing mutant AR degradation target primarily the protein quality control machinery. AR function and stability are controlled by the Hsp90/Hsp70-based chaperone complex. In this complex, association with Hsp90 stabilizes AR, while Hsp70 regulates the degradation of client proteins through the recruitment of chaperone-dependent ubiquitin ligases, such as C-terminus of Hsp70-interacting protein (CHIP) (Pratt et al. 2015). More specifically, activation of Hsp70 or inhibition of Hsp90 promotes UPS-mediated clearance of the mutant AR (Thomas et al.

2006; Wang et al. 2013). A recent study reported that a small molecule, YM-1, able to stabilize Hsp70 in its ADP-bound state and to promote binding to unfolded proteins, accelerates the degradation of mutant AR and rescues its toxicity in a Drosophila model (Wang et al. 2013). Association with co-regulators also influences the stability of AR. For example, AR is stabilized by interaction with the nuclear receptor coactivator 4 (NCOA4) (Hu et al. 2004). ASC-J9 and genistein promote the dissociation of AR and NCOA4 (Ohtsu et al. 2002; Qiang et al. 2013). Interestingly, both compounds decrease levels of mutant AR and attenuate disease manifestations in mouse models of SBMA (Qiang et al. 2013; Yang et al. 2007).

Recently, our study demonstrates that serine 96 dephosphorylation through PKA pathway activation increases polyQ-AR protein turnover, destabilizing the formation of aggregates and decreasing toxicity both in *in vitro* and *in vivo* SBMA models (**Polanco et al. 2016**). This non-invasive potential treatment for SBMA proved that the modulation of crucial post-translational modifications of polyQ proteins can be a valid strategy to undertake in next clinical trials.

1.2.3.4. Modulation of polyQ-AR toxic transactivation

Transcriptional dysregulation is an important downstream effect implicated in mutant AR toxicity in SBMA (Lieberman et al. 2002). Aberrant interactions between expanded polyglutamine proteins and transcription factors and coregulators have been well-described (McCampbell et al. 2000). Sodium butyrate, an inhibitor of histone deacetylases, improves SBMA motor phenotype, delays disease progression, and leads to an overall increase in histone acetylation in SBMA mice (Minamiyama et al. 2004). It has been published that transcriptional dysregulation in SBMA can also be counteracted by triptans (Minamiyama et al. 2012). Moreover, mutant AR expression is associated with upregulation of calcitonin gene-related peptide 1 (CGRP1), which leads to neuronal damage through stress kinase activation. The serotonin 1B/1D receptor agonist naratriptan was shown to prevent transcriptional induction of CGRP1 and to ameliorate the disease phenotype in SBMA mice. These results highlighted that the AR native function is dysregulated in SBMA and this could be exploited as target to develop new therapeutic strategies for SBMA and other polyQ diseases.

1.3. Physiological functions of neuronal AR

AR plays a fundamental role in neuroendocrine regulation of reproductive behavior and it is responsible of the masculinization of the brain. Early studies report that during rat development, testes secrete testosterone (T) with two waves, one in late gestation, around embryonic days 18-19, and one on the day of birth (Motelica-Heino et al. 1988; Weisz and Ward 1980), suggesting that this hormone plays a crucial role in the physiological development. Moreover, the analysis of gender disease prevalence, symptoms and specific mechanisms of neurological disorders and mental illnesses, including neurodegenerative diseases, reveal a strict relationship with sex steroids (Cahill and Larry 2006). For instance, Alzheimer's disease (AD) progression and development are different in males and females (Pike 2017). Women show a decrease in estrogen levels during aging, and the premature reduction of this steroid hormone is a risk factor for AD. Conversely, men possess very low concentration of estrogens throughout life, but T deprivation due to aging is predicted to increase AD incidence (Pike 2017). Males and females respond differently to stress cues, leading to distinct incidence of psychiatric illnesses, such as depression, schizophrenia and attention deficit hyperactivity disorder (Klein and Corwin 2002). Therefore, the molecular mechanisms involved in the gender-specific susceptibility to diseases and physiological changes in brain development need to be investigated.

There are sexually dimorphic regions in the brain of adult mammals that form thanks to steroid hormones, T and estrogen, and the action of both AR and estrogen receptor (ER) (Coumailleau et al. 2015; Shah et al. 2004). The aromatization of T leads to the production of estradiol, the female steroid hormone, which binds specifically to ER. Dihydrotestosterone (DHT) is a non aromatizable hormone, that binds exclusively to AR. For this reason, in the next paragraphs all the work presented to prove the role of AR function in neurons involve DHT treatments, or similar, in addition to the use of specific AR antagonists, such as flutamide.

1.3.1. Sexually dimorphic brain regions

The cognitive regions that are sexually dimorphic are part of the limbic system, responsible for the behavior, long-term memory and motivation, and cerebral cortex (***Fig. 16***) (Juraska

1991). There are two types of sexual dimorphism: structural and functional. The first one comprises all the differences at architectural level, the second one describes brain regions which differ for the neurotransmitter composition or the activated response to stimuli. An example of structural dimorphism is the observation that female cortical mantle results to be thicker compared to males when normalized for the brain total size, suggesting a dimorphic organization of the brain and in particular of the architecture of the cortex (Luders et al. 2006). MRI scans reveal also a gender-specific difference in the dimension of the grey and white matter, leading to evidences of the sexual dimorphism in various regions of the cerebrum (Allen et al. 2003). An example of functional dimorphism is the biochemical characteristics of hippocampus, which suggest differential response to activation of several neurotransmitter pathways (Madeira and Lieberman 1995).

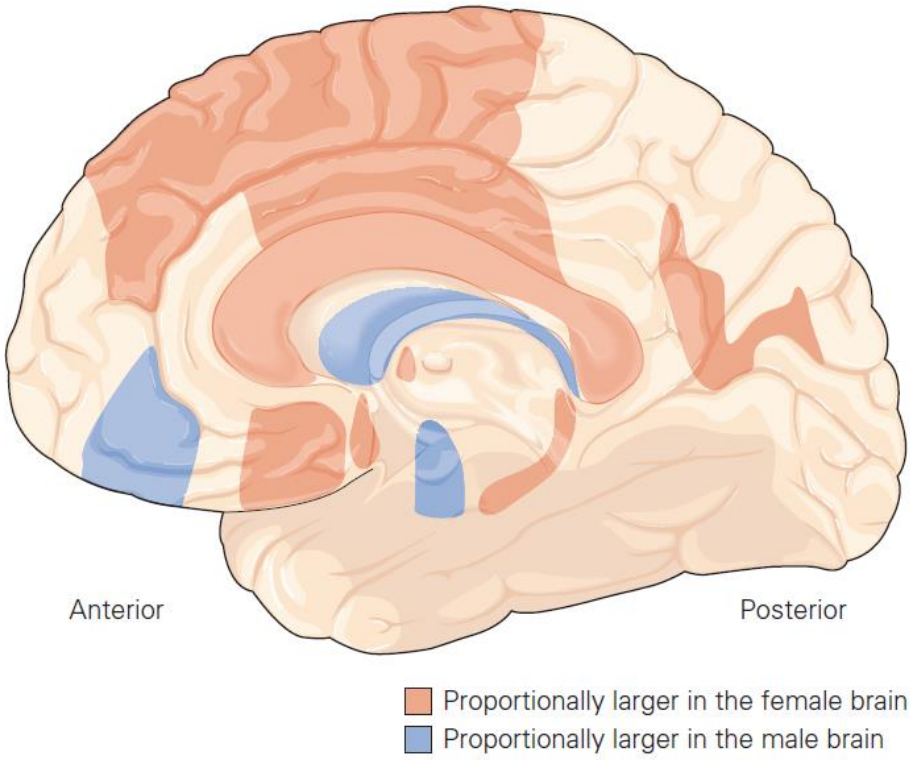


***Figure 16*. Sexual dimorphism in the human brain.** Through a magnetic resonance imaging (MRI) study, the volume of brain regions in adult men and women was measured. The volume of the regions was normalized to the cerebrum size. Many regions display sex differences, including some areas of the cortex implied in cognitive functions (Kandel et al. 2013).

The hippocampus is the brain region which belongs to the limbic system. Its major functions comprise long, short-term and general spatial memory (Rueckemann and Buffalo 2017), but

also it is involved in the regulation of reproductive, sexual and maternal behaviors (Meaney and Michael 2001). Indeed, its structure and function are sexually dimorphic (Cahill and Larry 2006). Hippocampus size and the number of pyramidal cells in the CA1 region are larger in females than in males (Madeira and Lieberman 1995). The major functional sexual dimorphisms are the number of benzodiazepine, alpha-adrenergic and high-affinity corticosterone receptors (Dulce Madeira and Lieberman 1995). Other gender-dependent aspects are hippocampal long-term potentiation (LTP), excitability of hippocampal neurons with the shaping of their dendrites and the increase of NMDA receptor binding (Cahill and Larry 2006). A region of the brain which is responsible for reproductive behaviors is the amygdala (Kim et al. 2012). Male amygdala is bigger than the one in females and there are substantial body of evidence for its functional sexual dimorphism. Indeed, the amygdala is also involved in the memory of emotional events, in particular stressful events (Ressler 2011). When an emotional cue is induced in humans, male individuals activate preferentially the right amygdala circuitry, in opposition to the female preference to use the left hemisphere (Canli et al. 2002). Many sexual differences arouse from the study of neurotransmitter pathways. Sexual dimorphism involves serotonin, GABA, acetylcholine, vasopressin, opioids and monoamines. Male rats have a lower serotonin concentration than females in the whole brain and hypothalamic homogenates, and it was shown that this hormone promotes the feminization of the preoptic area (POA) of the hypothalamus, another sexually dimorphic region of the brain (Madden et al. 2016). The enzyme responsible of the oxidation of monoamines is more expressed in several brain regions of women respect to men. Stress-induced response of the monoamine-rich neurons of the locus coeruleus was 30-times greater in females than in males (Curtis et al. 2006). GABA-induced stimulation of rat neurons has different effect in male and female substantia nigra, causing male neuronal depolarization and female neuronal hyperpolarization (Galanopoulou 2006). Recently, the sexually dimorphic gene expression pattern was unraveled, identifying four major players in sex typical behaviors, bombesin receptor subtype 3, cholecystokin A receptor, insulin receptor substrate 4 and synaptotagmin like 4 (Xu et al. 2012). This finding demonstrates the existence of differential gene expression programs in sexual dimorphic brain regions, that probably shape and influence specific gender-dependent brain traits. Serrano-Saiz and colleagues used the nematode *Caenorhabditis elegans* to map the gender-specific usage of neurotransmitters, revealing a generalized sexually dimorphic system. This simplified model could be a starting point for functional and developmental analysis of the male nervous system (Serrano-Saiz et al. 2017).

1.3.2. AR expression in the central nervous system

AR is a ubiquitous protein, with highly expression in reproductive tissues, including testes, prostate, ovaries and uterus, and non-reproductive tissues such as liver, adipose and muscle (Bookout et al. 2006). AR is also expressed in the CNS, even if in a lesser extent, and at different levels during development and according to neuronal subtypes (***Fig. 17***) (Camacho-Arroyo et al. 2018; Mhaouty-Kodja 2017; Tsai et al. 2015).

AR mRNA and protein expression are present in the POA and ventromedial hypothalamus, amygdala and bed nucleus of the stria terminalis (BNST) (Mhaouty-Kodja 2017). In the mouse hypothalamus, AR mRNA is detected in increasing concentrations from embryonic day 19 (E19) to postnatal day 7 (P7), whereupon it decreases in both sexes, but with a higher expression in males (Mogi et al. 2015). Juntti and colleagues analyzed a mouse model in which β-galactosidase (β-gal) was expressed under an AR-specific promoter, in order to follow during mouse development AR expression especially in the CNS (Juntti et al. 2010). They analyzed transgenic embryos and they detected AR expression at E15.5 and E17.5 in neurons of the arcuate and ventromedial nuclei of the hypothalamus, but not in BNST, POA and medial amygdala (MA). At postnatal day 1, when the first surge of testosterone occurs, they found only a slight AR expression in the previously described sexually dimorphic brain regions. At P4, when the testosterone level is returned to stable concentration, β-gal reactivity was more evident. Anyway, the higher AR expression was found in adult mice (Junnti et al. 2010).

AR was detected also in cerebellum, recently found to be part of the sexual behavior circuit in men (Perez-Pouchoulen et al. 2016). In this report, through immunohistochemistry method, they found the expression of AR in Purkinji neurons of adult rats, and the expression level differs according to the location and lobule.

The spinal nucleus of the bulbocavernosus (SNB) is composed of lumbar motoneurons which innervate the bulbocavernosus and levator ani muscles in the perineal zone of males. This apparatus is testosterone and AR-dependent. Smith and colleagues showed that AR expression in the SBN motoneurons started at PND 4, after the appearance of AR immunoreactivity in the muscles of this area, at E15 (Smith et al. 2012).

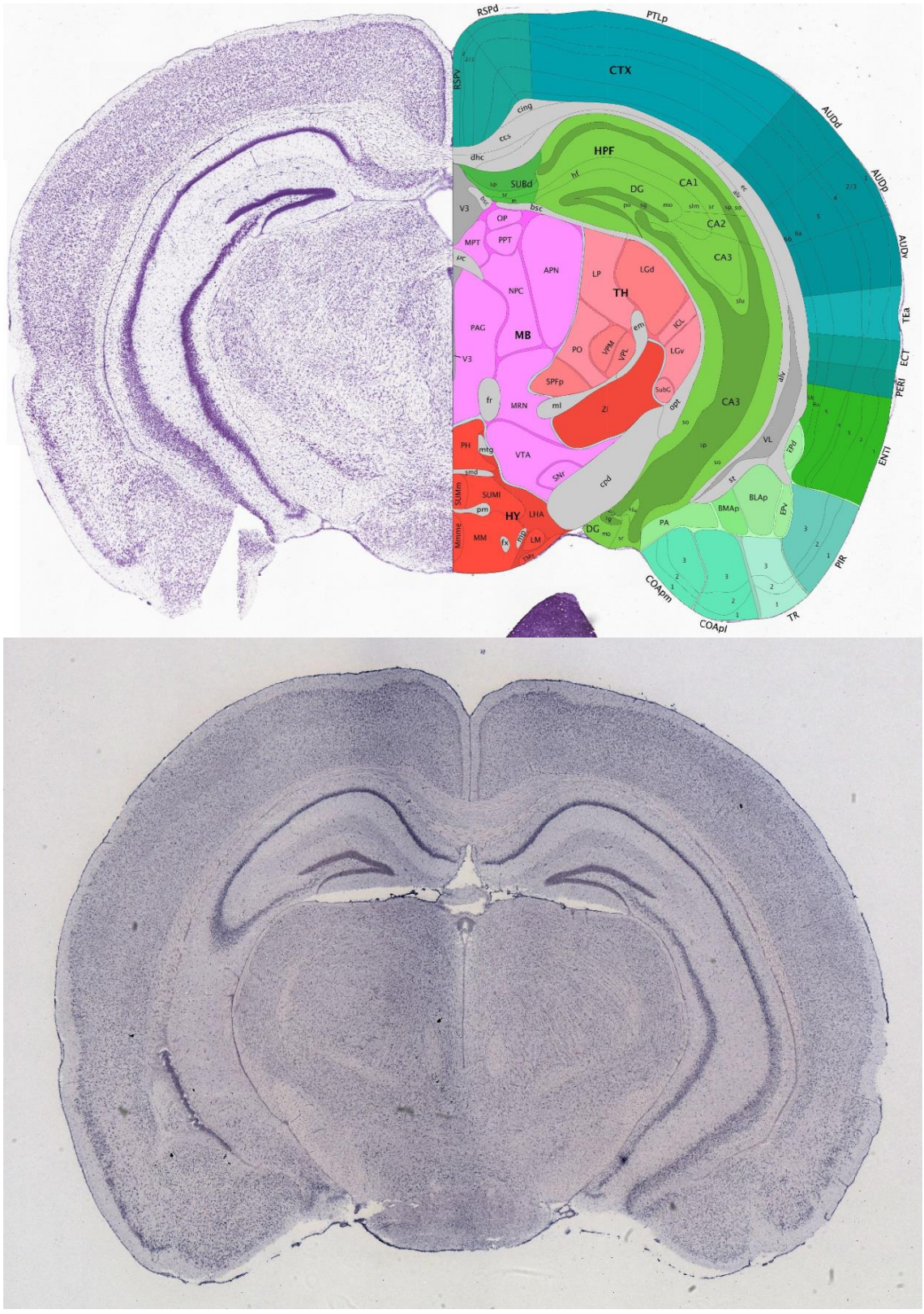


Figure 17. **AR expression in the adult mouse brain.** The upper panel represents Nissl staining of the left part of the coronal section of the brain, and the reference area in the right part. The lower image reports in situ hybridization of androgen receptor. (Allen Brain Atlas, 2018).

AR expression also occurs in the hippocampus, principally in cornu ammonis (CA) 1 and CA2/3 regions (Moghadami et al. 2016). Hippocampal AR mRNA in the developing male rat increases from PDN1 to PDN14, after which remains stable in the adulthood (Kimoto et al. 2010). In the mouse male hippocampus, AR mRNA is similarly expressed, with increased detection from PND 5 to PND 7 (Mogi et al. 2015). AR immunoreactivity can also be detected in the cerebral cortex. Mogi and colleagues showed AR mRNA expression in the cortex starting from E19 and increasing until PND7 in both sexes (Mogi et al. 2015). Another study detected the transcript from PND0 to PND21 in the mouse cortex and hippocampus (Tsai et al. 2015).

Taking together, these anatomical observations during developmental stages seem to suggest that AR may play an important role in adult brain response to specific sexual stimuli and in the maintenance of sexually dimorphic circuitries, but it is unlikely to be a major contributor of the masculinization of the brain at early stages in the studied models. However, specific functional and behavioral studies on AR function in sexual brain are discussed in detail in the next paragraph (1.3.3.).

Not only neurons express AR in the brain structures. AR expression was also detected in astrocytes of rat brain, but only in specific locations and at specific ages (Lorenz et al. 2005). There are evidences that astrocytes in the posterodorsal portion of the MA are sexually dimorphic in adult rats (Jonhson et al. 2013). Indeed, male rats have a higher number of astrocytes than females, and the masculinization of MA astrocytes is AR-dependent in its long-lasting effects (Jonhson et al. 2013). Recently, AR was implicated in CNS re-myelination exerted by oligodendrocytes (Bielecki et al. 2016). In this study, a mouse model in which AR was deleted from neurons, astrocytes and oligodendrocytes was applied. This ablation disrupted the capacity of myelin regeneration, suggesting an important role in this process for testosterone-AR signaling, even if the cell-autonomous component was not elucidated.

1.3.3. AR function in sexual behavior

Sexual behavior in rodents comprises two phases, the pre-copulatory and the copulatory phases. The pre-copulatory phase is characterized by olfactory cues exerted by feminine pheromones, which are detected by the male olfactory bulb, starting a signal cascade of transmission addressed to the medial amygdala, BNST and POA. The signal is also

transmitted from the hypothalamus to the spinal cord, in the SNB, area involved in erectile function. Then, the copulatory phase occurs, in which male rodents exhibit sexual arousal, thrusting, mounting and intromitting behaviors. Neural AR manipulation in rats reveals no difference in olfactory preference, normal anogenital investigation and normal Fos-activation pattern following exposure to bedding of female with estrus (Marie-Luce et al. 2013; Raskin et al. 2009; Swift-Gallant et al. 2016). In contrast, male sexual arousal is altered by AR activity. One of the brain structure involved in male sexual arousal is the medial amygdala. Flutamide injection directly targeting the medial amygdala was sufficient to lower the non-contact erections and the time spent in genital grooming (Bialy et al. 2011). Moreover, neural AR knock-out mice spend more time to initiate copulatory behavior (Raskin et al. 2009). AR-null male mutant mice display complete loss of male sexual behavior and severely impaired male aggressive behaviors. This phenotype was not rescued by DHT or estradiol treatments (Sato et al. 2004). In addition, also estrogen receptor α (ERα) $^{-/-}$ mice display impaired male-typical behaviors. To exclude a role of ER in male brain masculinization, mice were treated with DHT, leading to the rescue of the impaired male sexual behavior (Sato et al. 2004). Perinatal DHT treatment of female WT mice led to the DHT-dependent induction of typical male sexual behaviors in adultness. This female brain masculinization was repressed by AR inactivation. These observations confirm that AR function is critical for male-typical behaviors and brain masculinization (Sato et al. 2004).

1.3.4. AR function in cognition

As described above, AR is expressed in brain structures related to cognition. In order to evaluate the impact of AR function in learning and memory, male rats subjected to gonadectomy were treated with intra-hippocampal infusions of DHT (Edinger and Frye 2004). DHT treatment ameliorated learning and memory, assessed through behavioral tasks, such as the inhibitory avoidance task and the Morris water maze. Another proof of the importance of AR in cognition was demonstrated by the fact that the administration in the dorsal hippocampus of flutamide, provoked poorer performance in behavioral cognitive tasks (Edinger and Frye 2007). Recently, Picot and colleagues explored the role of AR in the temporal processing of information, revealing that the abrogation of AR function in the brain impaired this ability through the alteration of glutamatergic transmission in CA1 area of the hippocampus (Picot et al. 2016).

At the cellular level, AR stimulation through DHT leads to the expression of sarco and endoplasmic reticulum calcium ATPase 2 (SERCA2) in rat primary hippocampal neurons, which increases the Ca^{2+} stores in response to glutamate (Foradori et al. 2007). Moreover, the androgen-induced maintenance of spine synapse density in the CA1 and prefrontal cortex is mediated by AR action (Hajszan et al. 2007; Hatanaka et al. 2015). AR function was also implicated in activity-dependent long-term potentiation and long-term depression plasticity in CA1 area (Pettorossi et al. 2013). Another phenomenon in which AR is involved is the neurogenesis of the dentate gyrus, which is induced by DHT treatment. The DHT-dependent increase of hippocampal neurogenesis was blocked by flutamide administration (Hamson et al. 2013).

1.3.5. AR-mediated neuronal protection

Steroid hormones exert neuroprotective effects in acute neuronal injury and in neurodegeneration. In particular, starting from 2002, estrogen is the most studied hormone with neuroprotective effect (Simpkins et al. 2012). Estradiol is the principal estrogen hormone with very high affinity for ER (Kuiper et al. 1997). The binding of estradiol to ER is the principal mechanism involved in estrogen neuroprotection (Simpkins et al. 2012). Testosterone is extensively involved in neuroprotective mechanisms. The aromatization of testosterone through the action of aromatase leads to the production of 17β-estradiol. Aromatase is expressed in ovaries, adipose tissue, skin, testis, muscle, liver and also in the central nervous system (Moraga-Amaro et al. 2017). Hence, testosterone neuroprotective effect may arise from aromatase activity and binding to ER. To assess the specific AR involvement in neuroprotection activating pathways, the role of non-aromatizable androgens has to be addressed. For example, DHT is a non-aromatizable androgen that exclusively binds to AR with the higher affinity among androgen hormones. Another strategy is the use of selective AR antagonist, which block hormone binding. In human primary neurons insulted with serum deprivation, another non-aromatizable androgen, mibolerone, succeeded in the inducement of neuroprotection (Hammond et al. 2001). This effect was reverted by flutamide treatment. In an *in vitro* model of Alzheimer's disease, amyloid β peptide toxicity was prevented by androgen and estrogen treatments (Zhang et al. 2004). In this study, the selective inhibitions of both AR and ER, separately, blocked hormone-dependent neuroprotection. The same toxicity paradigm was used also by Nguyen and colleagues in which they observed that the AR-mediated neuroprotection induced by testosterone and DHT

treatments is exerted by MAPK/ERK signaling activation, probably via non-genomic AR actions (Nguyen et al. 2005). The AR-mediated neuroprotection was also proved in hippocampal slice cultures insulted with oxygen-glucose deprivation (Ishihara et al. 2016).

1.4. Molecular mechanisms of activity-regulated transcription

Neurons are excitable cells which undergo activity-dependent synaptic plasticity. A persistent experience-driven stimulus modifies neuronal synaptic strength causing the reinforcement or the elimination of synapsis during life. The idea that sensory input triggers gene expression to transduce sensory experience into long-lasting changes in the brain is supported by several works (West and Greenberg 2011). The mechanism through which brain plasticity is achieved starts with neuronal activity-regulated gene expression.

The first report of a fast-induced transcription in quiescent cells was the discovery of the regulation of *c-Fos* gene expression by external stimuli (Cochran et al. 1984; Greenberg and Ziff 1984). They discovered that mitogen-treated 3T3 fibroblasts rapidly activate robust *c-Fos* transcription within minutes. Several proto-oncogenes were linked to the immediate transcription, suggesting an essential role for this process in mitogenesis (Lamph et al. 1988; Ryder et al. 1988). The genes induced by rapid transcription were named immediate-early genes (IEGs). PC12 pheochromocytoma cells treated with nerve growth factor (NGF) and epidermal growth factor (EGF) were shown to rapidly activate transcription of several proto-oncogenes, including *Myc*, *Fos*, *Raf* and *Fgr* (Greenberg et al. 1985; Milbrandt 1986). In 1986, it was shown that the rapid gene expression of IEGs in differentiated PC12 treated with a nicotinic acetylcholine receptor agonist was dependent on calcium release after membrane depolarization (Greenberg et al. 1986). This mechanism required L-type voltage-sensitive calcium channels (L-VSCCs) activation. Additional experiments came in the following years, linking immediate transcription with stimuli inducing activation of post-mitotic cells. For example, *in vivo* treatment with a seizure-inducing stimulus caused a rapid increase of *c-Fos* expression in neurons of the cortex, hippocampus and limbic system (Morgan et al. 1987). This new concept led to the hypothesis that external stimuli may cause long-lasting effects on the gene expression, changing neuronal function and metabolism.

Differential cloning experiments revealed the list of upregulated IEGs in stimulated neurons. Nedivi et al. used subtractive and differential cloning strategy of cDNA derived from non-treated rat brains and kainate-activated dentate gyrus, in order to identify the activity-induced gene transcription (Nedivi et al. 1993). In the same year, another group performed a similar screening using metrazol-induced seizure paradigm in mice and discovering an

unprecedented regulation for known neuronal-specific genes by long-term potentiation (LTP) (Qian et al. 1993). These results reveal that stimuli that trigger neuronal activity induce a plethora of genes which are neuronal-specific and involved in synaptic function. Recently, new methods were developed to analyze gene expression profiles, such as microarray and RNA-seq techniques. These studies reveal that different stimuli activate specific subsets of genes which are down- or upregulated.

1.4.1. Models of transcriptional control of immediate early genes

The pattern of gene expression induced by changes of the activity state of a neuron varies depending on cell type and treatment (Flavell et al. 2008; Guan et al. 2005; Spiegel et al. 2014; Xiang et al. 2007). Although these evidences lead to the concept of spatial-, time- and developmental stage-specific regulation of transcriptional program, the IEGs induced after neuronal stimulation can be classified in two classes: i) the classical IEGs that are transcriptional factors activated in a broad range of cells generally upon external stimuli, such as growth factors, hormones and stress, and, ii) neuronally-enriched genes that regulate synapse development and function (***Fig. 18***). As I show in the next paragraphs, the first class is composed by genes such as *Fos*, *Jun*, *NPAS4* and more, the second class comprises, among the others, Homer, a family of scaffold proteins that binds to metabotropic glutamate receptors and has a function in synaptogenesis (Xiao et al. 2000), neuronal activity-regulated pentraxin (Narp), responsible for the clustering of AMPA receptors (O'Brien et al. 1999).

IEGs share some common features. IEGs expression is fast and transient. For example, *Fos* gene expression after stimulation reaches the maximum level of transcript production around 15 minutes and after 90 minutes it returns to normal threshold, in the absence of persistent stimulus (Greenberg and Ziff 1984). The quick expression of IEGs is protein synthesis-independent, thus inhibitors of translation are not effective in blocking IEGs transcription (Greenberg et al. 1986). The induction of their transcription is initiated by extracellular signals: growth factors, mitogens, signals triggering immune response, neural depolarization and stress (Herschman 1991). Fos protein family is eliminated partially by a ubiquitin-independent degradation through proteasome. This fact remarks their instability and the transient nature of IEGs. This characteristic is possible thanks to the action of an important regulator of IEG transcript degradation, Zfp36. It is an IEG demonstrated to be essential for the downregulation of *Fos* gene expression (Amit et al. 2007). Zpf36 is a zinc-finger protein that binds to AU-rich elements (AREs) in the 3'-UTR of mRNA, that induces deadenylation

and degradation of target mRNAs (Carballo et al. 1998). Another common feature is the presence of specific binding sites for common transcription factors which are over-represented in IEGs promoter regions. These transcription factors are serum-response factor (SRF), nuclear factor-kB (NF-kB) and cyclic AMP response element-binding (CREB) protein. In addition, the IEG promoters are enriched with TATA boxes, underlying a solid and redundant mechanism of gene expression (Tullai et al. 2007).

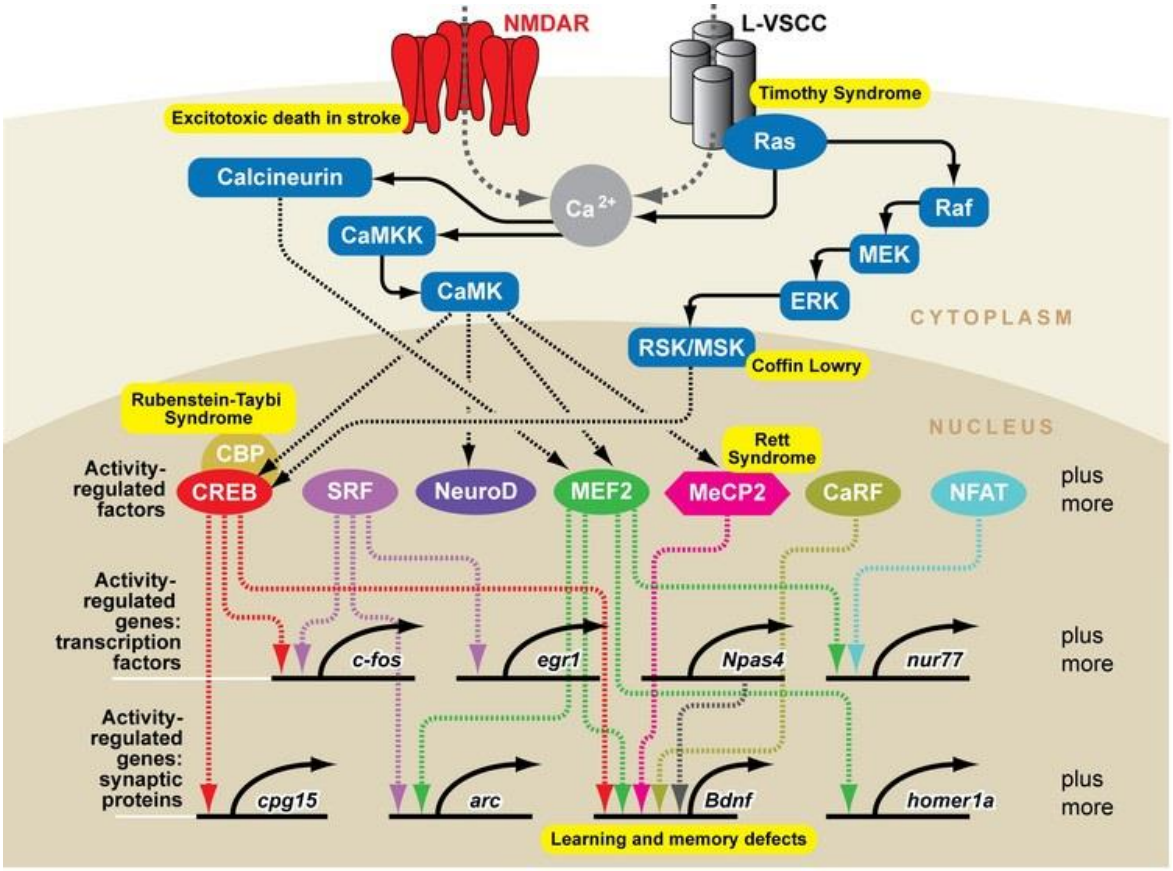


***Figure 18*. Signaling pathways initiated by neuronal activity which result in gene expression of activity-dependent transcription factors and synaptic proteins.** Calcium influx through neurotransmitter receptors or voltage-gated calcium channels activates calcium-regulated signaling enzymes. These factors activate pre-exiting transcription factors in the nucleus through post-translational modifications which trigger the expression of additional activity-regulated transcription factors and synaptic proteins (Adapted from Flavell and Greenberg 2008).

Rapid activation of IEGs expression is elicited by a characteristic chromatin structure, named bivalent chromatin, at the transcription starting sites (TSSs). It means that they have both active and repressive histone marks. For example, tri-methylation of lysine 4 on histone H3 (H3K4me3), an active chromatin marks, coexists in the same site with tri-methylation of

lysine 27 on histone 3, a repressive signature. This type of promoter was discovered as enriched in pluripotent embryonic stem cells (ESCs) (Bernstein et al. 2006). The IEGs TSSs are essentially repressed but occupied by RNA polymerase II (RNA pol II) poised for quick transcription (Rye et al. 2014). The promoter regions of IEGs are dynamically regulated by histone modifiers, such as the "writers" histone acetyl transferases (HATs) and the "erasers" histone deacetylases (HDACs). A specific HAT, named p300/CREB-binding protein (CBP), mediates this dynamic process (Crump et al. 2011).

In the next paragraphs, I describe the specific mechanisms of transcription of some IEGs.

1.4.1.1. Fos

The first gene that was characterized in its immediate expression after extrinsic stimuli was *Fos* gene, whose product is the oncogenic transcription factor Fos. This protein was firstly described in early '80s as v-FOS, the viral omolog of cellular Fos (c-Fos), isolated from Finkel-Biskis-Jinkins virus (Curran et al. 1982). Fos alone is not a functional transcription factor, but it dimerizes with members of the Jun family, forming the transcription factor activator protein 1 (AP-1) (Curran et al. 1985). The interaction between the two proteins occurs through a basic leucine zipper motif (Kouzatides and Ziff 1988). Jun can homodimerize to form AP-1, instead Fos forms an active heterodimer only in association with Jun. The homodimer Jun/Jun is less prone to bind DNA respect to the heterodimer Fos/Jun (Halazonetis et al. 1988). Fos/Jun heterodimer recognizes the consensus sequence TGACTCA, that is the TPA responsive element (TRE) binding site(Angel et al. 1987).

As *Fos* was the first IEG to be identified, its transcriptional regulation is extensively characterized, and provides a model for activity-dependent gene expression (***Fig. 19***). *Fos* transcription depends on transcription factors which are activated upon stimuli and bind to stimulus-response elements located in the proximal promoter. One of these sites was named serum response element (SRE), which is 300 bp upstream of the *Fos* TSS and it is required for induction of *Fos* upon serum and growth stimuli (Treisman 1985). The transcription factor which binds to SRE was named serum response factor (SRF). Another element was named calcium/cAMP-response element (CRE) and it is 60 bp upstream of the TSS (Hyman et al. 1988; Sheng et al. 1988). This site is required for stimulation of *Fos* transcription upon calcium and cAMP-releasing stimuli. Proteins which bind to CRE were named calcium-response element binding protein (CREB) family. A third element is present near CRE and

it was defined as retinoblastoma control element (RCE) (Udvadia et al. 1992). As the name suggests, this element is required for transcription activation of *Fos* by retinoblastoma tumor suppressor (rb) stimulated pathways. The transcription factor which binds to RCE was named Sp1 (Udvadia et al. 1992). *Fos* proximal promoter is primed by these elements, as well as by the pre-initiation complex formed by RNA pol II (Kim et al. 2010; Sheng et al. 1988). This characteristic is of primary importance for the very quick activation of Fos transcription.

To prevent the constituvely transcription of *Fos*, the promoter needs to be kept in a repressive state through specific histone modification of the chromatin. Indeed, silencing of the promoter is achieved via RCE, where the complex composed by Sp1, the transcriptional co-repressor Rb, the Brahma-related gene 1 (BRG1) which remodels the chromatin, and the scaffold protein calcium-responsive transactivator (CREST), binds. When the neuron is quiescent, HDACs bind to Rb and deacetylate the histones that surround the *Fos* TSS, keeping the promoter repressed (Qiu and Ghosh 2008). HDACs can also be recruited through its binding to ETS domain containing protein Elk1, which in turn binds to SRE (Yang et al. 2002). Another trait which may underlie an additional regulatory step for *Fos* orchestrated transcription is the presence of intragenic polymerase pause sites inside the gene (Lamb et al. 1990), common trait to several IEGs. Upon neuronal stimulation, HDACs dissociate from Rb thanks to its calcineurin-dependent dephosphorylation, leaving the promoter site. The neuronal depolarization also induces phosphorylation of HDACs, which causes the active export of HDAC4 and HDAC5 from the nucleus (Chawla et al. 1988; Qiu and Ghosh 2008). Alternative signaling pathways activate the recruitment of the histone acetyltransferase CBP by binding to CREB and CREST, acetylating histones at the promoter and triggering transcription (Impey et al. 2002; Qiu and Ghosh 2008). CREB phosphorylation at Ser133 by PKA elicits the binding of CBP to CREB (Sheng et al. 1991).

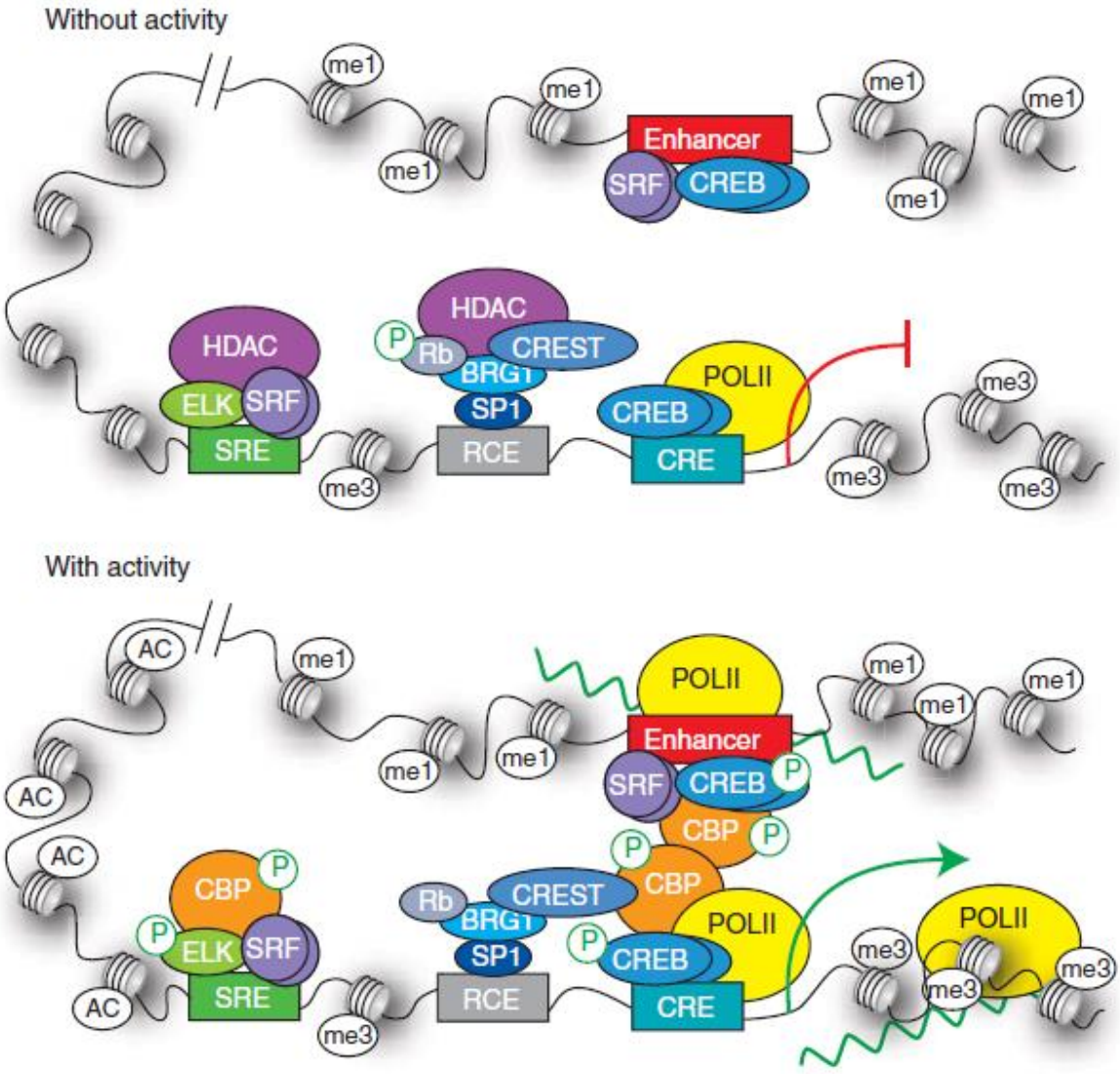


***Figure 19*. Fos transcription activation after neuronal stimulation.** The upper panel represents Fos promoter in the absence of neuronal activity, the lower panel shows the Fos promoter architecture and protein composition upon neuronal activity (West and Greenberg 2011).

Upon stimulation of neuronal activity, there is also the recruitment at the enhancer elements of both CBP and RNA pol II (Kim et al. 2010). These regions have a similar occupancy as the promoter region, pre-bound by activity-regulated transcription factors, including SRF, CREB and myocyte enhancer factor 2 (MEF2) (Flavell et al. 2008). Recently, Kim and colleagues discovered that RNA pol II produces short, non-coding enhancer RNA transcripts, called enhancer RNAs (eRNAs), starting from these intergenic non-coding elements (Kim et al. 2010). This new class of molecules is described in detail in paragraph 2.1.2.3.2..

1.4.1.2. Brain-derived neurotrophic factor (BDNF)

BDNF is a protein of the neurotrophin family, together with nerve growth factor (NGF). It is highly expressed in the nervous system and it has a fundamental role in the development of the CNS and in modulating plasticity (Poo 2001). BDNF mRNA level is rapidly increased after several environmental stimuli in specific activated brain areas. In particular, *in vivo* seizure activity induces the increase of BDNF mRNA level in the cerebral cortex and hippocampus (Zafra et al. 1990). This increase is induced also by normal physiological activity which generates hippocampal long-term potentiation (LTP) (Patterson et al. 1992). Another aspect which is regulated by neural stimulation is the trafficking to the plasma membrane of TrkB, the BDNF receptor (Meyer-Franke et al. 1998). In this way, BDNF-TrkB signaling is strictly tuned by neuronal activity. Specific mutations in the *Bdnf* gene leading to the decrease of BDNF protein levels and secretion, provokes deficits in brain development and plasticity (Genoud 2004; Laudes et al. 2012). These data suggest that the fine tuning of *Bdnf* gene expression is a requirement for its function.

In 1993, Timmusk and colleagues described the *Bdnf* gene, identifying four short 5' exons and one coding 3' exon, recognizing eight different Bdnf mRNAs with four different 5' ends and two alternative polyadenylation sites (Timmusk et al. 1993). They also identified five TSS at the beginning of these exons, proving the presence of at least five different promoters (Timmusk et al. 1993). More recently, the same group identified three more 5' exons (Aid et al. 2007). This lead to the modern definition of the *Bdnf* gene, composed by nine exons and regulated by at least eight distinct alternative promoters (***Fig. 20***). This promoter complexity allows *Bdnf* expression in a time- and spatial-specific manner, depending on the developmental stage, the brain region and the cell type (Liu et al. 2006). All the promoters result to be active in the CNS, but the more reactive transcript to neuronal activity is exon IV-containing Bdnf mRNA regulating by promoter IV (promoter III in the old nomenclature, (Tao et al. 1998)).

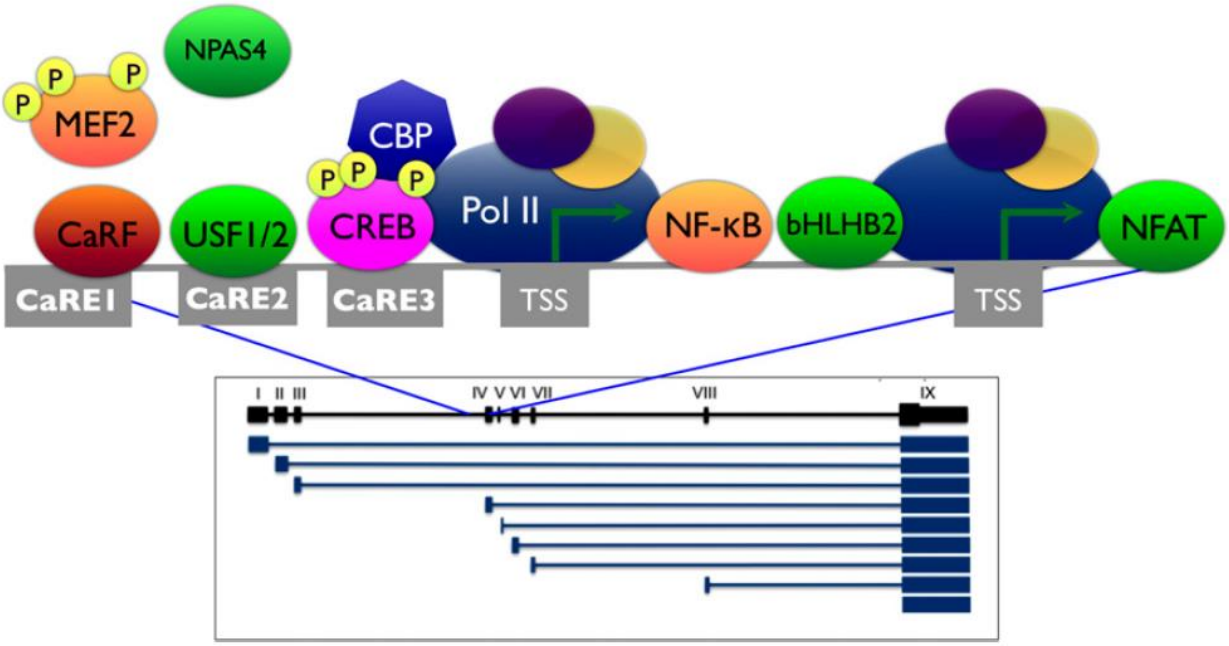


Figure 20. **Mechanism of activity-induced Bdnf promoter IV.** The lower box shows spliced mRNAs (blue) from the eight alternative promoters of Bdnf mouse gene (black). Black boxes represent the nine exons and thicker region of exon IX indicates the coding sequence. The upper panel represents the structure of promoter IV and the transcription factors which bind to the different motifs (Lyons and West 2011).

Promoter IV of the *Bdnf* gene comprises the 170 bp upstream of the exon IV. Within this regulatory region, there are three calcium responsive elements, CaRE1, 2 and 3. These CaREs are regulated by calcium response factor (CaRF), the upstream stimulatory factor 1/2 (USF1/2) and proteins of the CREB family (Chen et al. 2003; Shieh et al. 1998; Tao et al. 2002). Recently, it was added another player in *Bdnf* gene regulation, the activity-inducible transcription factor neuronal PAS domain protein 4 (NPAS4) (see next paragraph, (Pruunsild et al. 2011)). Nuclear factor kB (NF-kB) and basic helix-loop-helix protein B2 (bHLHB2) bind to an alternative TSS 100 bp downstream of the 5' end of exon IV (Jiang et al. 2008; Lipsky et al. 2001). Another factor which is implicated in the regulation of *Bdnf* gene expression upon N-methyl-D-aspartate (NMDA) treatment is the nuclear factor of activated T-cells (NFAT), which binds to an intergenic element 3' to the alternative TSS (Vashishta et al. 2009). ChIP experiments reveal the binding to *Bdnf* promoter IV of myocyte enhancer factor-2 (MEF2), but the precise binding site remains elusive (Hong et al. 2008). The function of the Bdnf-regulating transcriptional factors at promoter IV has been investigated through loss-of-function mouse models. $CaRF^{-/-}$ transgenic mice show brain region-specific

downregulation of Bdnf transcription (McDowell et al. 2010). Indeed, Bdnf exon IV-containing transcript and BDNF protein levels are specifically reduced in the frontal cortex of $CaRF^{-/-}$ mice compared to WT littermates and is completely unaffected in the hippocampus and striatum (McDowell et al. 2010). Anyway, the analysis of this knock-out mice reveals that CaRF is not required for activity-dependent *Bdnf* promoter IV transcriptional induction (McDowell et al. 2010). On the other side, the introduction of point mutations at CaRE3 site, which is required for CREB binding to *Bdnf* promoter IV, produce a mouse model in which neurons have normal basal BDNF protein level, but lack activity-dependent transcription from *Bdnf* promoter IV (Hong et al. 2008). Additionally, CaRE3 mutants fail to recruit to promoter IV the transcriptional factor MEF2, which binds to a different site from CaRE3. These experiments provide evidences for a model in which a multifactor transcriptional complex is recruited at *Bdnf* promoter IV and CREB mediates its nucleation.

1.4.1.3. Neural PAS domain protein 4 (NPAS4)

Neural PAS domain protein 4 (NPAS4), previously called NFX, belongs to the family of the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) proteins. It is a transcriptional factor which was identified by similarity to Sim2 protein, a transcriptional repressor involved in Down's Syndrome (Ooe et al. 2004). In the same period, another group identified NFX as a seizure-induced gene through an unbiased microarray analysis of mice treated with the convulsant pentylenetetrazol (Flood et al. 2004). NPAS4 forms heterodimers with another subfamily of bHLH-PAS proteins, Arnt-type proteins (Ooe et al. 2004). It is a transcriptional activator and it was shown that NPAS4 and SIM2 compete for the same target promoters (Ooe et al. 2004). It is an IEG selectively express in neuronal tissues and its expression is selectively induced by neuronal stimuli which trigger the release of calcium ions (Zhang et al. 2009). NPAS4 is essential for the regulation of GABAergic synapse development. Indeed, the overexpression of NPAS4 increases the number of GABAergic synapses in primary hippocampal neurons, instead the silencing through RNAi leads to the selective decrease of this type of synapse. Interestingly, in this model, the glutamatergic synapse number is unaltered, indicating a selective role of NPAS4 in inhibitory synapse development (Lin et al. 2008). Recently, it was shown that NPAS4 stimulation after the increase of intracellular calcium concentration causes the redistribution of inhibitory synapse on neurons, regulating the balance between inhibitory synapses on the cell body and on apical dendrites (Bloodgood et al. 2013). Experiments of ChIP-sequencing for NPAS4 revealed that this protein is

recruited not only to promoters, but also at enhancer regions (Kim et al. 2010). The specific role of NPAS4 at enhancers is not understood, but it was speculated by Greer and Greenberg, that NPAS4 may be act as a claw to prolong transcription and to permit the achievement of the critical threshold of expression to exert specific processes (Greer and Greenberg 2008). In 2014, it was discovered that NPAS4 has differential functions in excitatory and inhibitory neurons. Through genome-wide analysis of gene expression in activated inhibitory and excitatory neurons, they found that NPAS4 was induced as IEG in both neurons, but it selectively induces two different transcriptional programs of late-response genes to induce specific outcomes (Spiegel et al. 2014). These results prove that the induction of the same pool of IEGs regulate cell-specific outputs through the differential gene expression of cell-specific late-response genes.

1.4.2. New concepts in neuronal immediate early gene expression

The concept of immediate induction of neuronal activity-stimulated transcription revolutionized neurobiology of the last century. Incredibly advances have been done during the last years, adding little pieces to approach the answer to essential questions regarding neuronal plasticity.

1.4.2.1. Enhancer RNA (eRNA) in the regulation of neuronal-activity regulated transcription

As I show in the previous paragraph, in the past, the neuronal activity-dependent transcription was deeply characterized at promoter level. The discovery of enhancer-regulating gene expression of neuronal activity IEGs occurred at the very beginning, firstly in *Fos* gene (Deschamps et al. 1985), but the precise regulation kinetics and the functional meaning of the induction by enhancer elements was not elucidated.

Enhancers are fundamental cis-acting elements which regulate gene expression spatiotemporally. They were first described in the β-globin gene in cultured cells, as a sequence that cause an increase of 200-fold in β-globin expression (Banerji et al. 1981). From this discovery, almost every gene started to be linked to enhancer-regulated transcription. Recently, with the genome-wide analysis era, the precise mapping of these elements became easily detectable. The enhancer regions are characterized by the presence of differential variants of nucleosomes with particular histone modifications in the flanking areas. Indeed, enhancers are associated with the presence of the histone variants H3.3 and H2A.Z, which

are highly mobile, and this feature facilitates the access of transcription factors and other regulatory proteins in promoters, enhancers and insulator regions (Jin et al. 2009). The histone modifications that characterize active enhancers are histone 3 lysine 4 mono-di-trimethylation (H3K4me1-me2-me3) and histone 3 lysine 27 acetylation (H3K27ac) (Creyghton et al. 2010). This last histone modification is deposited by CBP/p300, which contains a catalytic histone acetyltransferase (HAT) domain (Kim and Shiekhattar 2015). H3K4me1 is the unique feature specific to enhancers, instead dimethylation of lysine 4 of histone 3 is found both in enhancers and promoters, the trimethylation is found to be enriched at active promoters (Bernstein et al. 2002; Santos-Rosa et al. 2002).

The genome-wide analysis through chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) techniques provide an overview of promoter and enhancer locations. The profiling of the chromatin occupancies of epigenetic marks H3K4me1 and H3K4me3, in addition to CBP, permitted the identification of numerous activity-regulated enhancers (Kim et al. 2010). In this pioneering study, it was shown that RNA Pol II was recruited to enhancers upon neuronal stimulation. From these sites, transcription occurred bidirectionally, leading to the production of short non-coding RNA, called enhancer RNAs (eRNAs) (Kim et al. 2010). In the same year, another group showed widespread transcription at enhancers using macrophages stimulated with lipopolysaccharide (de Santa et al. 2010). In the last years, several groups proved the existence of eRNAs (Andersson et al. 2014; Djebali et al. 2012; Hah et al. 2011; Wang et al. 2011). The role of eRNAs remains elusive, but it was proposed the notion that eRNAs may be a mere signature of functional enhancers (Andersson et al. 2014). This view is counteracted by the idea of a specific function for eRNAs, that was postulated from several studies. Kim and colleagues reported a specific pattern of eRNA expression from a subset of enhancers and a correlation between the abundance of eRNAs and transcription level of target mRNAs, suggesting a sub-classification of eRNA-producing enhancers at a certain time and with a certain stimulus (Kim et al. 2010). The study of TSSs reveals that enhancers and promoters share several features, such as the bidirectionality of transcription and similar composition of the canonical core transcriptional elements (Core et al. 2014). Experiments on the kinetics of promoter and enhancer transcription prove that eRNA precedes target mRNA expression (Arner et al. 2015). Lam and colleagues demonstrate that the inducement of full enhancer activity to target promoter requires eRNAs transcription, dictating a specific role of this non-coding RNA sub-class to conventional transcription machinery (Lam et al. 2013). Finally, *Fos* promoter is

regulated by multiple enhancers which are activated upon different stimuli to confer stimulus-specificity and robust expression of the protein (Joo et al. 2015). This mechanism is likely to be regulated by combinatorial eRNA interaction with the *Fos* promoter. These data indicate that eRNAs have fundamental functions in regulating time- and cell-specific programs of gene transcription.

1.4.2.2. Neuronal activity-induced double strand breaks

In the light of these discoveries, the transcription of neuronal activity-stimulated genes may require fast interaction between promoter and enhancer elements. The trigger for this mechanism could be the recent observation of activity-induced DNA breaks in the promoter of IEGs (Madabhushi et al. 2015). Several studies reported that different stimuli which trigger neuronal depolarization both *in vivo* and *in vitro* stimulate DNA double strand breaks (DSBs) (Crowe et al. 2006; Suberbielle et al. 2013). In the past, DNA DBSs were associated to cytotoxic events, such as ultraviolet light exposure, chemical agent and irradiation, and these damages lead to genomic instability that cause the development of cancer and neurodegenerative diseases (Crowe et al. 2006; Jackson and Bartek 2009; Madabhushi et al. 2015). DNA DSBs activate the response of various cellular effectors of the DSB repair machinery, such as DNA-dependent protein kinase (DNA-PKcs), ataxia-telangiectasia mutated (ATM) kinase and ATM- and Rad3-related (ATR) kinase. ATM kinase phosphorylates a particular histone variant that occupies DSB loci, H2A.X, making this post-translational modification a hallmark of DNA DSB (Burma et al. 2001). Madabhushi and colleagues exploit this signature to analyze whether DNA DSBs occur at particular sites in the DNA of stimulated neurons (Madabhushi et al. 2015). They found that NMDA-dependent stimulation of neurons led to DNA DSBs at specific loci, particularly to promoters of IEGs, including *Fos, FosB, Npas4, Nr4a1*, *Nr4a3* and *Egr1*. Several studies reveal that DNA DSBs may be a common mechanism of rapid induction of gene transcription followed by several stimuli, such as insulin and glucocorticoids (Trotter, King, and Archer 2015; Wong et al. 2009).

Another observation that adds a detail in the mechanism of neuronal activity-induced DNA DSBs is that the protein responsible for this DNA morphological modification is topoisomerase-IIβ (Top2B) (Madabhushi et al. 2015; Suberbielle et al. 2013). Topoisomerases are enzymes able to resolve torsional stress into the DNA caused by physiological processes, such as DNA replication, transcription and recombination (Pommier

et al. 2016). The resolution of the supercoiled DNA occurs through the generation of a transient DNA DSBs, the passage of one strand to another in order to relax the tension and the rapid seam of the two ends by topoisomerase itself (Pommier et al. 2016). Neuronal activity somehow impedes the completion of this mechanism, causing the formation of long-lasting DNA DSBs, which require the action of the DNA repair machinery to be resealed, through non-homologous end-joining (Madabhushi et al. 2015). Recently, it was observed that Top2B interacts with CCCTC-binding factor (CTCF), an architectural protein which mediates the interaction between enhancer and promoter regions, and Top2B-mediated DNA DSBs are enriched at CTCF binding sites (Canela et al. 2017). It was proposed that enhancer-promoter interaction is impeded by DNA architectural constraints which causes RNA Pol II pausing inside IEG promoters, at basal conditions (described above in paragraph 1.2.1.1., (Lamb et al. 1990; Madabhushi and Kim 2017)). When neuronal depolarization is induced, Top2B-induced DSBs relax the DNA supercoiling, permitting enhancer-promoter interaction and transcriptional initiation (***Fig. 21***).

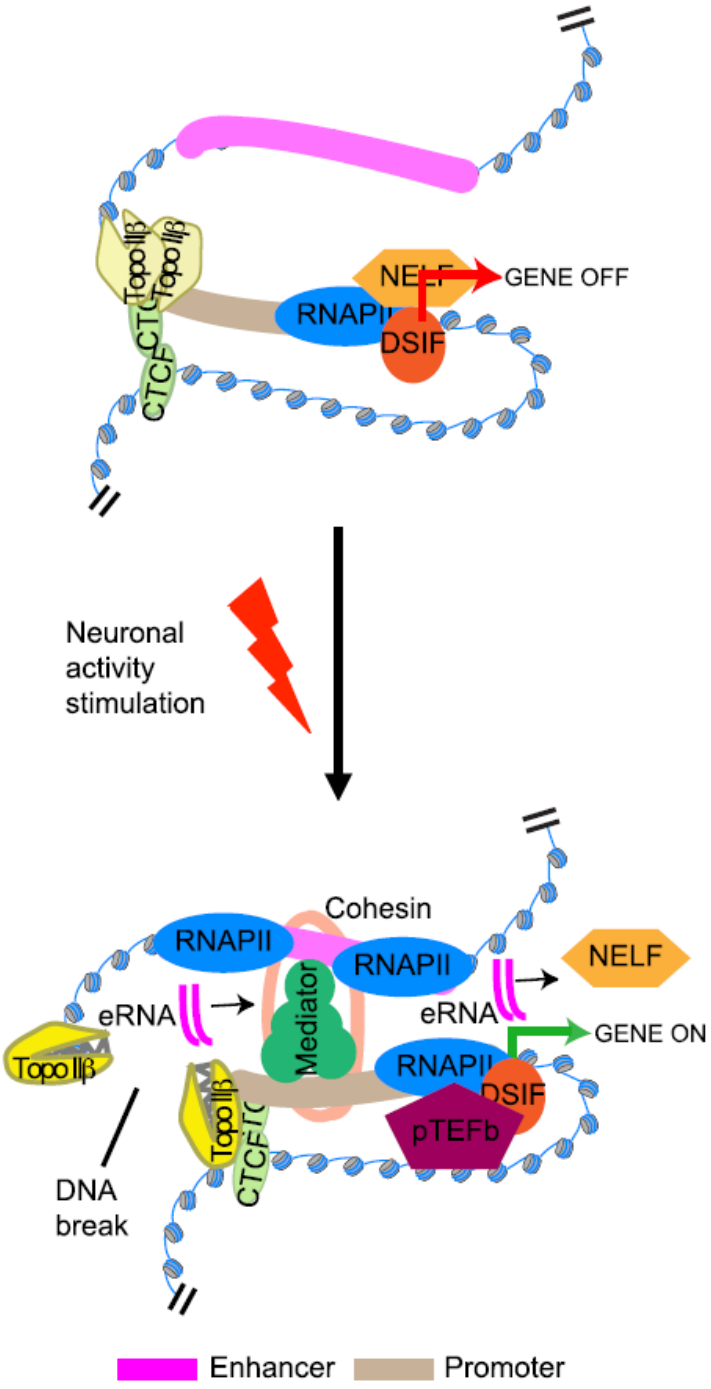


***Figure 21*. The role of DNA-strand breaks and enhancer RNA (eRNA) in IEG transcription.** Enhancer-promoter interactions are impeded by architectural constraints imposed by CTCF protein. Neuronal activity stimulation triggers Top2B-mediated DNA breaks to overcome the constraint, allowin enhancer-promoter interactions. This interaction initiates the production of eRNA from enhancer regions. Several observations suggest that eRNAs play a role in orchestrating the neuronal activity-dependent transcription (Adapted from Madabhushi and Kim 2017).

2. AIM OF THE THESIS

Phosphorylation is a fundamental mechanism of regulation of AR function. AR is extensively phosphorylated at several sites, with differential and combinatorial final effects. Phosphorylation can influence the stability, transcriptional activity, localization of the protein or can stimulate cell growth through the activation of additional pathways (Gioeli and Paschal 2012). Serine 96 was found to be a constitutive phosphorylation site (Coffey and Robson 2012), but we observed that neuronal cell types demonstrate an increase in the percentage of phosphorylated serine 96 versus total AR protein level compared to non-neuronal cell types (Polanco et al. 2016). Moreover, we identified the kinase responsible for serine 96 phosphorylation, namely CDK2. In cultured cells, CDK2-mediated phosphorylation at serine 96 increased the toxicity of polyQ-AR and the phospho-defective mutant at this site (S96A, i. e. serine 96 mutated to alanine) protected motor neuron-derived SBMA cells from DHT-induced cell death. Surprisingly, the treatment of SBMA mice with a compound that decreased serine 96 phosphorylation through the increase of $p21^{CIP1}$, a well-known CDK2 inhibitor, was efficient in rescuing motor phenotypes and increasing mouse lifespan (Polanco et al. 2016). The specific molecular mechanisms involved in dephosphorylated serine 96-induced protection in SBMA is unknown. In addition, the role of serine 96 phosphorylation/dephosphorylation events of neuronal AR in normal conditions is totally uncharacterized.

Taken together, these preliminary observations suggest that serine 96 has a pivotal importance in SBMA pathogenesis, but also a potential unexplored physiological role in neurons. For these reasons, the central hypothesis of this thesis is that AR function is controlled by CDK2-mediated serine 96 phosphorylation, which is finely tuned by neuronal activation, a level of regulation altered by polyQ expansion.

In order to test the central hypothesis, I pursued these three specific aims:

- Specific Aim 1: To identify AR as a neuronal activity-regulated transcription factor. We will test the hypothesis that AR transcriptional activity is regulated by neuronal stimulation through serine 96 phosphorylation. Using luciferase assays and genome-

wide analysis of gene expression through microarray, we will uncover AR-regulating gene patterns in activated neurons.

- Specific aim 2: To assess the role of CDK2-mediated polyQ-AR phosphorylation *in vivo*.

  We will test the hypothesis that genetic deletion of CDK2 reduces the polyQ-AR toxicity by decreasing phosphorylation at serine 96. By genetic modification of CDK2 expression in SBMA transgenic and knock-in mice, we will assess the effect of loss of CDK2 function in SBMA with measures of survival, motor function and biochemistry.

- Specific aim 3: To identify molecular pathways which alter polyQ-AR phosphorylation at serine 96.

  We will test the hypothesis that genetic and pharmacological inhibition of Cdc25 attenuates polyQ-AR toxicity. We will test overexpression of WT and catalytically dead form of Cdc25 and a compound identified by screening a library of kinase and phosphatase inhibitors, for its ability to attenuate polyQ-AR nuclear shuttling.

From Aim 1, I expect to link specific properties of vulnerable cells, i.e. neuronal excitability, to modification of the native structure through phosphorylation, which modifies function of the disease protein. With respect to Aim 2, I will provide proof-of-principle that CDK2 is a novel modifier of SBMA pathogenesis. From Aim 3, I expect to prove the therapeutic potential of novel genetic and pharmacologic inhibitors of CDK2-mediated serine 96 phosphorylation of polyQ-AR by targeting Cdc25.

3. MATERIALS AND METHODS

3.1. Plasmids

EGFP-N1 vector (Clontech, kindly provided by prof. Massimo Pizzato, CIBIO, University of Trento) was used to insert AR24Q at the N-terminal of EGFP by cloning through NheI/XhoI digestion. Mutagenesis for AR (S96A) were obtained by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent) using primers S96A-F: 5′-GCAGGGTGAGGATGGTGCTCCCCAAGCC CATCGTAGAGG-3′; S96A-R: 5′-CCTCTACGATGGGCTTGGGGAGCACCATCCTCACCCTGC-3′ (Polanco et al. 2016, mutagenesis made by Sara Parodi). CMV-Cdc25 vector was obtained by PCR cloning (Sara Parodi).

3.2. Cell culture and transfection

HEK293T (ATCC), and HeLa (ATCC) cells were cultured in Dulbecco's modified Eagles's medium (DMEM) with 10% of heat inactivated fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml) and L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO_2. MN-1 stably expressing AR24Q and AR100Q with and without S96A mutation (Sara Parodi) were cultured in Dulbecco's modified Eagles's medium (DMEM) with 10% of heat inactivated fetal bovine serum (FBS), geneticin G418 (140 ug/ml), penicillin/streptomycin (100 U/ml) and L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO_2. HEK293T were transfected with polyethylenimine (PEI) linear MW 25,000 Da (Sigma-Aldrich) according to well dimension. DNA:PEI ratio was 1:1. MN-1 cells were transfected with Lipofectamine 2000, according to manufacturer's instructions (Thermo Fisher Scientific).

3.2.1. Generation of HeLa cells stably expressing AR65Q-EGFP

HeLa cells stably expressing AR65Q-EGFP were generated after transfection with Lipofectamine 2000 (Invitrogen) with CMV-AR65Q-EGFP construct, conferring neomycin resistance. Cells were selected with 1.6 µg/µl of G418 (Sigma-Aldrich). Total cell population were analyzed through FACS Canto^{TM} A analyzer (BD Bioscience) and EGFP^{+} cell population were sorted through FACS Aria^{TM} II cell sorter (BD Bioscience) (in collaboration

with Cell Analysis and Separation Facility, CIBIO, University of Trento). $EGFP^+$ cells were expanded and analyzed for transgene expression by western blot and for protein nuclear shuttling through fluorescent microscopy after dihydrotestosterone (DHT) or EtOH (vehicle) treatments.

3.3. Transcriptional assay

HEK293T cells were transfected with AR vectors together with pARE-Luciferase and TK-Renilla. The day of the assay, cells were washed in PBS at room temperature (RT), then they were lysed in passive lysis buffer (PLB) coming from Dual-Luciferase assay kit (Promega) and rocked for 15 minutes at RT. Each sample (10ul) was transferred to a 96-well plate and luciferase substrate from the kit was added in each well. The plate was analyzed with Infinite 200Pro (Tecan instruments). After the acquisition, Renilla substrate from the kit was added and a second reading was carried out.

3.4. Mtt assay for cell viability

Cells were seeded at a concentration of 5000-200000 cells/well, according to the well format. Cells were incubated and treated for the described timing. The day of the assay, 1:10 MTT was added to the cell culture medium and incubated for 30'-1h, according to cell types. DMSO was added to solubilize the formed formazan crystals. The absorbance was recorded at 570 nm with Infinite 200Pro (Tecan instruments).

3.5. High-throughput screening of phosphatase and kinase inhibitors

HeLa AR65Q-GFP cells were plated into 384-well plates. The day after, cells were treated with 10 µM of phosphatase and kinase inhibitors, taken from SCREEN-WELL® Phosphatase inhibitor library (Enzo Life Sciences) and Kinase inhibitor library (Selleck Chemicals), respectively, for 5 hours. 1 hour later DHT was added. Then, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes, stained for DAPI (Thermo Fisher Scientific) and CellTracker™ (Thermo Fisher Scientific), to visualize nucleus and cytosol of the cells, respectively. Images were taken with High Content Imaging System Operetta (Perkin Elmer) and analyzed through Columbus Image Data Storage and Analysis system (Perkin Elmer).

3.6. Western blot

For biochemical analysis *in vivo*, brainstem, spinal cord, and quadriceps were pulverized using pestle and mortar. For Western blotting analysis, proteins were extracted from pulverized tissues using a lysis buffer containing 2% SDS, 150 mM NaCl, 2 mM EDTA, and 10 mM Hepes (pH 7.4) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates from brainstem and spinal cord were sonicated and centrifuged at 15,000 rpm for 15 min at room temperature. Lysates from quadriceps were homogenized (homogenizer RZR 2052 control, Heidolph) at 600 rpm (20 times); passed through syringes of 18-, 22-, and 25-gauge needles; and centrifuged at 15,000 rpm for 15 min at room temperature.

For biochemical analysis *in vitro*, cells were lysed in ice-cold PBS and scraped in 100 µl of RIPA lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0,5% sodium deoxycholate, 1% NP-40), supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysate was sonicated and centrifugated at 15000 rpm for 15 minutes at 4°C.

Protein concentration was measured using the bicinchoninic acid assay method. Equal amounts of protein were subjected to 7-10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membrane (BioRad). Immunoblotting was done in 5% non-fat dry milk in Tris-buffered saline for all antibodies as follows: AR (1:1000, sc-13062, Santa Cruz), phospho-Serine 96 AR (1:500, Biomatik), β-tubulin (1:5000, ab21057, Abcam) and CDK2 (1:1000, ab32147, Abcam). Immunoreactivity was detected using IRDye secondary antibodies for Odyssey Imaging system (LI-COR Biosciences), following manufacturer's instructions.

Quantifications were done using ImageJ 1.51j8 software (National Institutes of Health).

3.7. Animals

Animal care and experimental procedures were conducted in accordance with the University of Trento ethics committee and were approved by the Italian Ministry of Health. Mice were housed in filtered cages in a temperature-controlled room with a 12-hour light/12-hour dark cycle with ad libitum access to water and food and were fed a standard diet (Mucedola

4RF21). All the mice used in this study have been previously genotyped or derived from C57BL/6J mates.

3.7.1. Generation of transgenic mice

By random insertion, we generated transgenic mice to express high levels of full length human AR with pathogenic (AR100Q) polyQ tract in a ubiquitous manner. Transgene expression was driven by the cytomegalovirus (CMV) promoter and the chicken beta-actin (pCAGGS) promoter. These transgenic lines were then backcrossed to the C57Bl6J background for more than 10 generations before subsequent analysis of phenotype and pathology. The pronuclear injection involves collecting fertilized eggs at the single cell stage. For a short window of time the pronuclei containing the genetic material from the sperm head and the egg are visible within the cytoplasm. At this stage, a linearized DNA construct is injected into the male pronuclei. The injected eggs are then transferred into the oviducts of 0,5 day post coitum (dpc) pseudo-pregnant CD1 foster mice. The injection background strain we used was C57Bl6/NCrl (Charles River).

3.7.2. Genotyping

The genotype of the mice was determined according to the following protocols. Genomic DNA was extracted from a small piece of mice's tails or ears and amplified using RED Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich). Briefly, genomic DNA was extracted by adding to the tails the Extraction and Tissue Preparation Solutions in a ratio of 4:1. The samples were incubated 10' at room temperature and 3' at 95°C to allow tissue digestion. After, an equal volume of Neutralization Solution B was added to the mix and the samples were mixed by vortexing. An aliquot of the neutralized extract is then combined with the RED Extract-N-Amp PCR Reaction Mix, already containing all the PCR reagents needed for a proper amplification. To identify the different mouse genotypes, we used the following combinations of primers:

1. TG AR100Q mice: two primers TG Forward (5'-CTTCTGGCGTGTGACCGGCG-3') and TG Reverse (5'-TGAGCTTGGCTGAATCTTCC-3') was added to the PCR reaction. PCR conditions were set at 94°C for 30'' for denaturation step, at 60°C for 1' 30'' for annealing step and at 72°C for 1' 30'' for extension step, all steps repeated for 30 cycles. The PCR products were run on a 1% agarose gel.
2. CDK2 mice: $CDK2^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were distinguished by PCR product amplifications with the following primers: 5'-CAAGTTGACGGGAGAAGTTG-3' (CDK2a), 5'-

ACGAACAGCCCTGGACCCCTC-3' (CDK2b) and 5'-GCGATAAGCTTCGAGGGACC-3' (CDK2c). CDK2a in combination with CDK2b were used to detect the WT CDK2 allele (WT PCR). CDK2a in combination with CDK2c were used to detect the knock-out allele (KO PCR). PCR conditions were set at 94°C for 30'' for denaturation step, at 60°C for 30'' for annealing step and at 72°C for 1' 30'' for extension step, all steps repeated for 35 cycles. The PCR products were run on a 1% agarose gel for KO PCR and 2% agarose gel for WT PCR.

3. SBMA knock-in mice: two primers AR113Q Forward (5'-CCACGTTGTCCCTGCTGGGCCCCAC-3') and AR113Q Reverse (5'-GACACTGCTTTACAACTCCTTGGC-3') were added to the mix to allow the discrimination between human and murine AR. PCR conditions were set at 94°C for 25'' for denaturation step, at 67°C for 30'' for annealing step and at 72°C for 30'' for extension step, all steps repeated for 27 cycles. The PCR products were run on a 2% agarose gel.

3.7.3. Rotarod test

Motor coordination was measured by rotarod analysis (Ugo Basile Instruments). TG AR100Q, TG AR100Q/CDK2$^{+/-}$, TG AR100Q/CDK2$^{-/-}$ mice received a weekly session which included three test trials at 15-30 rpm progressive speed for a maximum period of 300 seconds. A recovery time of 300 seconds was added between each test. Mice were trained the week before starting the test. The highest of recordings for each mouse was used to analyze rotarod performance. SBMA knock-in mice performed the same test but with different protocol: 15-40 rpm progressive speed for a maximum period of 300 seconds.

3.7.4. Hanging wire test

For hanging wire muscle force assessment, a simple grid was used, in which mice were allowed to grasp and immediately gently reversed. The test lasts maximum 60 seconds and we counted the seconds the mice lasted hanging on the grid. Three test trials were performed every week and the average of the recordings for each mouse was used to analyze muscle force.

3.7.5. Grip strength test

For grip strength muscle force, a grip strength meter (Ugo Basile Instruments) was used to measure forelimb grip strength. The grip strength meter was positioned horizontally, and the

mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the smooth metal triangular pull bar with their forelimbs only and then were pulled backward. The force applied to the bar at the moment the grasp was released and recorded as the peak tension. Mice received a weekly session which included three test trials, the highest of recordings for each mouse was used to analyze muscle force production.

3.7.6. Tissue collection

For tissue collection, 3 TG AR100Q, 3 TG AR100Q/CDK2$^{+/-}$ and mice 3 TG AR100Q/CDK2$^{-/-}$ mice were sacrificed at 8 weeks of age. Mice were euthanized by CO_2 inhalation. After euthanasia, tissues were flash-frozen in precooled isopentane in liquid nitrogen, then stored at -80°C until further processing.

3.8. Primary cortical cell culture and transfection

Primary cortical neurons were cultured from embryonic E15.5 C57BL/6J mice as previously described (9). In brief, the cortices were dissected out, digested in Papain solution (20 U Papain, 5mM EDTA and 30 mM Cysteine in 1x Eagles' Balanced Salt Solution (EBSS)) for 20 min at 37°C. This was followed by a DNase I treatment for 3 min. The dissociated cells were centrifuged at 1000 x g for 5 min. Supernatant was discarded and the digestion was blocked with a solution containing Soybean Trypsin inhibitor and bovine serum albumin in EBSS. Following a centrifugation of 1000 x g for 10 min the cells were plated in Neurobasal medium supplemented with B27, PenStrep (100 units), L-Glutamine (2mM) and AraC (100 µM). Half of the media was replaced with fresh media every three days. Primary cortical neurons were transfected with Lipofectamine 2000 (Thermo Fisher Scientific), according to manufacturer's protocol.

3.9. Microarray

Total RNA was extracted with TRIzol (Invitrogen).

RNA quality was analyzed by microfluidic gel electrophoresis on RNA 6000 Nano chip using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA). RIN values ranged from 7 to 9.50, indicating high quality total RNA.

Cyanine-3 (Cy3) labeled cRNA was prepared from 0.2 µg RNA using the One-Color Low Input Quick Amp Labeling Kit (p/n 5190-2331, Agilent Technologies) according to

manufacturer's instructions (Agilent Technologies, USA), followed by RNeasy column purification (QIAGEN, Valencia, CA). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, USA). 1.65 µg of Cy3-labeled cRNA (specific activity: 14.3±3.1 pmol Cy3 per µg cRNA) was fragmented at 60°C for 30 minutes in a reaction volume of 55 µl containing 1x Fragmentation Buffer and 2x GE (Gene Expression) Blocking Agent (Gene Expression Hybridization Kit, p/n 5188-5242, Agilent Technologies) following manufacturer's instructions. On completion of the fragmentation reaction, 55 µl of 2x Hi-RPM Hybridization Buffer were added to the fragmentation mixture and hybridized to Agilent Mouse GE 4x44K V2 Microarray kit (G4846A, Agilent Technologies) for 17 hours at 65°C in a rotating hybridization oven. After hybridization, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 and 1 minute at 37°C with GE Wash buffer 2 (Gene Expression Wash Buffer Kit, p/n 5188-5327 Agilent Technologies).

Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505C, Agilent Technologies) using the AgilentHD_GX_1Color Profile (Scan Area: 61x21.6 mm; Scan resolution: 5 µm, dye channel set to 100% Green PMT) of Agilent ScanControl software 8.1.3 (Agilent Technologies). The scanned images were analyzed with Feature Extraction Software 10.7.3.1 (Agilent Technologies) using default parameters (protocol GE1_107_Sep09).

Statistical analysis of the genome-wide expression data was done by Erik Dassi (CIBIO, University of Trento).

3.10. Statistical analysis

All experiments were done at least 2-3 times. The statistical analysis was performed through GraphPad Prism 7 software or Statistica 10.0 software.

4. RESULTS

4.1 AR is a transcription factor regulated by neuronal activity through serine 96 modulation

4.1.1. Stimulation of neuronal activity modifies AR phosphorylation of serine 96

AR function is a mechanism highly regulated by phosphorylation events. As described in the introduction (paragraph 1.2.2.1.2.), AR protein is resolved in a 7% SDS-PAGE as a doublet of about 110 and 112 kDa (Blok et al. 1998). The upper isoform corresponds to phosphorylated AR, while the lower isoform is the unphosphorylated AR (Polanco et al. 2016). The analysis of the expression in HEK293T cells of different phosphorylation mutants of AR shows that only the mutation at serine 96 abrogates the formation of the upper isoform of AR. The phosphodefective alanine mutant (S96A) runs as the lower 110 kDa band, instead the phosphomimetic aspartic acid mutant (S96D) runs as the upper 112 kDa band of both normal and polyQ-AR (Polanco et al. 2016). This means that phosphorylation at serine 96 of AR is necessary to form the upper isoform of about 112 kDa detectable by SDS-Page electrophoresis. Moreover, pituitary adenylyl cyclase activating polypeptide (PACAP) and forskolin treatment decreased the accumulation of the upper phosphorylated isoform through the activation of PKA/AC pathway (Polanco et al. 2016).

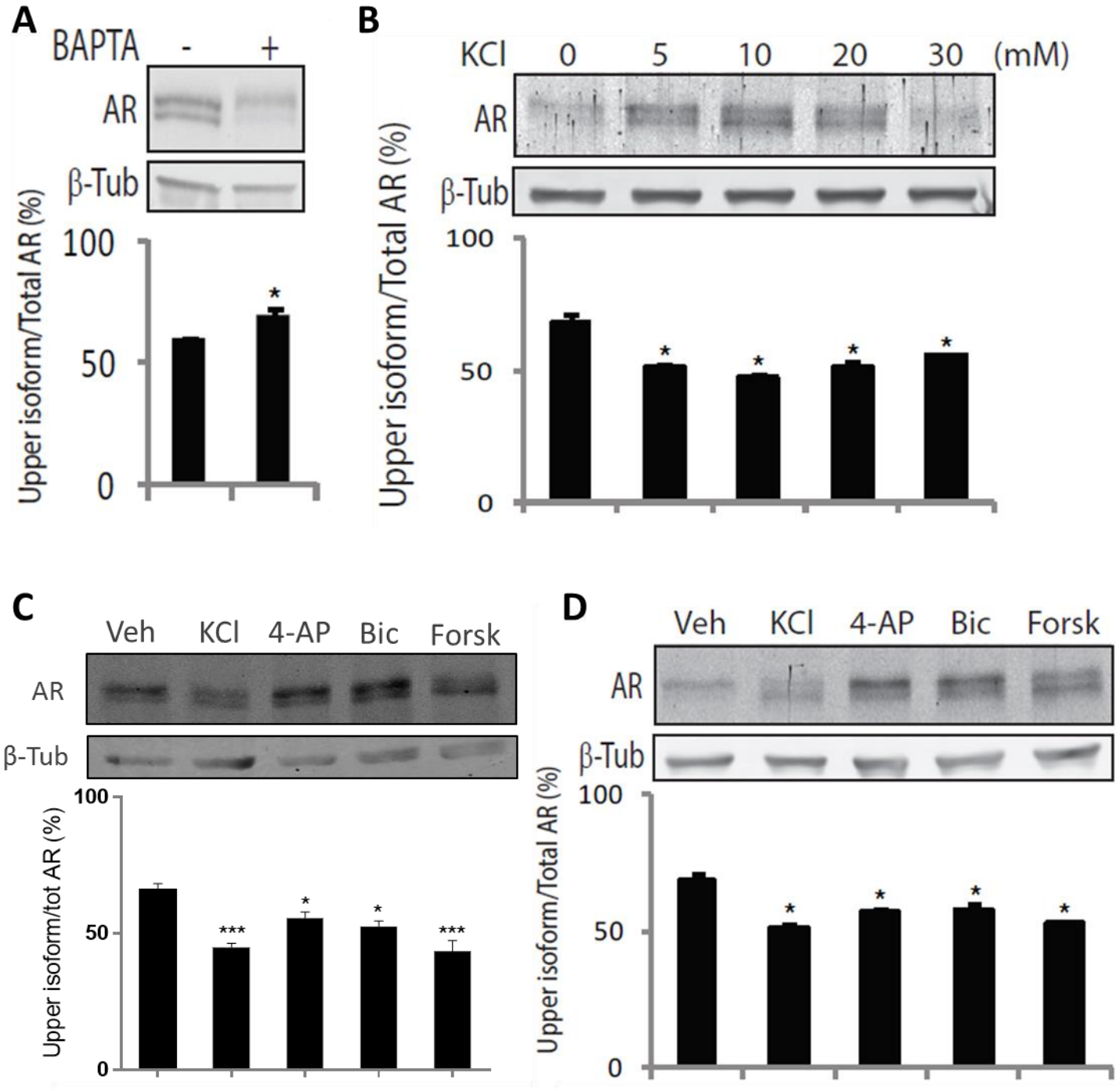


Figure 22. Phosphorylation of serine 96 is altered by different stimuli that trigger neuronal activation. A) Western blotting analysis in HEK293T cells expressing polyQ-AR and treated with the calcium chelator BAPTA. B) C) and D) Western blot analysis of primary cortical neurons overexpressing polyQ-AR and treated for 1 hour with increasing concentrations of KCl, 100 µM of 4-amynopyridine, 15 µM of bicuculline or 10 µM of forskolin. In A) t-test was performed and asterisk represents $p<0.05$ with respect to control condition. In B) C) and D) One-way ANOVA test with Tukey's post-hoc test was performed, and asterisks represent: *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ with respect to control condition. Graphs, mean ± SEM, N=2-3.

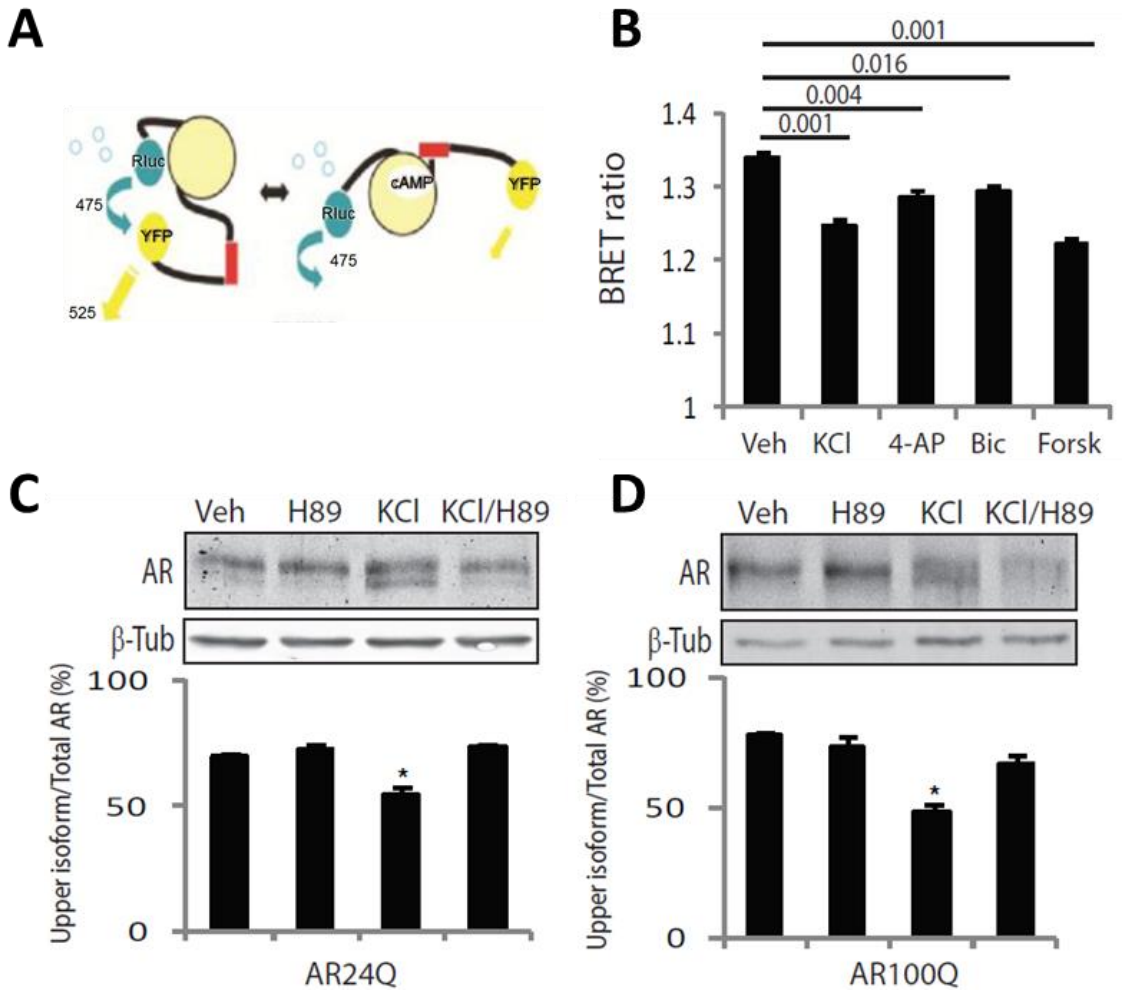
I noticed that treatment of HEK293T cells overexpressing polyQ-AR with 55 glutamines with 1, 2 – Bis (2-aminophenoxy) ethane - N, N, N′, N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) increased the accumulation of the upper phosphorylated isoform of AR (***Fig. 22A***). This experiment suggested that dephosphorylation of AR at serine 96 is calcium-dependent. Neuronal activity is a process that involves the release of calcium ions in the cytosol of neurons. Thus, I hypothesized that neuronal depolarization modifies AR phosphorylation at serine 96.

To address this hypothesis, I produced primary cortical neurons from transgenic mice overexpressing AR with 24Q and 100Q. Neuronal activity was triggered in DIV8 cultures with different stimuli. To induce membrane depolarization of all the neurons in culture I used potassium chloride (KCl) stimulation. A gradient of KCl concentrations was applied, and I checked the migration of the AR. 5-30 mM of KCl exposition for 1h was sufficient to trigger the dephosphorylation of serine 96 of expanded AR (***Fig. 22B***). I stimulated neuronal activity through the treatment of neurons with 4-aminopyridine, a non-selective voltage-dependent K^+ channel blocker, which causes an increase in neuronal conduction (Smith et al. 2009). I treated the primary cultures also with bicuculline, a competitive antagonist of $GABA_A$ receptors, known to induce seizure-like phenotype in *in vitro* models (Chang et al. 2015). These stimuli were used in order to recapitulate a more physiological trigger of neuronal activity. These treatments led to the decrease of the accumulation of the upper phosphorylated isoform of non-expanded and expanded polyQ-AR (***Fig. 22C-D***, respectively). I used forskolin treatment as positive control for the dephosphorylation of AR at serine 96 (Polanco et al. 2016). These data suggest that neuronal activity trigger the dephosphorylation of non-expanded and expanded serine 96 AR.

4.1.2. Neuronal activity-stimulated dephosphorylation of serine 96 requires active PKA

We showed that cAMP release elicits the dephosphorylation of AR at serine 96 (Polanco et al. 2016). Then, I asked whether the stimuli used to trigger neuronal activity increased the release of cAMP in our system. To test this, in collaboration with Dr. Stefano Espinoza, we used a bioluminescence resonance energy transfer (BRET) cAMP biosensor (Barak et al. 2008). The sensor is composed by a N-terminal truncated variant of the exchange protein activated by cAMP (EPAC), a *Rotylenchulus reniformis* luciferase at the N-terminus and a

yellow fluorescent protein (YFP) at the C-terminus. EPAC binds to cAMP inducing a conformational change that increase the distance between the luciferase donor protein and the YFP acceptor, resulting in a decrease in bioluminescent signal (***Fig. 23A***). We used as positive control of cAMP release 10 µM forskolin (Polanco et al. 2016). We transfected primary cortical neurons with the sensor and we treated them with KCl, 4-AP and bicuculline to induce neuronal activity and monitor the following cAMP fluctuations in real time. The applied stimuli decreased the BRET signal, thereby providing a quantitative measure of increased release of cAMP (***Fig. 23B***). Next, I tested whether PKA activation is essential for serine 96 dephosphorylation. I treated primary cortical neurons expressing AR with 24 or 100Q with 20 mM KCl, with or without 10 µM H89, a selective inhibitor of PKA. Treatment with PKA inhibitor for 1 hour blocked KCl-induced dephosphorylation of serine 96, indicating that PKA activity is downstream in the pathway involved (***Fig. 23C-D***).



Caption in the next page (p. 75).

Figure 23. **Neuronal activity-dependent dephosphorylation of serine 96 is blocked by PKA inhibition.** A) Schematic representation of the bioluminescence resonance energy transfer (BRET) cAMP biosensor used in B). B) Primary cortical neurons were transfected with the BRET cAMP biosensor and treated with the indicated compounds. C) and D) Western blotting analysis of primary cortical neurons overexpressing normal and polyQ-AR, respectively, treated for 1 hour with 20 mM KCl and 10 μM H89. One-way ANOVA test with Tukey's post-hoc test was performed and asterisks represents $p<0.05$. Graphs, mean ± SEM, N=3.

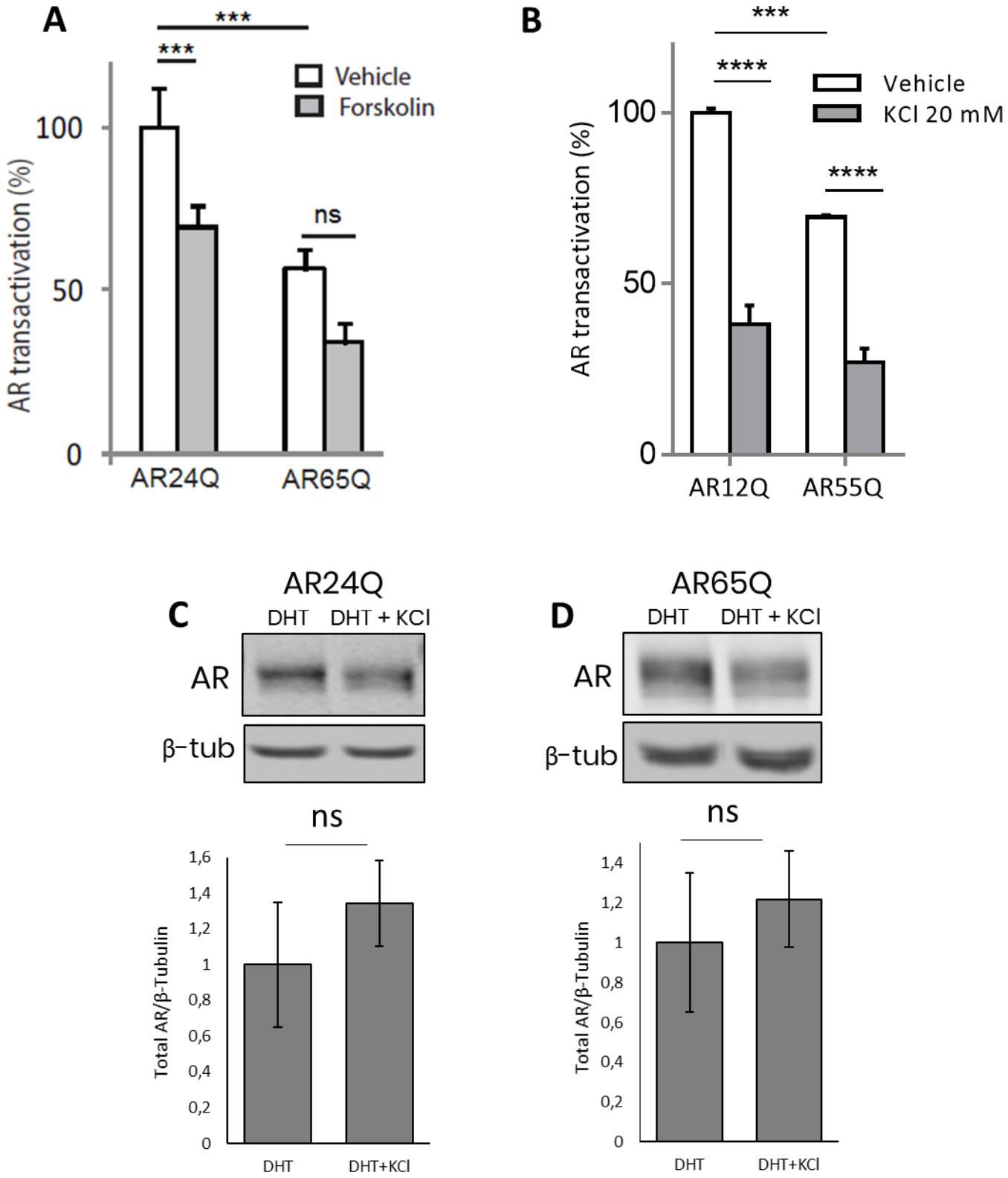
4.1.3. AR transactivation is altered by stimulation of neuronal activity

AR transcriptional activity is known to be modified by several post-translational modifications, the most abundant of which is phosphorylation (Gioeli and Paschal 2012). I asked whether modulation of serine 96 phosphorylation regulates AR transactivation. To address this question, I used *in vitro* luciferase assays. I transfected primary cortical neurons with non-expanded and expanded polyQ-AR, together with a reporter construct, in which transcription of the firefly luciferase reporter gene is controlled by a promoter recognized by AR (androgen-responsive element, ARE). In addition, I transfected primary cultures with a renilla-expressing vector, in order to normalize the firefly luciferase signal on transfection efficiency.

Transfected mouse primary cortical neurons were treated with forskolin, in order to check the transcriptional activity of AR when serine 96 was dephosphorylated (Polanco et al. 2016). Treatment with this cAMP release-activating compound decreased luciferase signal by 2,3-fold, which indicates diminished binding to ARE promoter by normal AR. PolyQ-AR responded similarly to forskolin treatment compared to normal AR, but the difference with respect to vehicle-treated cells was not significant (***Fig. 24A***).

Next, I treated neurons with and without 20 mM KCl for 6 hours, in order to appreciate differences in luciferase signal after dephosphorylation of AR at serine 96 (protocol adapted from Spiegel et al. 2014). The cultures were treated with 10 nM DHT for 24h prior to depolarization, in order to trigger AR translocation into the nucleus and AR binding to specific regions of chromatin. Indeed, AR transactivation signal was induced by 32-fold upon DHT treatment compared to the vehicle-treated neurons (data not shown). Moreover, expanded polyQ-AR transactivation was reduced by 2-fold compared to non-expanded AR

(***Fig. 24B***), as expected (Scaramuzzino et al. 2015). Stimulation of neuronal activity decreased significantly non-expanded and expanded AR transactivation (***Fig. 24B***). The results collected showed that stimuli which trigger the dephosphorylation of serine 96 are involved in non-expanded and expanded AR transcriptional activity. This level of regulation is present in depolarizing neurons overexpressing non-expanded and expanded AR.



Caption in the next page (p. 78).

***Figure 24*. Forskolin and KCl reduce normal AR transactivation.** A) and B) Primary cortical neurons were transfected with normal and polyQ-AR and treated with 10 μM forskolin, 20 mM KCl or vehicle for 6 hours. C) and D) Western blot analysis of AR protein levels in the same conditions as in A) and B). One-way ANOVA with Tukey's post-hoc test was performed in A) and B); unpaired two-tailed t-test was performed in C) and D): *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001. Graphs, mean ± SEM, N=3.

4.1.4. Transcriptional activation of AR is modulated by serine 96 phosphorylation

Taking into consideration the data obtained so far, I hypothesized that serine 96 phosphorylation and dephosphorylation change AR transcriptional activity. In order to target the question in neurons, I exploited a motor neuron-derived cell culture (MN-1 cells) previously established in our laboratory (Polanco et al. 2016; Scaramuzzino et al. 2015). These cells were transduced with viral vectors expressing AR24Q and AR100Q in which serine 96 was mutated into phospho-defective alanine (S96A). I checked AR transactivation capacity through luciferase assay. The cells were transfected with the reporter vectors, as previously described. Then, I treated the cells with 10nM DHT for 24 hours, after which luciferase assay was performed. AR transactivation was decreased by expansion of polyQ tract, as expected, but the difference compared to normal AR was not significant. The S96A AR variant exhibited decreased AR transactivation compared to WT AR24Q. AR100Q S96A showed decreased activity as well, but the difference between the mutant and the WT isoform was not significant (***Fig. 25A***). These results add confidence to the hypothesis that serine 96 phosphorylation is implicated in the regulation of AR transactivation in physiological and disease conditions.

In order to test the role of serine 96 phosphorylation in a more physiological context and to link this modulation to neuronal activity, I exploited excitable mouse primary cortical neurons. Primary cortical neurons were transfected with non-expanded and expanded polyQ-AR with S96 mutated into alanine. Then, neurons were treated with 10nM DHT for 24h, the last 6 hours with or without 20mM KCl. The substitution of serine 96 into alanine in neurons provoked AR transactivation decrease, even if the effect was less evident, confirming the previous data obtained in MN-1 cell culture (***Fig. 25B***). PolyQ-expanded AR with S96A mutation seemed to lose this level of regulation (***Fig. 25B***). The treatment with KCl reduced non-expanded AR transactivation, as previously shown. Substitution of serine 96 into alanine

prevented this effect (***Fig. 25C***). These results suggest that serine 96 phosphorylation plays a pivotal role in AR transactivation in neurons and it is influenced by neuronal activity.

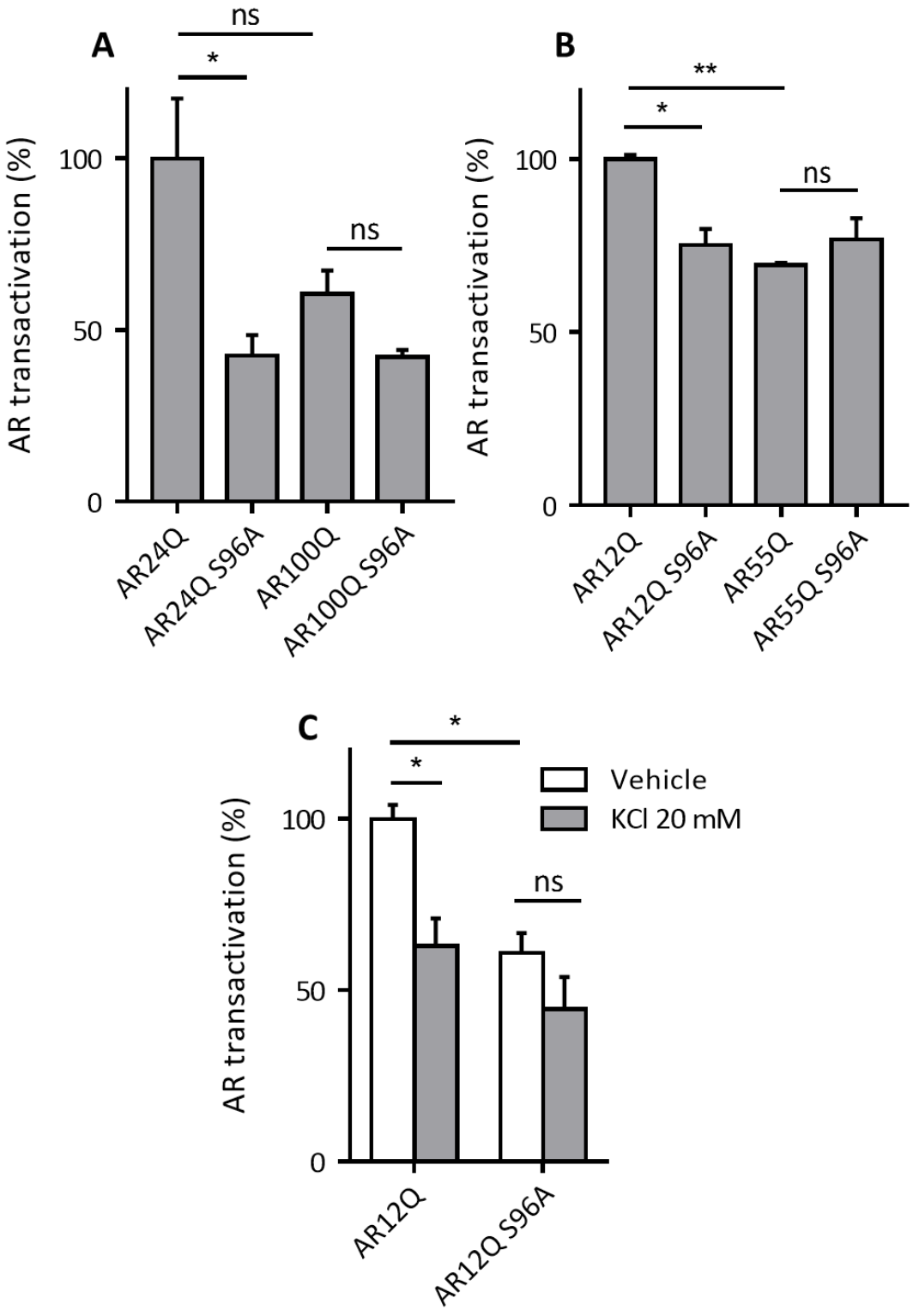


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***Figure 25*. Phospho-defective mutation at serine 96 alters AR transactivation and is resistant to KCl treatment.** A) Transcriptional assay in MN-1 cells expressing stably AR24Q and 100Q with and without S96A mutation, treated with 10 nM DHT for 24 hours. B) and C) Transcriptional assay in primary cortical neurons transfected with AR12Q and 55Q with or without S96A mutant, treated with 10 nM DHT for 24 hours and 20 mM KCl for 6 hours, where indicated. In A) and B) One-way ANOVA with Tukey's post-hoc test was performed, *=p<0.05 and **=p<0.01. In C) two-way ANOVA test with Sidak's test for multiple comparisons were performed. Graphs, mean ± SEM, N=3-4. The reported experiments are representative of two-three independent experiments.

4.1.5. Microarray analysis of stimulated AR-expressing neurons reveals an androgen-regulated gene expression program

The results described above show that AR transactivation is influenced by serine 96 phosphorylation, which is modified by neuronal activity. I hypothesized that this has an important regulatory role in mature neurons. To address this hypothesis, I analyzed the transcriptional profile of stimulated mature neurons expressing AR and treated with either vehicle or DHT, to compare gene expression profile in cells in which AR is inactive to cells in which AR activity is induced by androgens. I cultured primary cortical neurons derived from a transgenic mouse model in which AR with 24 glutamines was overexpressed. I used non-expanded AR to mimic the physiological activity of AR in cultured neurons. At DIV8, mature neurons were treated for 6 hours with and without 10 nM DHT, and for 1 or 6 hours with or without 20 mM KCl and we performed microarray analysis. The final conditions were: vehicle, DHT, KCl 1h, DHT-KCl 1h, KCl 6h and DHT-KCl 6h (***Fig. 26A***).

Analysis is represented by the visualization of the number of differentially expressed genes (DEGs) derived from the comparison between the conditions (***Fig. 26B***). DHT treatment, compared to vehicle, altered the expression of 5 genes, i.e. neural epidermal growth factor-like like protein 2 (*NELL2*), chromosome transmission fidelity factor 8 (*CHTF8*), calcium binding protein 5 (*Cbp5*), phosphatidylinositol glycan anchor biosynthesis class T (*Pigt*) and prolin-rich 7 *(Prr7*). Treatment with KCl for 1 hour induced the downregulation of 80 genes and the upregulation of 168 genes. As expected, KCl treatment was sufficient to stimulate the expression of several immediate early genes (IEGs), including *Fos*, *Fosb*, *Egr4*, *Jun*, *Klf4*, *Myc*, *Npas4*, *NR4A1*, *NR4A2*, *NR4A3*, which were previously shown to be transcribed upon neuronal activation (Ataman et al. 2016). Upon 6 hours of KCl treatment, IEGs induced the transcription of late responsive genes (LRGs), which are specific for neuronal subtypes (Hill

and Treisman 1999). First, I conducted an unbiased analysis looking at gene ontology enrichment using GOrilla tool (Gene Ontology enRIchment anaLysis and visuaLizAtion tool, Eden et al. 2009). The complete gene ontology terms enrichments for the described list of genes are reported in ***Table 2*** (Appendix). I took into consideration the lists of both up- and down-regulated genes in the different compared conditions. After 6 hours of KCl treatment the terms which resulted to be more enriched were related to the "activation of transcription by RNA polymerase II" and the positive regulation of cellular processes in general, including RNA biosynthetic process, macromolecule biosynthetic process and nitrogen compound metabolic process. Among the list, we noticed the presence of "signal transduction" processes with "regulation of protein phosphorylation", in which the most specific terms were "regulation of MAPK cascade" and "regulation of ERK1 and ERK2 cascade", a typical signaling pathway activated by neuronal stimulation (Bading et al. 1993; Ghosh and Greenberg 1995). Indeed, the gene ontology analysis of cell components revealed the enrichment of the term "nucleus".

An unexpected result is the absence of DEGs derived from the comparison between DHT-KCl treatments, at both 1 and 6 hours, and KCl alone. I noticed that the lists of DEGs results from the comparison between KCl versus vehicle and DHT-KCl versus DHT-treated neurons at 1 and 6 hours were different, and I decided to take into consideration these lists in order to decipher the role of AR activation in stimulated neurons. I excluded all the DEGs which were present in both lists, and I selected as DHT-induced genes only the DEGs that were present exclusively in the DHT-KCl versus DHT-treated neurons DEG list. Summary Venn diagrams of the comparisons between the described conditions above are reported in ***Figure 27***. Gene ontology enrichment analysis was carried out on both up- and down-regulated genes, derived from the described selection process. As expected, AR activation induced the "regulation of transcription from RNA polymerase II promoter" term enrichment, which is the global regulation of transcriptional activity in cells. I noticed the enrichment of "regulation of cyclin-dependent protein serine/threonine kinase activity" term. The genes related to this enrichment are *Cdkn1a* (cyclin-dependent kinase inhibitor 1a, also known as $p21^{CIP1}$), *Cdkn1b* (cyclin-dependent kinase inhibitor 1b, or $p27^{KIP1}$), *Ccnd3* (cyclin d3), and *Ccnd2* (cyclin d2). The presence of the two CDK inhibitors and *Cdc14b* (cell division cycle 14b) gene led to the enrichment of "mitotic cell cycle arrest". Surprisingly, AR activation provokes the "regulation of nervous system development", in which the subordinate enriched classes are "regulation of neurogenesis" and "regulation of neuron differentiation". Another

specific enrichment term in stimulated neurons in which AR was active was “negative regulation of neuron death”, in accordance with the protective neuronal effect of androgens (see introduction, paragraph 1.3.3.) (Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008)(Pike et al. 2008).

Taken together, these observations suggest that AR may play an unprecedented role in regulating long lasting outcomes in AR-expressing neurons due to neuronal firing.

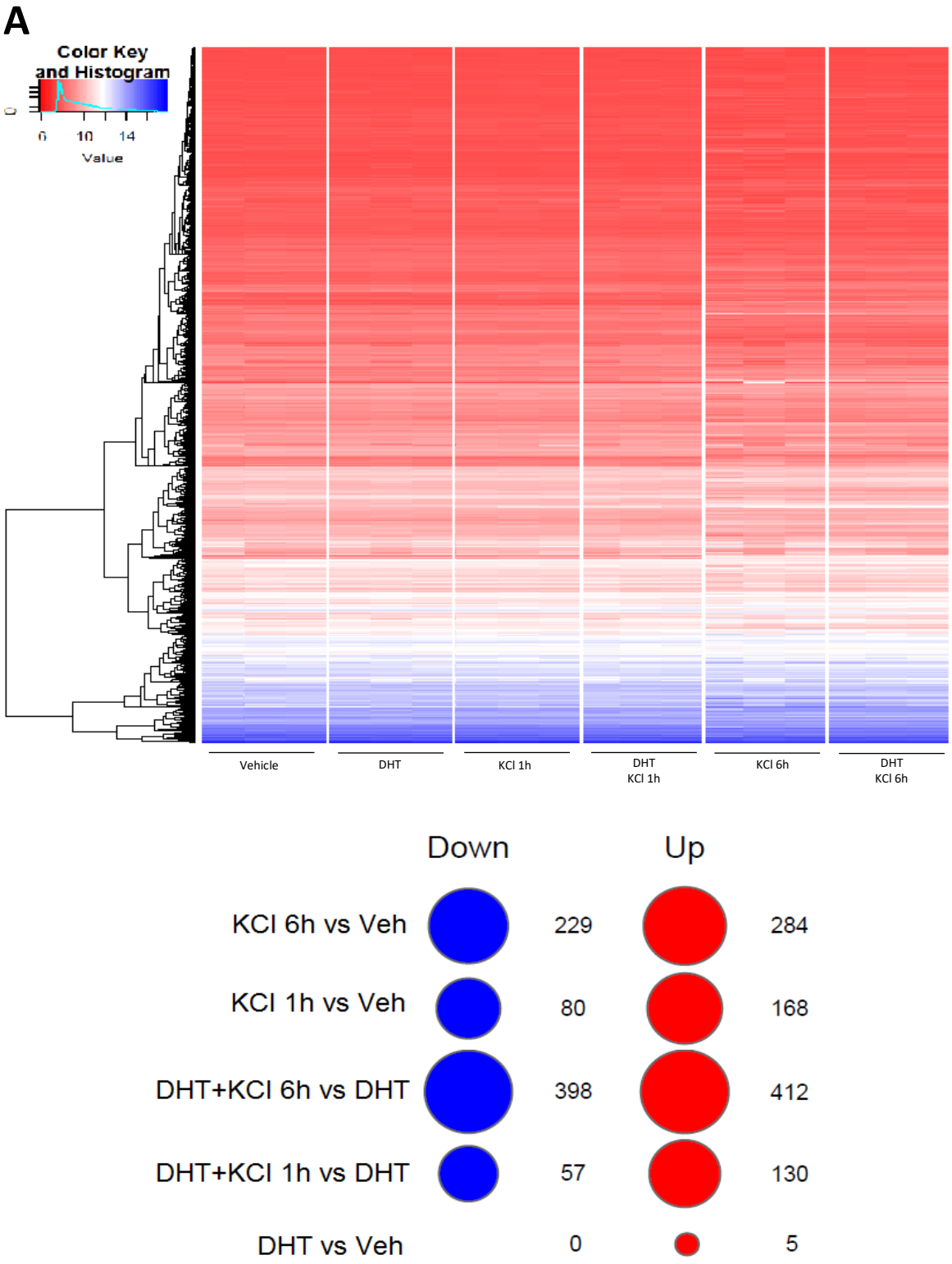

***Figure 26*. Microarray analysis of stimulated mature neurons.** A) Heatmap representing the up- (red) and down-regulated (blue) genes in mature neurons treated with 10 nM DHT for 6 hours and 20 mM KCl for 1 and 6 hours. B) Schematic representation of the differentially expressed genes (DEGs) derived from the comparisons between the indicated conditions.

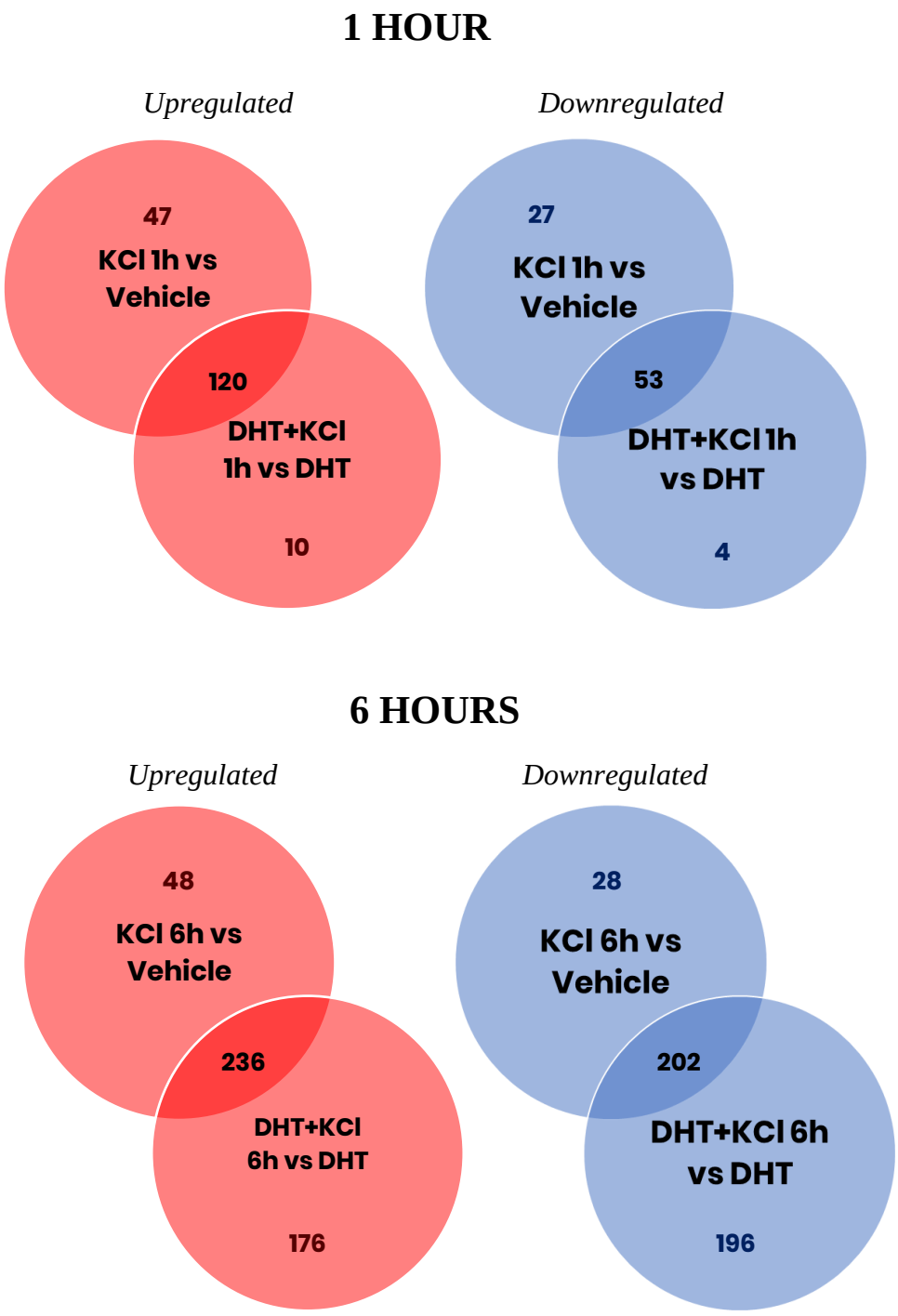

Figure 27. **Venn diagrams representing the number of DEG resulted between the comparisons of the indicated conditions.** The comparisons between DEGs derived from DHT-treated with vehicle-treated neurons at different time points revealed that only a fraction of genes is specifically regulated by AR activation. Upregulated genes are representing in red Venn diagrams, downregulated genes in blue. Numbers represent the cohorts of genes inside the different groups.

4.2. CDK2 is a novel modifier of SBMA pathogenesis

4.2.1. Both deletion and haploinsufficiency of CDK2 decrease mutant polyQ-AR level and serine 96 phosphorylation in an SBMA mouse model

CDK2 was recently characterized by our group as a novel kinase for non-expanded and expanded AR at serine 96 (Polanco et al. 2016). Serine 96 phosphorylation in SBMA knock-in mice was decreased by the administration of an analog of pituitary adenylate cyclase activating polypeptide (PACAP). This decrease correlated with an amelioration of motor phenotype and increment of lifespan (Polanco et al. 2016).

In order to decipher the role of CDK2 *in vivo* in SBMA pathogenesis, I crossed a mouse model in which one allele of CDK2 gene was deleted ($CDK2^{+/-}$, Ortega et al. 2003) with SBMA transgenic mice, a model generated and analyzed in the last years in our laboratory (TG AR100Q mice, Chivet et al., manuscript in preparation). $CDK2^{-/-}$ mice are generated by crossing heterozygous CDK2 mice. They are viable, but the frequency was slightly below the Mendelian ratio, suggesting prenatal lethality at low penetrance (Berthet et al. 2003). $CDK2^{-/-}$ mice do not demonstrate any sign of neurodegeneration or muscular defects.

I generated SBMA transgenic mice with haploinsuffiency and deletion of CDK2, in order to determine whether CDK2 plays a major role in SBMA pathogenesis, monitoring the effect on phenotype. I crossed TG AR100Q mice with the heterozygous CDK2 mouse model, obtaining SBMA mice with haploinsufficiency of CDK2 (TG AR100Q/$CDK2^{+/-}$). Then, I crossed the positive F1 generation with heterozygous CDK2 mice to obtain SBMA mice with CDK2 knockout (TG AR100Q/$CDK2^{-/-}$).

First of all, I validated the haploinsufficiency and the deletion of CDK2 in spinal cord. CDK2 levels correlated with the number of alleles containing CDK2 gene (***Fig. 28A***). Next, I assessed the impact of CDK2 on AR levels in disease-related tissues, i. e. brainstem and spinal cord. I noticed that the CDK2 deletion reduces AR protein levels by 1,36- and 1,25-fold in these two tissues, respectively (***Fig. 28B-C, spinal cord and brainstem, respectively***).

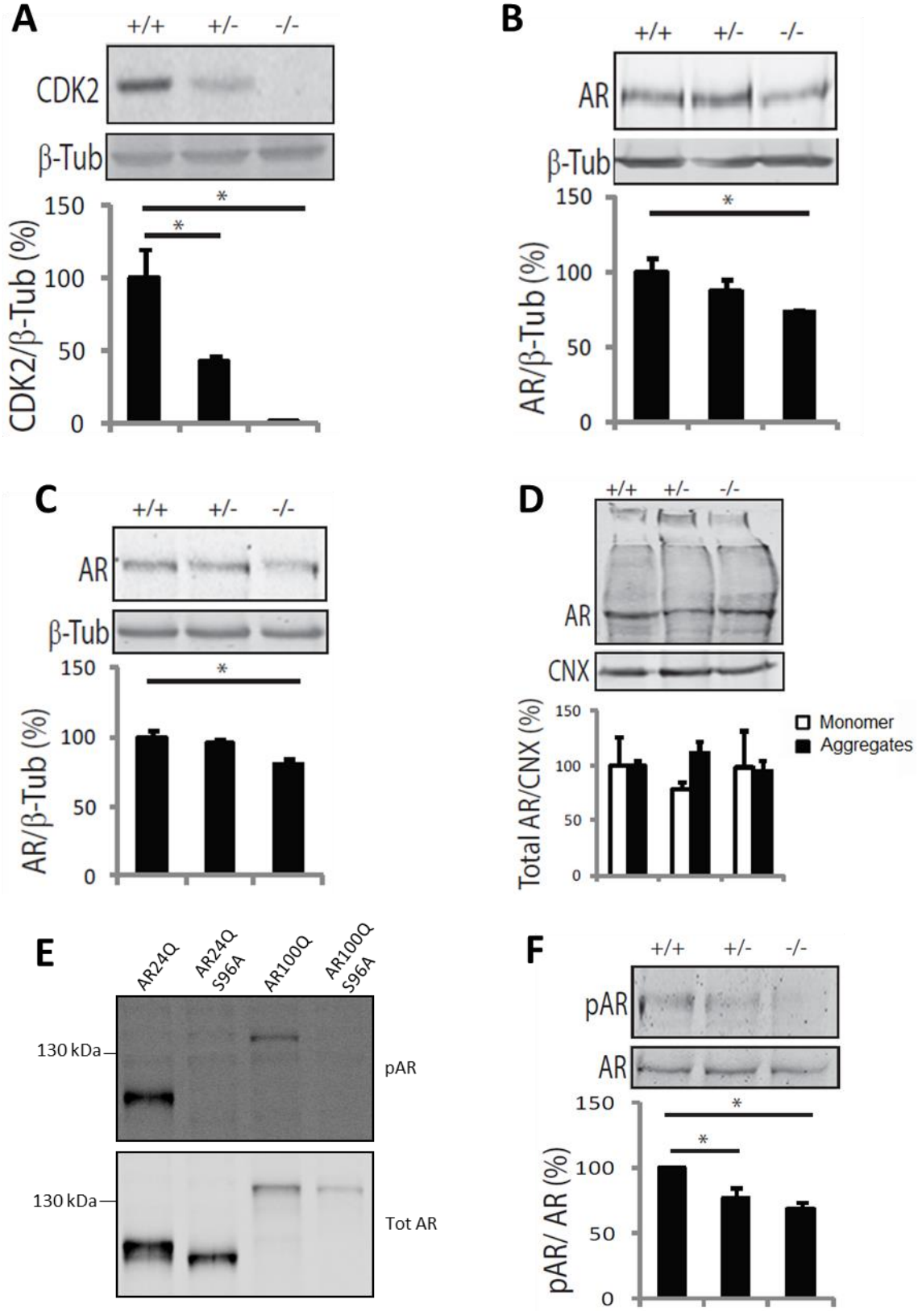

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Figure 28. **CDK2 heterozygosity or deletion in SBMA transgenic mice decrease AR levels and phosphorylation of serine 96.** A) Western blotting analysis of CDK2 protein levels in the spinal cord of $CDK2^{+/-}$ and $^{-/-}$ mice. B-C) Western blotting analysis of polyQ-AR in spinal cord and brainstem, respectively, of TG AR100Q mice with CDK2 heterozygosity and deletion. D) Western blotting analysis of poly-Q AR monomer and aggregates in quadriceps of the indicated mouse genotypes. E) Western blotting analysis of phosphorylated serine 96 AR and total AR of MN-1 cells stably expressing AR24Q and AR100Q with and without alanine substitution at serine 96. F) Western blot analysis of serine 96 phosphorylation of polyQ-AR in brainstem of the indicated mouse genotypes. One-way ANOVA test with Tukey's post-hoc test was performed, and asterisks correspond to $p<0.05$. Graphs, mean ± SEM, N=2-3.

Additionally, I assessed AR levels in skeletal muscle, i. e. quadriceps. This tissue is a primary target of the disease (Sambataro and Pennuto 2012). Indeed, it is characterized by atrophy, related to the deposition of polyQ-AR aggregates. Aggregates are easily detectable in the stacking part of SDS-PAGE gel, yet they are insoluble in 2% SDS lysis buffer. I analyzed polyQ-AR monomeric and aggregated species, and I found that CDK2 haploinsufficiency and deletion did not alter polyQ-AR aggregation and total protein levels in this tissue (***Fig. 28D***).

Next, I asked whether reduced CDK2 modifies serine 96 phosphorylation. I performed western blotting analysis with a specific phosphorylated serine 96 polyclonal antibody. To test the specificity of the phosphorylation antibody, I performed western blot analysis of motor neuron-derived cells (MN1) that stably express AR24Q and AR100Q, with and without alanine substitution of serine 96. The phospho-specific antibody against serine 96 AR recognized the non-mutated AR, but not the phospho-defective serine 96 AR (***Fig. 28E***). The haploinsufficiency and deletion of CDK2 decreased the level of phosphorylated serine 96 compared to TG AR100Q mice with physiological CDK2 levels in brainstem (***Fig. 28F***).

These results are consistent with the model whereby AR turnover is regulated by CDK2-dependent serine 96 phosphorylation, with decreased protein stability upon serine 96 dephosphorylation (Polanco et al. 2016).

4.2.2. Loss of CDK2 function ameliorates the phenotype of SBMA transgenic mice

Based on the results described above, I hypothesized that a decrease in polyQ-AR levels could have an impact on phenotype of SBMA mice. Therefore, I challenged the mice with three behavioral tasks for motor phenotype. First, I assessed motor coordination with rotarod test. The mice were monitored starting from 6 weeks of age through the death of the animals. The loss of motor coordination of TG AR100Q mice starts at 8 weeks of age. CDK2 haploinsufficiency in SBMA mice delayed this deficit, at 8 weeks, 9 weeks and 10 weeks of age (***Fig. 29A***).

Starting from the eleventh week of age, the difference between WT and TG AR100Q/CDK2$^{+/-}$ mice becomes significant. CDK2 deletion in SBMA mice resulted in an additional amelioration of loss of motor coordination. TG AR100Q/CDK2$^{-/-}$ mice performance is similar to that of WT mice in rotarod test until 11 weeks of age. From 12 weeks of age, the motor coordination of TG AR100Q/CDK2$^{-/-}$ degenerates (***Fig. 29A***). In summary, rotarod deficits were delayed by CDK2 haploinsufficiency and deletion in SBMA mice, with a greater improvement decreasing CDK2 levels.

I assessed muscle strength by hanging wire test. TG AR100Q mice demonstrate muscle deficits starting from 10 weeks of age. The muscle phenotype was ameliorated by CDK2 haploinsufficiency and deletion. TG AR100Q/CDK2$^{+/-}$ and $^{-/-}$ mice started the muscle force decline at week 12 (***Fig. 29B***). I applied another behavior paradigm for muscle force assessment, the grip strength test. The muscle force of TG AR100Q at 6 weeks of age was decreased compared to WT. This muscular impairment was rescued by haploinsufficiency and deletion of CDK2 in SBMA mice (***Fig. 29C***).

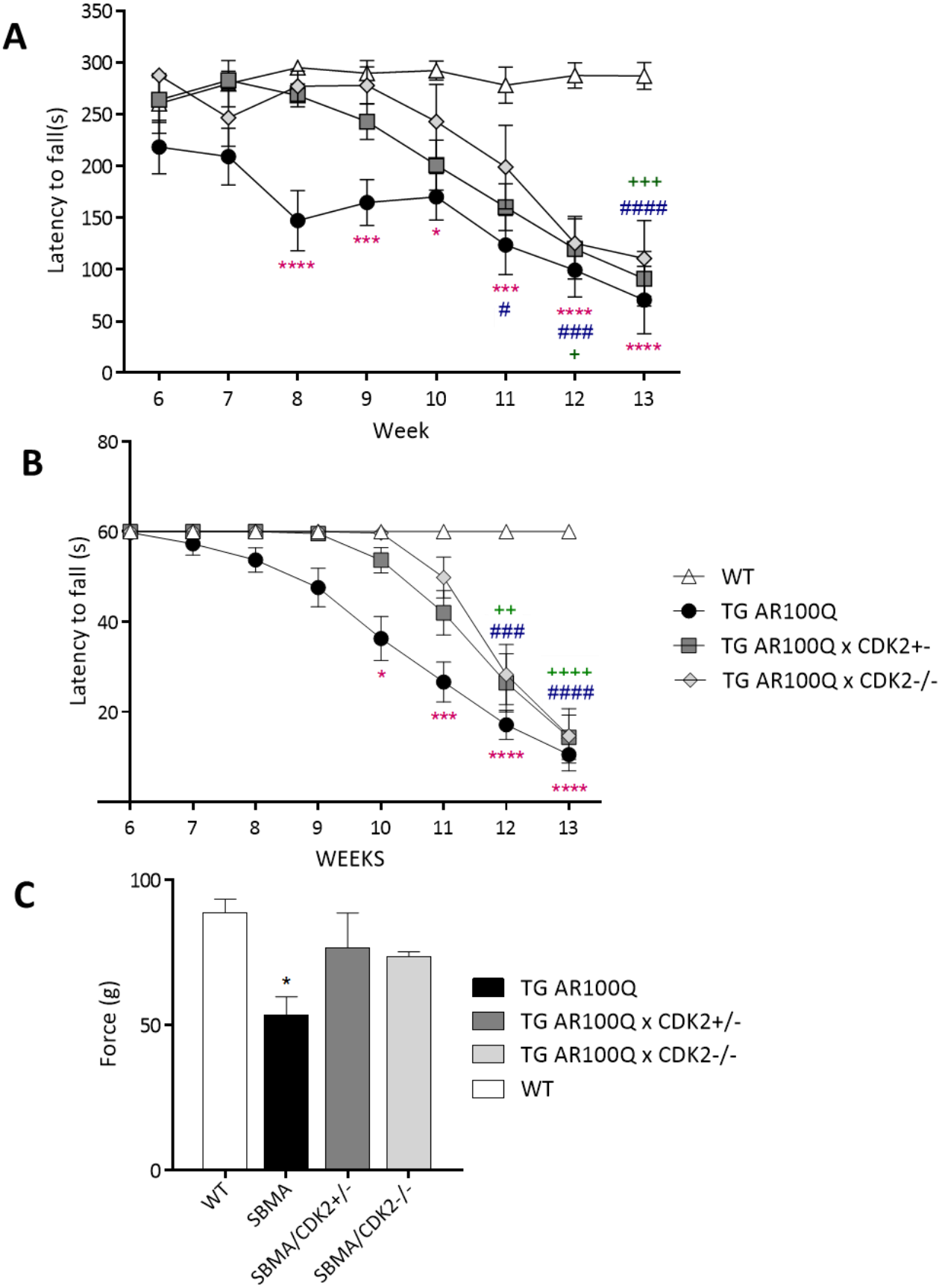

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***Figure 29*. CDK2 heterozygosity and knock-out ameliorate SBMA phenotype in mice.** A) Rotarod analysis of motor coordination of TG AR100Q mice with haploinsufficiency and deletion of CDK2. WT, N=7; TG AR100Q, N=13; TG AR100Q x $CDK2^{+/-}$, N=13; TG AR100Q x $CDK2^{-/-}$, N=7. B) Hanging wire analysis of muscle force of the indicated mouse genotypes. N as in B) except for TG AR100Q, N=24. C) Grip strength analysis of muscle force of the indicated mouse genotypes. N=3 for all the genotypes. In A) and B) One-way ANOVA test with Tukey's post hoc test was performed. One symbol, $p<0.05$; two symbols, $p<0.01$; three symbols, $p<0.001$; four symbols $p<0.0001$. * TG AR100Q vs WT, # TG AR100Q x $CDK2^{+/-}$ vs WT and + TG AR100Q x $CDK2^{-/-}$ vs WT.

4.2.3. CDK2 deletion in SBMA mice modifies body weight but not weight increase compared to SBMA mice

TG AR100Q mice are characterized by a lower body weight compared to WT, starting from 6 weeks of age (***Fig. 30A***). I analyzed the weight increase of TG AR100Q mice compared to WT, fixing as reference value the body weight at 6 weeks of age. TG AR100Q mice gain weight slowly compared to WT. They gain weight until week 9, then they gradually lose weight until their death (***Fig. 30B***). I monitored the body weight of TG AR100Q/$CDK2^{+/-}$ and $^{-/-}$. TG AR100Q/$CDK2^{+/-}$ weigh comparably to TG100Q during time. TG AR100Q/$CDK2^{-/-}$ mice appear smaller than TG AR100Q and TG AR100Q/$CDK2^{+/-}$ mice, from 6 weeks of age onwards (***Fig. 31A***). We noticed that all the three transgenic genotypes followed the same curve of gain and loss of body weight. Interestingly, the weight increase of TG AR100Q was different compared to WT mice from 9 weeks of age onwards. Instead, the weight increase of TG AR100Q/$CDK2^{+/-}$ and $^{-/-}$ was significantly different compared to WT mice from 10 weeks of age onwards (***Fig. 31B***).

4.2.4. Haploinsufficiency or deletion of CDK2 did not extend the lifespan of SBMA mice

I monitored the lifespan of transgenic mice with CDK2 haploinsufficiency and deletion. TG100Q mice have a severe phenotype characterized by loss of body weight, muscle weakness, which appear around two months of age, with kyphosis and partial paralysis in the late stage of disease. The loss of 20% of total body weight was set as criterion for humane

endpoint. The median survival of TG100Q was 13 weeks. CDK2 haploinsufficiency and deletion did not alter the median survival of SBMA mice (***Fig. 32A***).

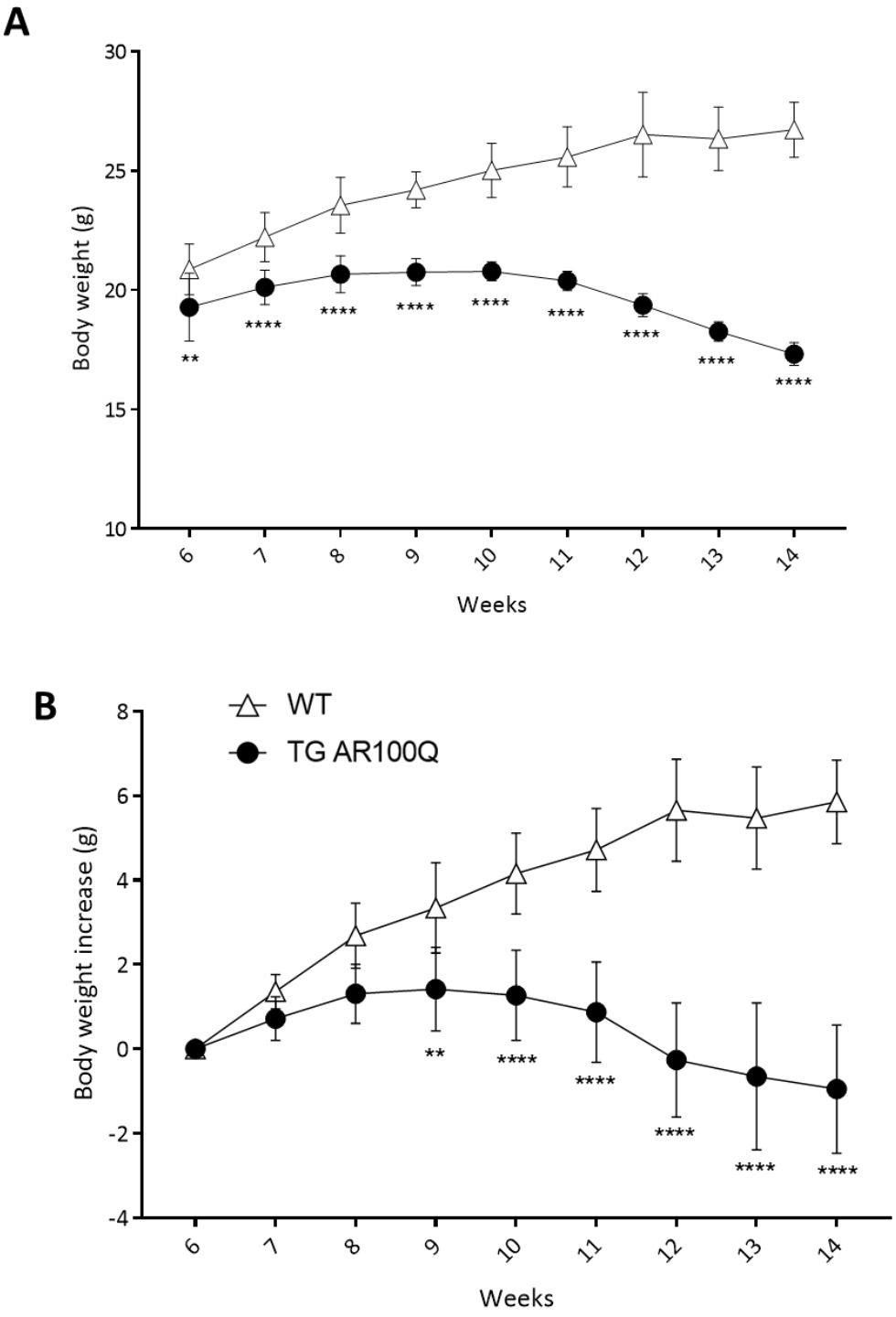

Figure 30. **Body weight and body weight increase of TG AR100Q is lower compared to WT.** A) and B) Body weight and weight increase analysis of WT versus TG AR100Q mice. Two-way ANOVA test with Sidak's test for multiple comparisons was performed. **, p<0.01; ***, p<0.001; **** p<0.0001. WT, N=7; TG AR100Q, N=13. Graphs, mean ± SD.

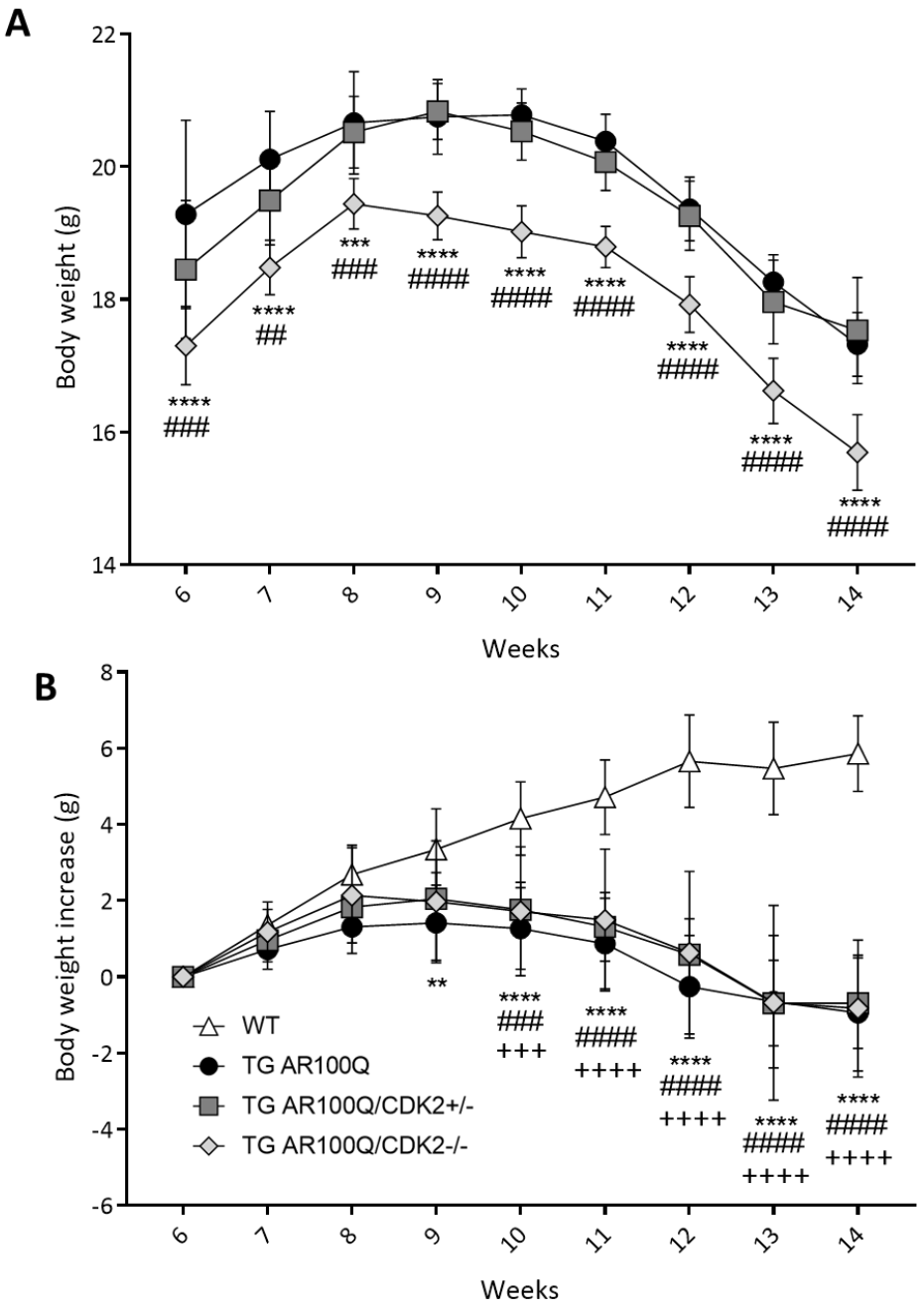

Figure 31. **Body weight of TG AR100Q, but not the weight increase, is altered by CDK2 haploinsufficiency and deletion.** A) Body weight analysis of the three transgenic mouse lines. Two-way ANOVA test was performed. B) Body weight increase analysis of the three transgenic mouse lines versus WT mice. Two-way ANOVA with Sidak's test for multiple comparisons test was performed. One symbol, $p<0.05$; two symbols, $p<0.01$; three symbols, $p<0.001$; four symbols $p<0.0001$. * TG AR100Q vs WT, # TG AR100Q x CDK2$^{+/-}$ vs WT and + TG AR100Q x CDK2$^{-/-}$ vs WT, in B). In A), * TG AR100Q/CDK2$^{-/-}$ vs TG100Q, # TG AR100Q/CDK2$^{-/-}$ vs TG AR100Q/CDK2$^{+/-}$. WT, N=7; TG AR100Q, N=13; TG AR100Q x CDK2$^{+/-}$, N=13; TG AR100Q x CDK2$^{-/-}$, N=7. Graphs, mean ± SD.

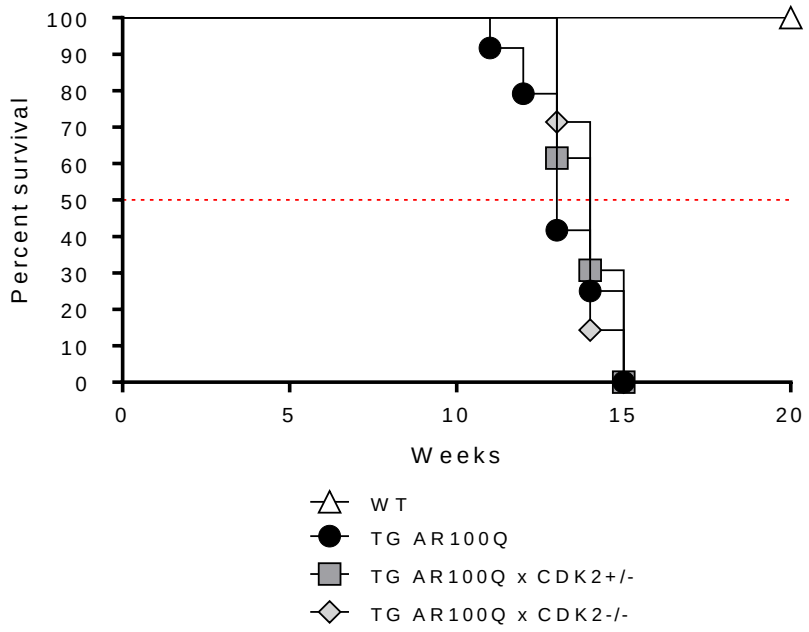

***Figure 32*. Survival of SBMA x CDK2+/- *and* -/- mice is *not* improved compared to SBMA mice.** Kaplan-Meier analysis of survival of TG AR100Q with haploinsufficiency and deletion of CDK2. WT, N=7; TG AR100Q, N=24; TG AR100Q x CDK2+/-, N=13; TG AR100Q x CDK2-/-, N=7. Gehan-Breslow-Wilcoxon test was performed.

4.2.5. CDK2 haploinsufficiency and deletion in knock-in SBMA mouse model do not alter survival and rotarod performance

In addition to transgenic mice, we crossed knock-in SBMA mouse model (AR113Q, Yu et al. 2006) with CDK2$^{+/-}$ mice, as previously described, in order to obtain AR113Q/CDK2$^{+/-}$ and $^{-/-}$ mice. The original colony of AR113Q mice had a median survival of around 4 months. In Polanco et al. 2016, we used AR113Q mice for the preclinical trial and the median survival increased by about 10 weeks, reaching 6 months. This phenomenon is probably due to the gradual restriction of polyQ tract seen in these mice. I monitored the survival of AR113Q line for this experiment and I found that the median survival reached 41 weeks, i. e. more than 10 months (***Fig. 33A***). AR113Q/CDK2$^{+/-}$ and $^{-/-}$ showed a median survival of 45 weeks, but the difference between AR113Q and AR113Q/CDK2 mice survival curves did not reach statistical significance (***Fig. 33B***).

We monitored motor coordination through rotarod behavioral test. CDK2 haploinsufficiency and deletion did not alter AR113Q mouse performance starting from 20 weeks until 1 year of age (***Fig. 33C***).

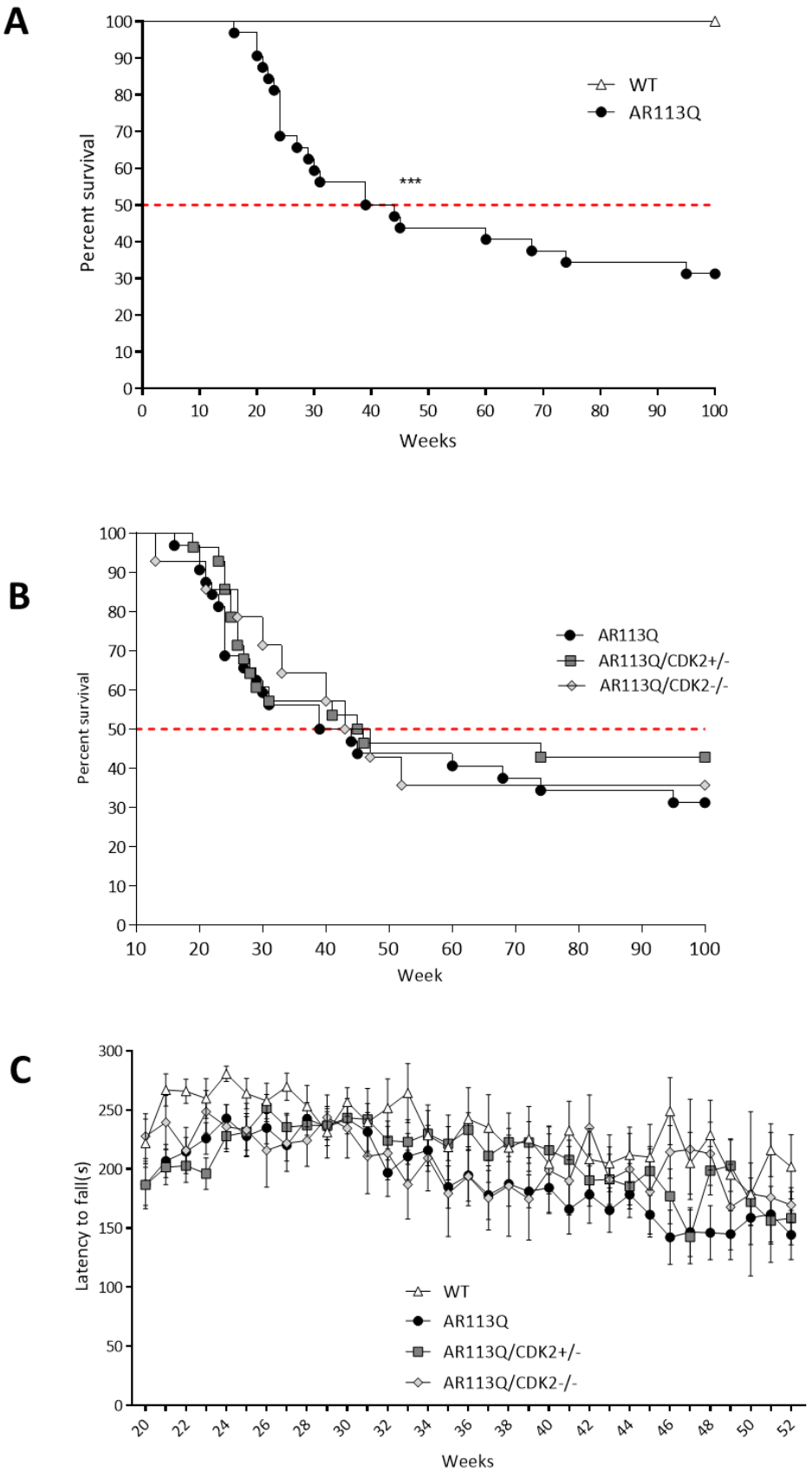

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***Figure 33*. Lifespan and motor coordination of knock-in SBMA mouse model are not perturbed by CDK2 heterozygosity and deletion.** A) Kaplan-Meier analysis of survival of AR113Q versus WT mice. WT=12 and AR113Q=32. B) Kaplan-Meier analysis of survival of the three knock-in mouse lines. AR113Q=32, AR113Q/CDK2+/-=28, AR113Q/CDK2-/-=14. C) Rotarod analysis of motor coordination of WT and the three knock-in mouse lines. WT=6-11, AR113Q=13-27, AR113Q/CDK2+/-=9-28 and AR113Q/CDK2-/-=2-9. Gehan-Breslow-Wilcoxon test was performed in A) and B), and *** is $p<0.001$. Two-way ANOVA test was performed in C).

4.3. CDC25 modulation modifies polyQ-AR toxicity, serine 96 phosphorylation and transcriptional activity

4.3.1. High-throughput screening of phosphatase and kinase inhibitors reveals CDC25 as a modifier of AR shuttling

Toxicity of mutant polyQ-AR is elicited by its nuclear translocation (Montie et al. 2009)(Nedelsky et al. 2010). AR shuttling is regulated by several molecular pathways that involve modulation of AR phosphorylation state (Gioeli and Paschal 2012). For this reason, I performed a high-throughput (HT) screening of phosphatase and kinase inhibitors, monitoring AR shuttling in order to find compounds that are potentially protective. I generated a HeLa cell line sub-clone that stably expresses poly-Q AR fused with GFP (AR65Q-GFP cells, ***Fig. 34A***). Positive AR-GFP expressing cells were selected after imaging via the Operetta® High Content Imaging System software (***Fig. 34B***). We validated the robustness and reproducibility of the assay treating cells plated in a 384-well plate with DHT (***Fig. 34A and C***). AR shuttling index was expressed as the average ratio between GFP intensity in the nucleus versus GFP intensity in the cytosol of the positive cell. DHT treatment increased AR nuclear translocation by two-fold compared to vehicle-treated cells (***Fig. 34D***). The most representative tool to evaluate the quality of a HT screening is Z-factor. A value between 0.5 and 1 is considered as excellent assay (Zhang 1999). The HT assay I performed resulted in a Z-factor of 0.502. Another parameter designed to assess robustness of HT assays is the strictly standardized mean difference (SSMD) (Zhang et al. 2007). An excellent assay with an extremely strong positive control has a SSMD>7. The presented HT assay resulted in a SSMD equal to 7,144. These parameters based on the preliminary set-up prove that the assay presents a signal window between positive and negative controls which is large enough to detect significant differences derived from inhibitor treatments (***Fig. 34E***).

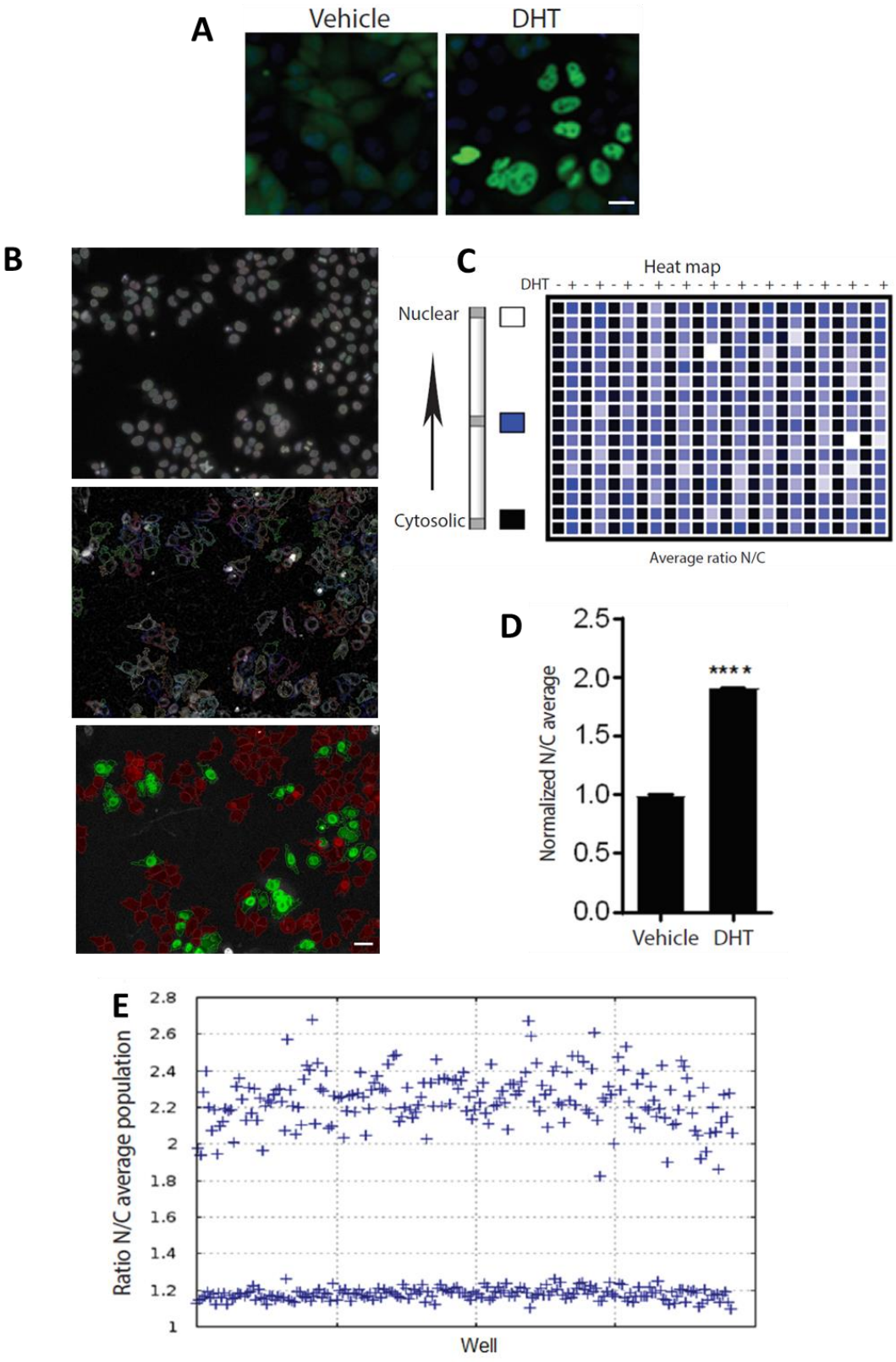

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***Figure 34*. High-throughput screening set-up for the identification of phosphatases and kinases that alter AR nuclear shuttling.** A) HeLa cell line stably expressing GFP-tagged polyQ-AR treated for 4 hours with vehicle or 10 nM DHT. Scale bar, 25 μm. B) Operetta® High Content Imaging System software analysis of the described cells: identification of nuclei, identification of cytosol and selection of polyQ-AR positive cells. Scale bar, 40 μm. C) Preliminary high-throughput screening set-up. Cells were treated with vehicle and 10 nM DHT for 4 hours. D) Normalized quantification of nuclear versus cytosolic (N/C) GFP intensity of positive polyQ-AR subpopulation in the preliminary high-throughput screening. Two-tail t-test was performed and asterisks represent $p<0.0001$. E) N/C ratio of the average population of single wells.

Next, I screened a library of 33 phosphatase and 273 kinase inhibitors on polyQ-AR nuclear translocation (***Table 3***, list of compounds). I treated the cells with 10 μM kinase and phosphatase inhibitors for 5 hours. One hour later, I added 10 nM of DHT or EtOH to the cells for 4 hours in total. From the evaluation of the standard deviations which separates the mean of the positive control from the mean of the different treatments, I selected as potential hits of the screening 6 phosphatase and 17 kinase inhibitors. These compounds decreased or increased the nuclear localization of polyQ-AR. We focused our attention on phosphatase inhibitors.

The phosphatase inhibitors that decreased nuclear localization of polyQ-AR with DHT treatment were NSC-95397, sanguinarine chloride, 9,10-phenanthrenequinone, NSC-663284, and shikonin (***Fig. 35A***). The target phosphatases of these compounds are cell division cycle 25 (Cdc25), protein phosphatase 2C (PP2C), CD45 tyrosine phosphatase, Cdc25 and PTEN, respectively. Importantly, the compound which increased nuclear localization of polyQ-AR was gossypol, a new selective inhibitor of calcineurin (***Fig. 35B***). The images which recapitulate the effect of the treatments are reported in ***figure 35C***.

I noticed that two phosphatase inhibitors out of 6 target Cdc25, a known activator of CDKs. These inhibitors are NSC-663284 and NSC-95397. Considering these facts, I decided to analyze further effects of these two compounds on AR biology.

Moreover, calcineurin could be a possible player in neuronal activity-dependent dephosphorylation of AR at serine 96, because its regulation by calcium signaling. In this dissertation, I focused on the straightforward effect of Cdc25 on the already characterized pathway involving CDK2.

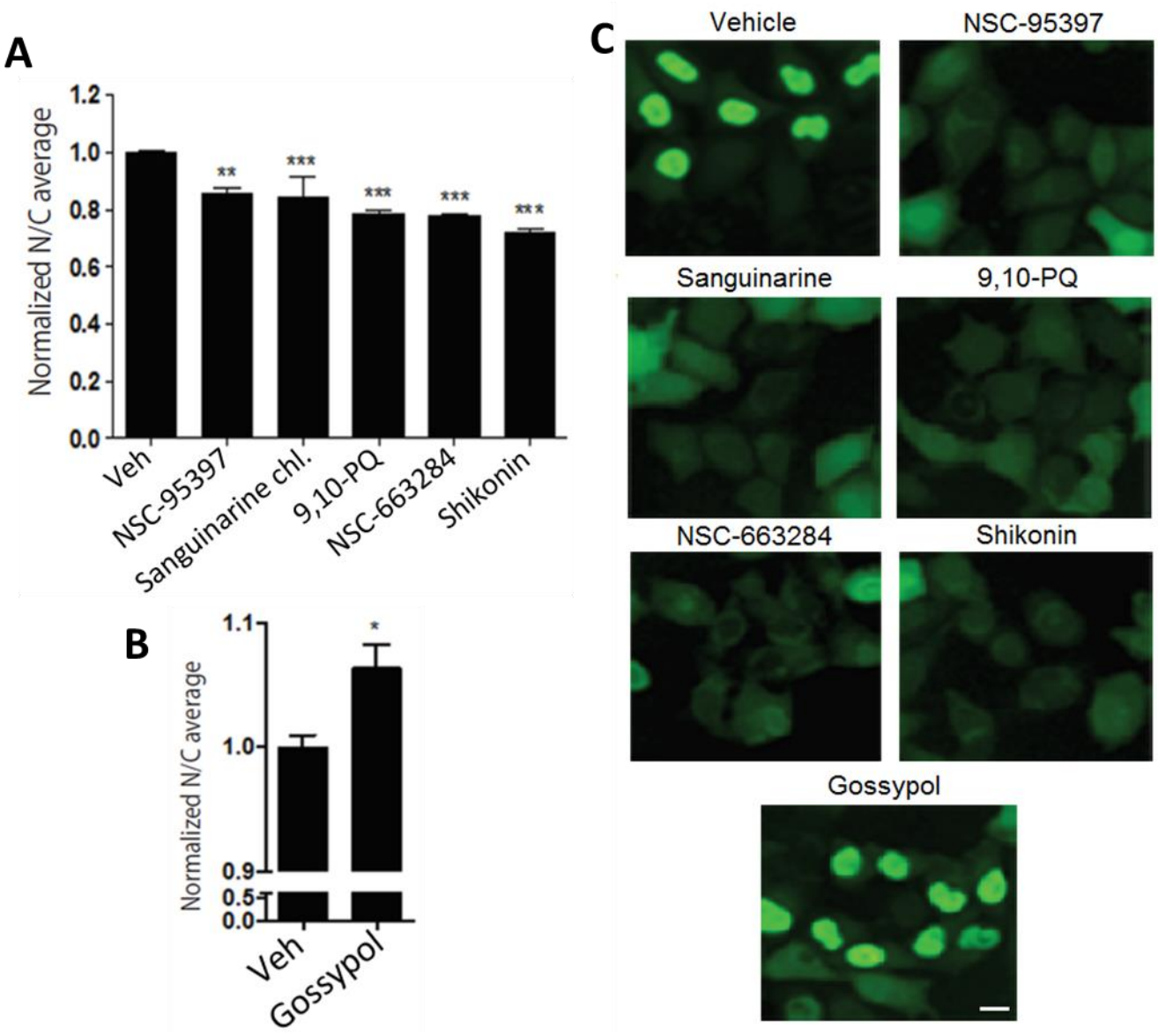
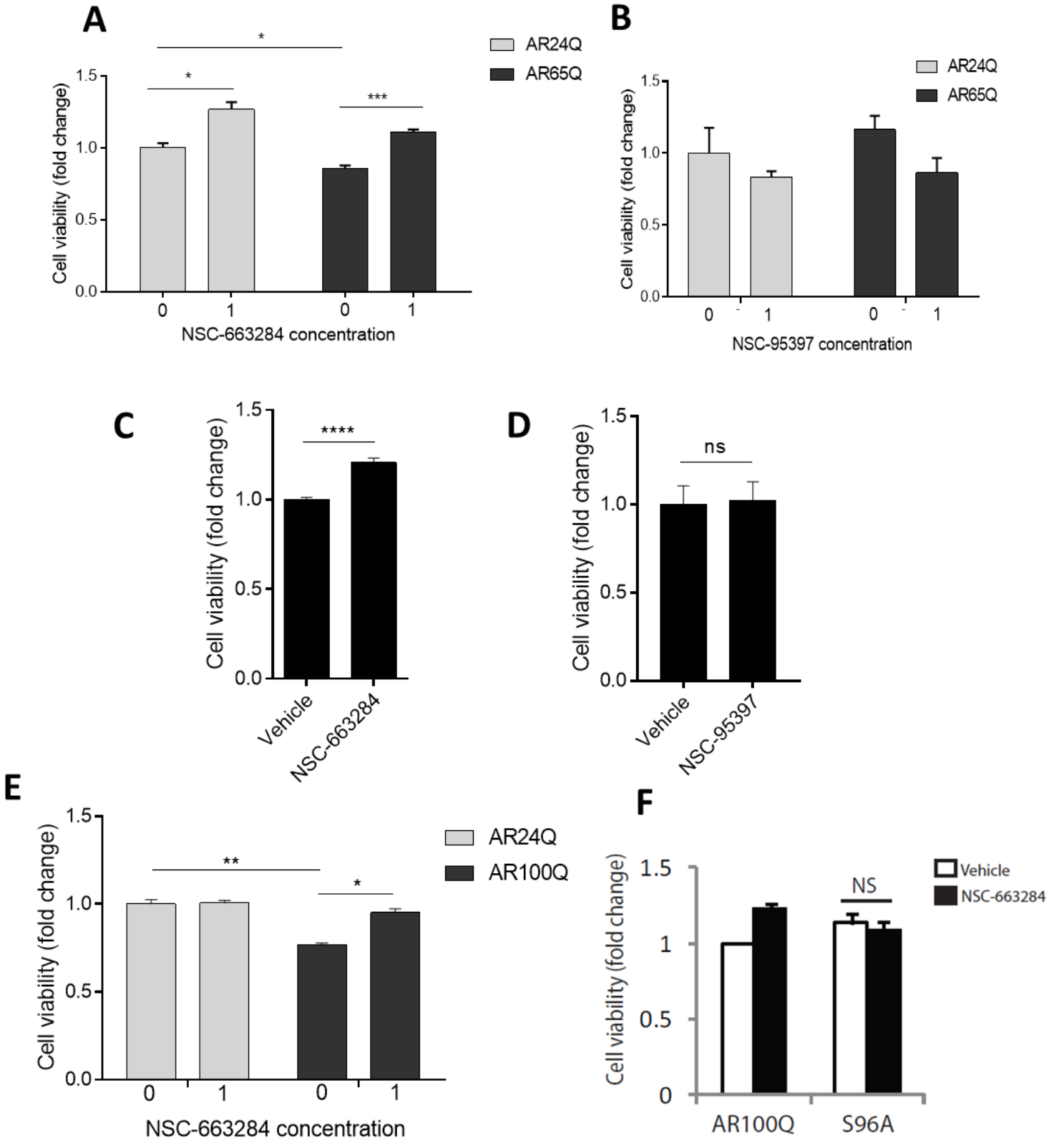


***Figure 35*. 6 phosphatase inhibitors modify the nucleus versus cytoplasm localization of polyQ-AR after DHT treatment.** A) and B) analysis of N/C localization of polyQ-AR expressing cells after 5 hours of the indicated phosphatase inhibitors at a concentration of 10 µM, followed by 4 hours DHT treatment at 10 nM. One-way ANOVA test with Tukey's post-hoc test was performed in A), unpaired two-tailed t-test was performed in B). * is $p<0.05$, ** is $p<0.01$, *** is $p<0.001$. C) Representative images of the indicated treatments. Scale bar, 25 µm.

4.3.2. NSC-663284 treatment increases the viability of polyQ-AR expressing cells

In order to test whether Cdc25 inhibition modifies cell viability, I treated cells with NSC-663284 and NSC-95397 inhibitors. I pre-treated cells overexpressing normal and polyQ-AR with 10 µM of DHT for 48 hours and 0 and 1 µM of NSC-663284 for the last 18 hours. This treatment led to the increase of cell viability in both overexpressing AR cell lines (***Fig. 36A***). Noteworthy, DHT treatment decreased the viability of polyQ-AR overexpressing HEK293T cells compared to control cells. I confirmed with three independent experiments the protection derived from 1 µM NSC-663284 treatment on polyQ-AR expressing cells (***Fig. 36B***). I treated the cells with 0 and 1 µM of NSC-95397. This compound did not induce any difference in cell survival of both non-expanded and expanded AR-overexpressing cells (***Fig. 36C***). NSC-95397 treatment did not alter cell viability of HEK293T overexpressing AR with 65 glutamines in three independent experiments (***Fig. 36D***).



Caption in the next page (p. 102).

Figure 36. **NSC-663284 inhibition of Cdc25 increases polyQ-AR expressing cell viability**. A) and B) MTT assay of HEK293T cells transfected with AR24Q and AR65Q, treated 48 hours with 10 μM DHT and 18 hours with NSC-663284 or NSC-953897. Two-tailed unpaired t-test was applied, and asterisks represent: *, $p<0.05$; ***, $p<0.001$. B) and D) MTT assay of HEK293T cells transfected with AR65Q, treated 48 hours with 10 μM DHT and 18 hours with 1 μM NSC-663284 or NSC-95397. Two-tailed unpaired t-test was applied, and asterisks represent: ****, $p<0.0001$. E) MTT assay of MN1- cells stably expressing AR24Q and AR100Q, treated 72 hours with 50 μM DHT and 18 hours with 1 μM NSC-663284. One-way ANOVA test with Tukey's post-hoc test was performed, and asterisks represent: *, $p<0.05$; **, $p<0.01$. F) MTT assay performed on MN-1 cells overexpression AR100Q and AR100Q S96A mutant, treated as previously described. Graphs, mean ± SEM, N=3.

Then, I conducted the same experiment with different cell lines. I exploited a cell line created in our lab in which motor neuron-derived cells (MN-1) were transduced with viruses expressing either AR with 24Q or 100Q (Scaramuzzino et al. 2015). I used this cell type in order to evaluate the effect of Cdc25 inhibition in a setting more similar to the disease. I pre-treated the cells with 50 μM DHT for 72 hours. I applied 0 or 1 μM NSC-663284 for the last 18 hours in AR24Q and AR100Q expressing cells. The inhibition of Cdc25 induced an increase of MN1- cell viability specifically in AR100Q-expressing cells, which resulted to be sensitive to DHT-induced toxicity (***Fig. 36E***).

Finally, I used MN-1 stably overexpressing AR100Q with serine 96 mutated into alanine (S96A), in order to prevent the phosphorylation at this site. I pre-treated the cells with 50 μM DHT for 72 hours in order to induce toxicity. Then for the last 18 hours, I treated the cells with and without mutation at serine 96, with 1μM NSC-663284. As expected, AR100Q S96A expressing cells displayed a higher cell viability compared to AR100Q cells (Polanco et al. 2016). NSC-663284 treatment was protective against DHT-induced cell death but did not increase the viability of the S96A mutant cells (***Fig. 36F***). These results suggest that NSC-663284-induced protection is achieved only with a phosphorylable AR at S96.

4.3.3. Cdc25 inhibition alters phosphorylation of AR at serine 96

The phosphatase inhibitor screening showed that NSC-663284 treatment decreases polyQ-AR nuclear localization, a phenomenon that correlated with increased cell viability. I asked which post-translational modifications are involved in this effect. NSC-663284 inhibits specifically Cdc25 but could have an indirect effect on AR biology through different pathways. The site which is more likely to be altered by NSC-663284 is serine 96. Indeed, this site is targeted by CDK2, and inhibition of Cdc25 and the subsequent inhibition of the activating dephosphorylation of CDK2 at threonine 14 and tyrosine 15 could diminish the accumulation of the upper phosphorylated isoform of AR (Lammer et al. 1998).

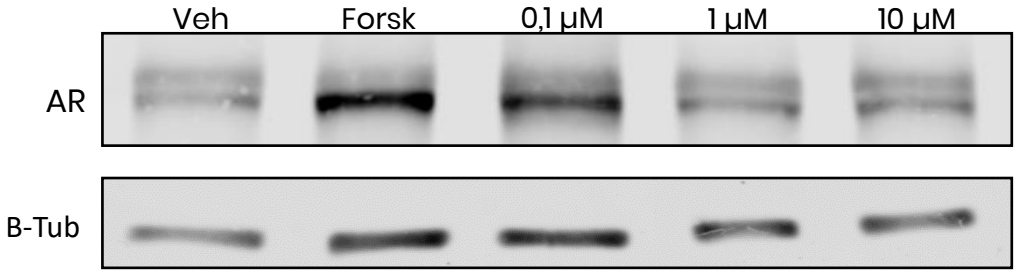


Figure 37. **NSC-663284 treatment decreases the accumulation of phosphorylated upper isoform of AR.** A) Western blotting analysis of phosphorylated and unphosphorylated AR at serine 96 of HEK293T cells transfected with polyQ-AR and treated with 10 µM forskolin (Forsk) or indicated concentrations of NSC-663284 for 5 hours. Loading control: β-Tubulin (β-Tub).

To test this hypothesis, I overexpressed polyQ-AR in HEK293T cells and I treated them with increasing concentration of NSC-663284 for 5 hours. I used forskolin as positive control for the dephosphorylation of AR at serine 96 (Polanco et al 2016). I performed a western blot analysis to separate 110 and 112 kDa AR isoforms. Treatment with 0.1 µM of NSC-663284 for 5 hours decreases the accumulation of the upper phosphorylated isoform at serine 96 of AR, similarly to forskolin treatment (***Fig. 37***). The other concentrations have minor effects on serine 96 phosphorylation.

4.3.4. Cdc25 inhibition modifies polyQ-AR transactivation activity

Phosphorylation is one the best characterized post-translational modifications that modulates AR transactivation (Gioeli and Paschal 2012; Palazzolo et al. 2007). For this reason, I asked whether the inhibition of Cdc25 through NSC-663284 changes AR transcriptional activity.

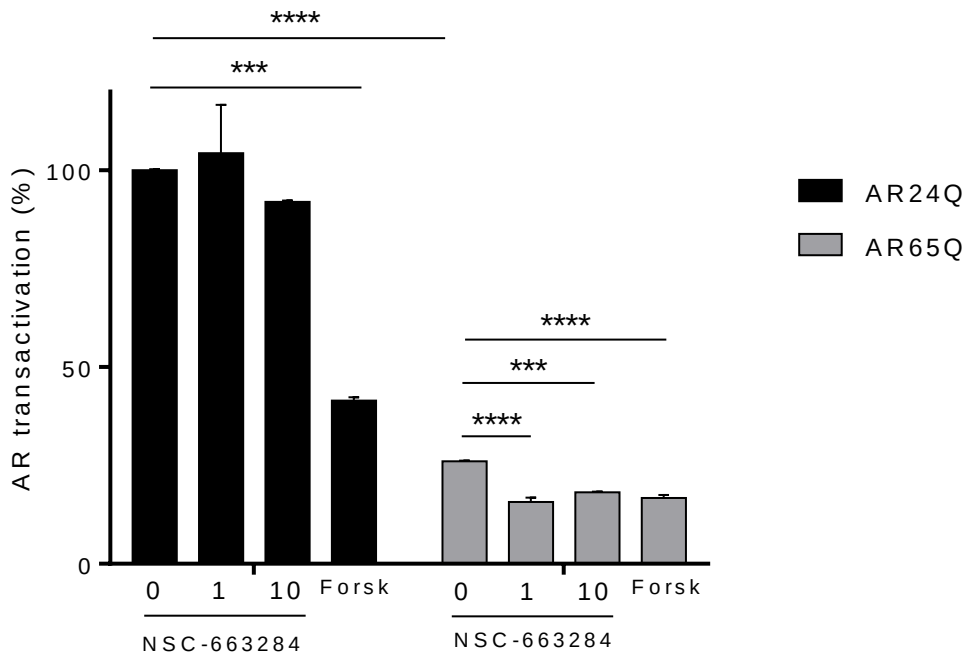


***Figure 38*. Cdc25 inhibition through NSC-663284 alters polyQ-AR transactivation.** Luciferase assay of HEK293T cells transfected with AR24Q and AR65Q, treated with 10 nM *D*HT for 24 hours, 10μM forskolin for 6 hours or with the indicated concentrations of NSC-663284. One-way ANOVA test with Tukey's post-hoc test was performed and asterisks represent: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; **** $p<0.0001$. Graphs, mean ± SEM. The reported experiment is representative of three independent experiments.

I transfected HEK293T cells with vector expressing either AR24Q or 65Q, together with the reporter constructs. I treated the cells for 24 hours with 10 nM of DHT, and with 0.1 and 10 μM of NSC-663284 for the last 6 hours. I selected these concentrations to mirror the conditions found to be protective for cell viability (1 μM) and found to alter AR cell localization (10 μM). Normal AR transactivation was not affected by NSC-663284 treatment. PolyQ-AR transcriptional activity was significantly decreased with 1 and 10 μM NSC-663284, with a reduction of 30% and 40%, respectively (***Fig. 38***). A similar decrease in AR transactivation was obtained in this experimental set-up from treatment with forskolin, as previously shown in mouse cortical primary neurons (***Fig. 24A***). These observations are in

accordance with a model in which AR is dephosphorylated at serine 96 by NSC-663284-induced Cdc25 inhibition, and this post-translational modification alters its transcriptional activity.

4.3.5. Cdc25C overexpression increases the accumulation of the upper isoform of AR and increases AR transactivation

The results described above show that inhibition of Cdc25 activity leads to decreased toxicity, dephosphorylation of serine 96 and a decrease of polyQ-AR transcriptional activity. Based on these observations, I asked whether overexpression of Cdc25 reverses these effects. Cdc25 isoform A and B were reported to be a corepressor and a coactivator of AR, respectively (Chiu et al. 2009, Ngan et al. 2003). To date, there are no evidences that Cdc25 isoform C is involved in AR biology. For these reasons, I decided to test the role of Cdc25C in regulating AR. Henceforth, Cdc25C is abbreviated as Cdc25.

To analyze AR phosphorylation at serine 96, I transfected polyQ-AR with mock or Cdc25 overexpressing vector in HEK293T cells. Overexpression of Cdc25 increased the accumulation of the upper phosphorylated isoform of polyQ-AR compared to mock-transfected cells (***Fig. 39A***).

In order to check whether Cdc25 modifies AR transcriptional activity, I performed luciferase assays. I transfected normal and polyQ-AR in HEK293T together with mock, Cdc25 or Cdc25 catalytically dead (mut) expressing vectors. The overexpression of Cdc25, but not Cdc25 mut, led to an increase in the luciferase activity driven by AR transactivation (***Fig. 39B***).

Taken together these observations suggest that Cdc25 modifies AR biology, altering serine 96 phosphorylation state, probably via CDK2 activation.

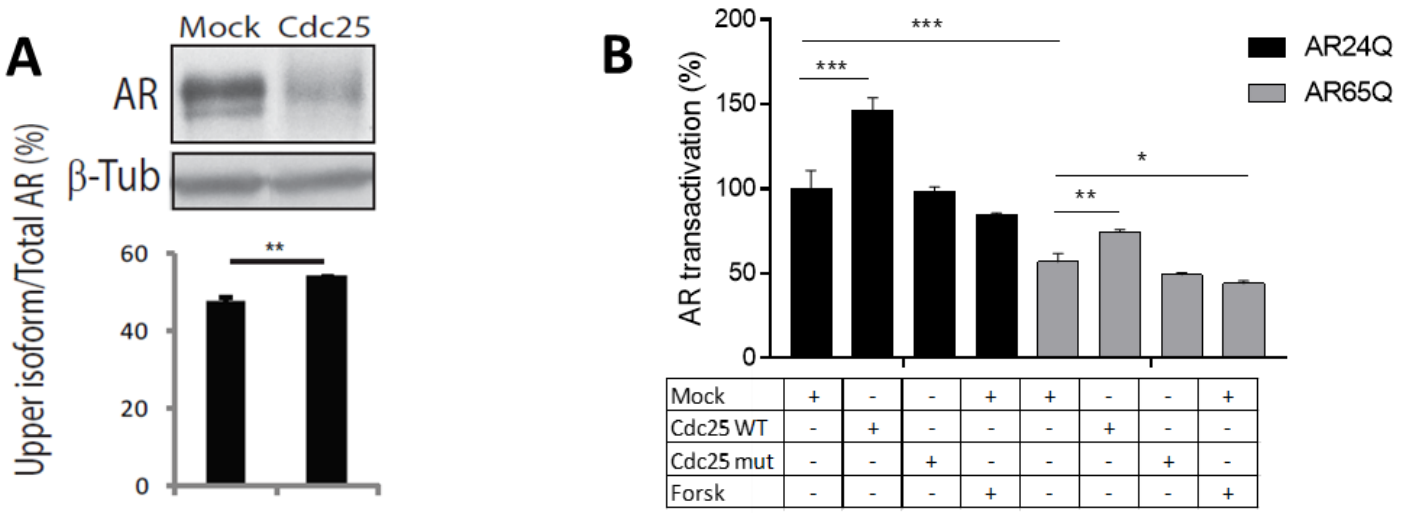


***Figure 39*. Cdc25 overexpression increases serine 96 phosphorylation and alters AR transcriptional activity.** A) Western blotting analysis of HEK293T cells transfected with AR55Q and with either mock or Cdc25 overexpressing vectors. Quantification is resulted from two independent experiments. Two-tailed t-test was performed and asterisks represent: **, $p<0.01$. B) Luciferase assay of HEK293T cells transfected with the indicated vectors and treated 6 hours with either vehicle or 10 µM forskolin. The reported experiment is representative of three independent experiments. One-way ANOVA test with Tukey's post-hoc test was performed, and asterisks represent: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Graphs, mean ± SEM.

5. DISCUSSION

Here, I showed that serine 96 phosphorylation has a central role in the modulation of polyQ-AR toxicity. In addition, it is a regulatory post-translational modification of AR physiological function. I showed that neuronal activity regulates AR transcriptional capacity through the dephosphorylation of serine 96. At the same time, I investigated the impact of serine 96 phosphorylation exerted by CDK2. I discovered that loss of CDK2 function results in a delay of SBMA motor phenotype onset *in vivo*, providing evidence that CDK2 is a novel modifier of SBMA. In addition, through a high-throughput screening of phosphatase inhibitors, I identified a Cdc25 inhibitor that diminished serine 96 phosphorylation, regulating transcriptional activity and decreasing polyQ-AR toxicity. Moreover, I identified a compound, gossypol, an inhibitor of calcineurin, that modifies polyQ-AR nuclear translocation. This last observation suggests a role in the regulation of this pathway also by the calcium-regulated phosphatase calcineurin. In the future, I will test the hypothesis that calcineurin dephosphorylates non-expanded AR upon neuronal activity.

Taken together these observations led to the proposed working model (***Fig. 40***).

5.1. AR transcriptional activity is regulated by neuronal activation

AR is expressed ubiquitously throughout the body, yet to a different extent among tissues. AR plays essential roles in the development of sexual glands and the maintenance of male fertility (Smith and Walker 2014). Moreover, AR has a central function in muscle development, remodeling and metabolism, increasing muscle mass and strength and provoking exercise-induced muscle hypertrophy (Inoue et al. 1994). From late 50s, AR was suggested to have a role in the central nervous system. AR mRNA is present in the forebrain, midbrain and spinal cord (Simerly et al. 1990). The protein is highly expressed in cortex and hippocampus, normally found in the nucleus, but in amygdala and cerebral cortex it was also detected in the axons and dendrites (DonCarlos et al. 2003). AR was involved in neuroprotective mechanisms. Indeed, androgens exert beneficial effects on neuronal trophic sustenance by acting directly through the AR. In hippocampal neurons, neuroprotection was observed against several apoptosis-inducing insults, such as β-amyloid, staurosporine and apoptosis activator II. This effect was seen both with testosterone and DHT treatment (Nguyen et al. 2010). DHT is a non-aromatizable androgen, exclusively bound to AR, and

this selectivity excludes an estrogen receptor-dependent effect. AR activity is also neuroprotective in motor neurons. It causes sexually-specific decrease of motor neuron cell death to model structural specialization during development and promotes protection from cell death after injury (Pike et al. 2008). In 2001, it was demonstrated that toxicity induced by serum deprivation in human primary neurons was rescued by treatment with the androgens and this treatment was not effective with a selective antagonist of AR (Hammond et al. 2001).

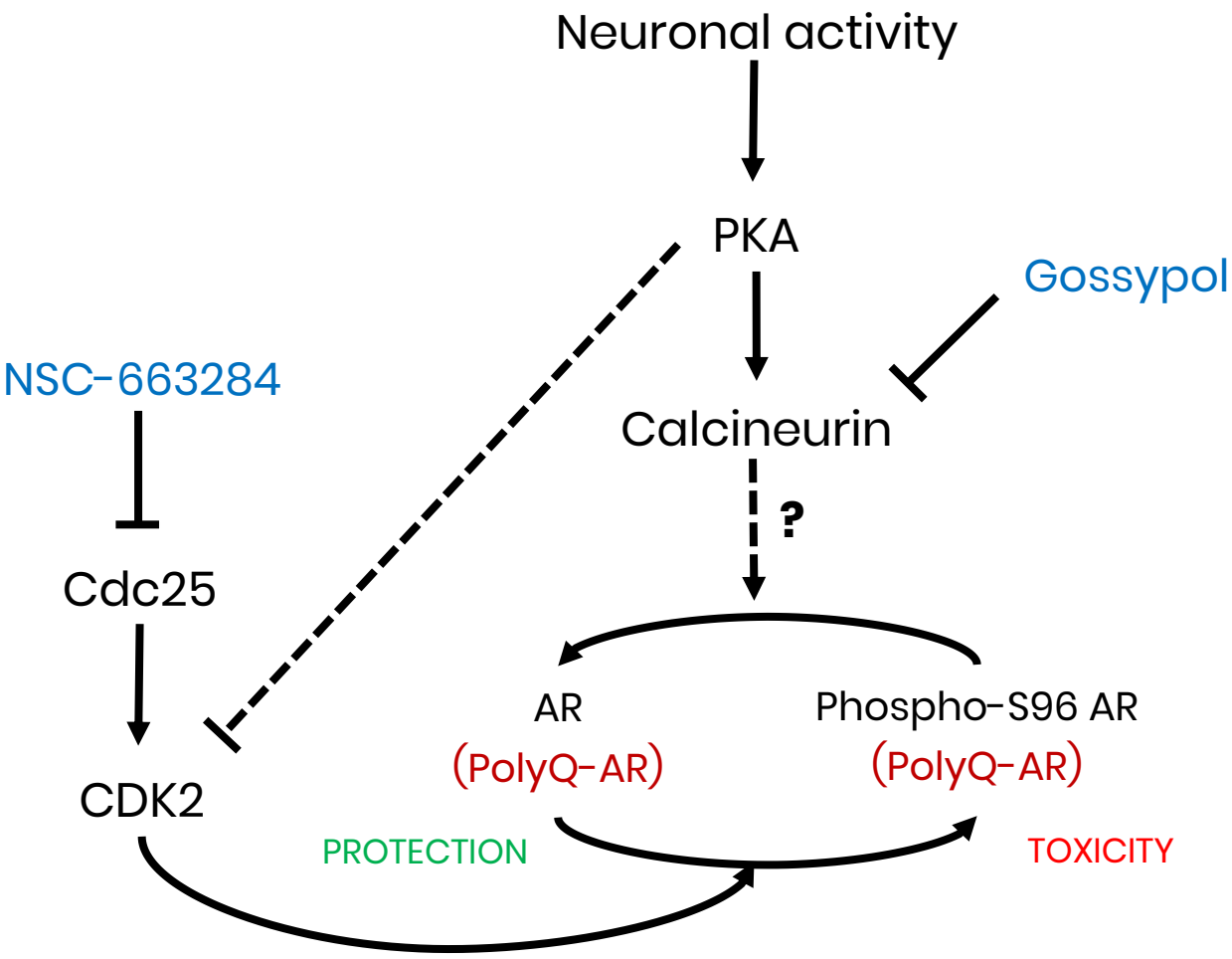


Figure 40. Working model. Neuronal activity through the activation of PKA trigger the dephosphorylation of AR (polyQ-AR) at serine 96. The hypothesis is that this dephosphorylation is triggered both by calcineurin activation due to calcium influx into the neuron, and inhibition of CDK2 (Polanco et al. 2016). I found one compound, NSC-663284, which counteracts the toxic phosphorylation of polyQ-AR at serine 96. I identified also another compound, gossypol, an inhibitor of calcineurin, which is potentially involved in the pathway, but further analyses have to be performed.

AR was also seen as a key factor in neurons and glial cells in myelin repair after cuprizone injury (Hussain et al. 2013). Moreover, androgens act through AR to induce spinogenesis in the hippocampus, inducing specific serine/threonine kinases, such as MAPK, PKA and PKC (Hatanaka et al. 2015). Little is known about the specific molecular mechanisms that underlie the qualitative effects triggered by androgens in an AR-dependent manner. For example, androgens are responsible for the masculinization of the brain: there are brain areas, including the nucleus of the preoptic area and ventromedial nucleus of the hypothalamus, that are sexually dimorphic (Ciofi et al. 2007). This evidence implies that AR plays primary roles in different subpopulations of neurons, whose specific molecular mechanisms involved are not yet well-defined. Neurons are excitable cells in which depolarization of the neuronal plasma membrane triggers an action potential. Key events in this process are intracellular calcium release and subsequent synaptic exocytosis. The discovery that AR is a transcription factor regulated by neuronal depolarization opens a new scenario for AR functional regulation in neurons. Additionally, a similar regulation of AR functions may occur in tissues other than the nervous system where calcium is a fundamental secondary messenger, such as skeletal muscle (Tu et al. 2016). AR was already shown to be implicated in calcium signaling. Gong and colleagues used prostate cancer cells to prove that calcium influences AR expression (Gong et al. 1995). They showed that both intracellular calcium increase or decrease diminish AR mRNA and protein levels, but the pathways involved remain elusive. AR was also shown to regulate the expression of sarco/endoplasmic reticulum calcium ATPase 2 (SERCA2), a calcium pump found to be localized in the endoplasmic reticulum (Foradori et al. 2007). SERCA2 catalyzes the transport of calcium ions from the cytosol into the endoplasmic reticulum, regulating basal calcium gradient (Guerrero-Hernandez et al. 2010).

In a model of excitability using primary neuronal cultures, I discovered that AR is regulated by neuronal depolarization through the dephosphorylation of serine 96. Surprisingly, I highlighted a link between neuronal depolarization, calcium release, dephosphorylation of serine 96 and AR transcriptional activity. This discovery could be the start for new insights into the molecular mechanisms regulating AR function in neurons. Given the activity-induced regulation of AR transactivation capacity, I hypothesized that AR activation through DHT treatment in AR-expressing neurons led to different gene induction and repression with respect to neurons in which AR is not activated. To analyze genome-wide differences in gene expression, I performed microarray analysis. I wanted to probe the immediate early gene expression machinery and the late-response gene induction due to early changes in gene

expression. For this reason, I treated excitable AR-expressing neurons with two time-points, one hour and six hours of depolarization (Spiegel et al. 2014). I am validating the obtained results, but a first analysis suggests the involvement of AR in the activation of a gene expression program that regulates important neuronal pathways.

I found that AR in neurons activates consistently five genes: neural epidermal growth factor-like like protein 2 (*NELL2*), chromosome transmission fidelity factor 8 (*CHTF8*), calcium binding protein 5 (*Cbp5*), phosphatidylinositol glycan anchor biosynthesis class T (*Pigt*) and proline-rich 7 (*Prr7*). NELL2 is a protein that is highly expressed in neural tissues and is composed of sex epidermal growth factor (EGF)-like domains (Watanabe et al. 1996). This domain is characterized by 40-50 amino acids and contains residues for Ca^{2+} binding (Handford et al. 1991). It was demonstrated that NELL2 interacts with an isoform of protein kinase C activated by calcium, phospholipid and diacylglycerol, proving that NELL2 is a cytosolic protein (Kuroda and Tanizawa 1999). NELL2 has protein motif similarities with trombospondin-1, a membrane protein partially secreted into the extracellular compartment (Chen et al. 2000). Indeed, an isoform of NELL2 was shown to be a secreted protein, suggesting a trophic role in neurons (Aihara et al. 2003). Interestingly, NELL2 drives motor neuron differentiation and the secreted isoform stimulates proliferation of the adjacent cells (Nelson et al. 2004). CHTF8 is part of the Ctf18 replication factor C (RCF) complex, important for sister chromatid cohesion and loading of the DNA replication processivity factor PCNA (Mayer et al. 2001). This gene was shown to be reduced in prostate and renal tumors (Sun et al. 2002). Cbp5 binds calcium and it has similarities with calmodulin protein (Haeseleer et al. 2002). Its expression was shown to be retina-specific (Haeseleer et al. 2000). Pigt is a subunit of the glycosylphosphatidylinositol (GPI) transamidase complex, responsible of GPI transfer to target proteins (Ohishi et al. 2001). Mutations in Pigt have been linked to an autosomal recessive intellectual disability (Kvarnung et al. 2013). Prr7 is a synaptic protein found to interact with the postsynaptic density-95 (PSD95) and NMDA receptor (Murata et al. 2005). Recently, it was associated with NMDAR-dependent excitotoxicity. Indeed, Prr7 blocks the ubiquitination of c-Jun, impeding its degradation, and this mechanism mediates excitotoxic neuronal death (Kravchick et al. 2016). Although AR-mediated transcriptional upregulation of these genes has to be characterized in detail, a first functional analysis suggests that AR induces gene expression alterations linked to important pathways for neuronal survival and differentiation.

5.2. CDK2 is a novel modifier of SBMA

AR is regulated mostly by phosphorylation (Koryakina et al. 2014). AR has several phosphorylation sites which are consensus site motifs for CDKs. These sites are serine or threonine, followed by proline (Kennelly and Krebs 1991). We showed that CDK2 is the kinase that specifically targets serine 96, and that phosphorylation at this site increases polyQ-AR toxicity (Polanco et al. 2016). In SBMA cells, PKA activation induced protected cells from the toxicity of polyQ-AR mainly through dephosphorylation of AR at serine 96. We exploited this information to develop a new strategy to pharmacologically decrease serine 96 phosphorylation *in vivo*. We used PACAP analogs to activate PKA and ultimately modify polyQ-AR phosphorylation and toxicity. This treatment was successful in ameliorating the motor phenotype and increasing lifespan of SBMA knock-in mice. In this work, the specific role of CDK2 in the disease pathogenesis was not elucidated. We analyzed the effect of PACAP analog on the canonical CDK inhibitors, $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (Sherr and Roberts 1999). We discovered a correlation between PKA pathway activation and increase of $p21^{CIP1}$ protein levels. Accordingly, we concluded that the inhibition of CDK2 through $p21^{CIP1}$ was downstream of PACAP treatment.

Our results demonstrate a key role of CDK2 in SBMA. I generated transgenic SBMA mice with deletion of one or both CDK2 alleles. The deletion of CDK2 in transgenic SBMA mice resulted in a delay of the motor phenotype. This mouse line begins to show disease manifestations at 8 weeks of age, when the mice start to lose motor coordination and muscle strength. Deletion and, importantly, haploinsufficiency of CDK2 improved the overall motor ability throughout lifespan. I observed an amelioration of rotarod and hanging wire performance, thereby proving that CDK2 is a novel modifier of SBMA pathogenesis. CDK2 deletion in all tissues is likely to have secondary effects that are not linked to AR biology. On the other hand, CDK2 heterozygosity preserves the biological function of the protein, still maintaining the rescue of TG AR100Q mice phenotype. A limitation of our experimental paradigm is the impossibility to decipher which specific tissues are the major player in the rescue. The targeted deletion of the protein in cell type- and time-specific manner could unravel the question. I did not observe any difference in survival of SBMA mice by knocking down CDK2. Nevertheless, an intervention on CDK2 activity to modulate serine 96 phosphorylation could lead to an amelioration of the quality of life of SBMA patients, although without an extension of lifespan. Importantly, I showed that the rescue in the

phenotype correlates with the phosphorylation state of serine 96, confirming the results that we have previously obtained (Polanco et al. 2016). In addition, I used SBMA knock-in mice, in which mouse AR was replaced by the human sequence of AR with a polyQ expansion of 113 glutamines (AR113Q mice) (Yu et al. 2006). Yu and colleagues showed that AR113Q mice have decreased body weight and weaker muscles compared to WT littermates and a median survival of 15 weeks. In the present study, the colony changed its characteristics, probably due to AR polyQ tract shortening. Indeed, the median survival was increased by 26 weeks, reaching 41 weeks, and the grip strength and rotarod performance was comparable to that of WT littermates during 1 year of monitoring. For these reasons, I could not use the SBMA knock in mice to test the effect of CDK2 deletion.

CDK2 has previously been implicated in neurodegenerative diseases. It was shown that tau, a protein implicated in Alzheimer's disease and other neurodegenerative diseases called tauopathies (Kovacs 2015), is phosphorylated by CDK2 (Baumann et al. 1993). Another pathway regulated by CDK2 is the accumulation of RNA regulatory element (TAR) DNA binding protein 43 (TDP-43) into stress granules (Moujalled et al. 2015). CDK2 phosphorylates a heterogenous nuclear ribonucleoprotein, hnRNP K, that is involved in TDP-43 subcellular localization. CDK2 inhibition decreases TDP-43 localization into stress granules, acting on hnRNP K (Moujalled et al. 2015). These observations suggest that CDK2 has important roles in neurons. CDK2 function in cell cycle progression is well-established, but its role in post-mitotic neuronal cells remains to be investigated. The evidence described above suggests a role for CDK2 not only in physiological processes, but also in pathological conditions, such as neurodegenerative diseases.

5.3. Cdc25 is a potential target for SBMA therapy development

Cell division cycle 25 (Cdc25) is another well-established player in cell cycle regulation. CDK2 and other CDKs have two conserved regulatory phosphorylation sites, tyrosine 15 (Tyr15) and threonine 14 (Thr14) (Pines 1999). Wee1/Mik1/Myt1 complex phosphorylates these residues, keeping the CDKs in an inactive state. To permit cell cycle progression, Cdc25 phosphatase dephosphorylates Tyr15 and Thr14, releasing CDKs inhibition (Pines 1999). Considering that CDK2 phosphorylation is toxic in SBMA, the inhibition of a CDK2 activator, such as Cdc25, should have protective effect.

Mammalian cells express three Cdc25 genes, namely Cdc25A, B, and C (Galaktionov and Beach 1991). Although all the three isoforms are able to dephosphorylate Tyr15 and Thr14, they have specific roles in the cell cycle progression. Cdc25A regulates early and late cell cycle transitions. Indeed, microinjection with antibodies against Cdc25A arrests cells in G1 phase, blocking their entry in S-phase (Hoffmann et al. 1994). The overexpression of Cdc25A induces mitosis, suggesting a role for this protein in S phase progression (Molinari et al. 2000). Its basal protein level increases from G1 to S phase and from S to G2/M phase, suggesting a broad role throughout the cell cycle (Hayes and Harper 2010). Cdc25B and C have limited roles in cell cycle progression. The microinjection with antibodies against Cdc25B and C causes G2 phase arrest, indicating that these proteins are essential for the transition from G2 to M phase (Lammer et al. 1998). AR has been the subject of thousands of studies because of its important role in the pathogenesis of prostate cancer (Heinlein and Chang 2004). Recently, it was shown that AR activity, phosphorylation and localization are regulated in a cell cycle-dependent manner (Koryakina et al. 2015). Indeed, it was already shown that AR transactivation is regulated by Cdc25, suggesting a mechanism of cell cycle-dependent regulation (Chiu et al. 2009; Ngan et al. 2003). It was demonstrated that Cdc25B acts as a coactivator of AR in prostate cancer cell line LNCaP (Ngan et al. 2003). Conversely, Cdc25A was shown to be a corepressor of AR in prostate cancer cells (Chiu et al. 2009). In both cases, the regulation of AR function by Cdc25 isoforms was independent from their phosphatase activity. Moreover, they proved the direct interaction between AR and Cdc25A and B. Only Cdc25A possesses the AR-interaction consensus site FXXLF, where F is phenylalanine, L is leucine, and X any amino acid (He et al. 2002). In this study, I employed Cdc25C genetic manipulations. Cdc25C has not been implicated in AR biology before and its amino acidic sequence comprises one LXXLL site, a motif that may mediate the direct interaction between AR and Cdc25C. The reported evidences support a more intriguing scenario in which Cdc25C-mediated inhibition of CDK2 prevent serine 96 phosphorylation, decreasing DHT-mediated toxicity in polyQ-AR cells.

NSC-663284 is a non-selective inhibitor of Cdc25 phosphatase activity. It inhibits all the three Cdc25 proteins with different half maximal inhibitory concentration values (IC_{50}) (Brisson et al. 2005). According to biochemical studies, NSC-663284 inhibits Cdc25A and C with a similar IC_{50}, slightly more specific for Cdc25C. The observations that NSC-663284 treatment led to the opposite effects exerted by Cdc25C overexpression suggest that the phosphatase activity of this protein is required. NSC-663284 was proposed as anticancer

drug, even if its rapid metabolism has limited its application (Guo et al. 2007). More experiments need to be performed, but in the light of our discoveries, I will propose in the future to repurpose anticancer drugs, such as NSC-663284 analogs, for the treatment of SBMA.

6. CONCLUSION AND FUTURE PERSPECTIVES

In this thesis, I explored the role of serine 96 phosphorylation of AR in non-pathological and disease conditions.

Neuronal activity is a process involved in long-lasting experience-dependent plasticity of the brain, which permits adaptation of an individual according to the incoming environmental cues. I identified AR as a neuronal activity-regulated transcription factor, which acts through the dephosphorylation of serine 96. Using genome-wide analysis of neuronal transcriptome, I highlighted neuronal-stimulated gene targets that are regulated specifically upon AR activation. These genes are putative effectors of AR-mediated response in stimulated AR-expressing neurons. These results open up the scenario in which AR influences the functional outcome in neurons in response to sexual and behavioral stimuli.

Serine 96 is phosphorylated by CDK2, a kinase classically involved in the regulation of the cell cycle, with emerging roles also in post-mitotic cells, such as neurons, as shown here. I showed that the genetic manipulation of CDK2 is protective for SBMA mice. The delay of the motor phenotype correlated with a decrease in the phosphorylation at serine 96. Although these encouraging results, the tissue-specific contributions to the phenotype amelioration is precluded in such model. The generation of conditional CDK2 null mice for tissue-specific knock down of CDK2 will help to unravel the cell-autonomous effects on the physio-pathological role of AR phosphorylation at serine 96 in neurons.

Through an unbiased high-throughput screening of phosphatase and kinase inhibitors on polyQ-AR-expressing cells, I identified a compound, NSC-663284, with protective properties and targeting serine 96. NSC-663284 is a selective inhibitor of Cdc25, a phosphatase responsible for the activation of CDK2. In mammalian cells, there are three different *Cdc25* genes, and NSC-663284 is a non-specific inhibitor of all the three isoforms. In my study, I reported for the first time that the isoform C is implicated in AR biology, with an impact on polyQ-AR toxic effect. In the near future, the aim is to identify analogues of NSC-663284 with higher selectivity for Cdc25C.

7. APPENDIX

***6.1. Table 2*. Gene ontology of the DEGs derived from the indicated comparisons of conditions.**

KCl 1h vs Vehicle

Processes

GO term	Description	Corrected p-value
none	none	none

Functions

GO term	Description	Corrected p-value
GO:0043565	sequence-specific DNA binding	3.23E-1

Components

GO term	Description	Corrected p-value
none	none	none

KCl 6h vs Vehicle

Processes

GO term	Description	Corrected p-value
GO:0006357	regulation of transcription from RNA polymerase II promoter	2.31E-6
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	1.27E-4
GO:1902680	positive regulation of RNA biosynthetic process	1.28E-4
GO:0045893	positive regulation of transcription, DNA-templated	9.62E-5
GO:1903508	positive regulation of nucleic acid-templated transcription	7.69E-5
GO:0010628	positive regulation of gene expression	1.49E-4
GO:0010557	positive regulation of macromolecule biosynthetic process	1.61E-4
GO:0009891	positive regulation of biosynthetic process	1.41E-4
GO:0031328	positive regulation of cellular biosynthetic process	1.25E-4
GO:0051254	positive regulation of RNA metabolic process	1.4E-4
GO:0010604	positive regulation of macromolecule metabolic process	1.38E-4
GO:0009893	positive regulation of metabolic process	2.75E-4
GO:0051173	positive regulation of nitrogen compound metabolic process	4.49E-4
GO:0031325	positive regulation of cellular metabolic process	5.92E-4

GO term	Description	Corrected p-value
GO:0051172	negative regulation of nitrogen compound metabolic process	1.96E-3
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	2.78E-3
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	2.92E-3
GO:0051253	negative regulation of RNA metabolic process	4.04E-3
GO:0030154	cell differentiation	6.43E-3
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	6.24E-3
GO:1902679	negative regulation of RNA biosynthetic process	6.84E-3
GO:0045892	negative regulation of transcription, DNA-templated	6.53E-3
GO:1903507	negative regulation of nucleic acid-templated transcription	6.24E-3
GO:0048522	positive regulation of cellular process	6.69E-3
GO:0010558	negative regulation of macromolecule biosynthetic process	7.58E-3
GO:0009966	regulation of signal transduction	7.37E-3
GO:0060255	regulation of macromolecule metabolic process	7.71E-3
GO:0070887	cellular response to chemical stimulus	8.32E-3
GO:0048518	positive regulation of biological process	8.35E-3
GO:0031324	negative regulation of cellular metabolic process	8.09E-3
GO:0045595	regulation of cell differentiation	8.12E-3
GO:0032502	developmental process	8.96E-3
GO:0010646	regulation of cell communication	9.95E-3
GO:0023051	regulation of signaling	9.65E-3
GO:0048519	negative regulation of biological process	9.67E-3
GO:0031401	positive regulation of protein modification process	9.91E-3
GO:0009890	negative regulation of biosynthetic process	1.11E-2
GO:0031327	negative regulation of cellular biosynthetic process	1.08E-2
GO:0043408	regulation of MAPK cascade	1.17E-2
GO:0051247	positive regulation of protein metabolic process	1.17E-2
GO:0080090	regulation of primary metabolic process	1.15E-2
GO:0051171	regulation of nitrogen compound metabolic process	1.12E-2
GO:0048523	negative regulation of cellular process	1.41E-2
GO:0051241	negative regulation of multicellular organismal process	1.4E-2
GO:0010605	negative regulation of macromolecule metabolic process	1.51E-2
GO:0051246	regulation of protein metabolic process	1.48E-2
GO:2000113	negative regulation of cellular macromolecule biosynthetic process	1.5E-2
GO:0070372	regulation of ERK1 and ERK2 cascade	1.55E-2
GO:0019220	regulation of phosphate metabolic process	1.88E-2
GO:0051174	regulation of phosphorus metabolic process	1.85E-2
GO:0031323	regulation of cellular metabolic process	2,00E-02

GO term	Description	Corrected p-value
GO:0019222	regulation of metabolic process	2,00E-02
GO:0032270	positive regulation of cellular protein metabolic process	2.05E-2
GO:1902531	regulation of intracellular signal transduction	2.22E-2
GO:0048856	anatomical structure development	2.21E-2
GO:0001932	regulation of protein phosphorylation	2.22E-2
GO:0042325	regulation of phosphorylation	2.18E-2
GO:0031399	regulation of protein modification process	2.16E-2
GO:0007165	signal transduction	2.25E-2
GO:0010468	regulation of gene expression	2.87E-2
GO:0032268	regulation of cellular protein metabolic process	3.55E-2
GO:0043207	response to external biotic stimulus	3.55E-2
GO:1901362	organic cyclic compound biosynthetic process	3.68E-2
GO:0006351	transcription, DNA-templated	3.75E-2
GO:0097659	nucleic acid-templated transcription	3.69E-2
GO:0035914	skeletal muscle cell differentiation	3.64E-2
GO:0048869	cellular developmental process	3.8E-2
GO:0032496	response to lipopolysaccharide	3.93E-2
GO:0002237	response to molecule of bacterial origin	3.87E-2
GO:0048511	rhythmic process	3.92E-2
GO:0071310	cellular response to organic substance	4.07E-2
GO:0002682	regulation of immune system process	4.04E-2
GO:0032501	multicellular organismal process	4.11E-2
GO:0051252	regulation of RNA metabolic process	4.07E-2
GO:2000177	regulation of neural precursor cell proliferation	4.29E-2
GO:1901700	response to oxygen-containing compound	4.5E-2
GO:0034654	nucleobase-containing compound biosynthetic process	4.83E-2
GO:0018130	heterocycle biosynthetic process	4.77E-2
GO:0019438	aromatic compound biosynthetic process	4.71E-2
GO:0048583	regulation of response to stimulus	4.71E-2

Functions

GO term	Description	Corrected p-value
GO:0043565	sequence-specific DNA binding	1.41E-4
GO:0000982	transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding	1.69E-4
GO:0005515	protein binding	1.96E-4
GO:1990837	sequence-specific double-stranded DNA binding	1.76E-4
GO:0000976	transcription regulatory region sequence-specific DNA binding	1.41E-4
GO:0001067	regulatory region nucleic acid binding	3.08E-4
GO:0044212	transcription regulatory region DNA binding	2.64E-4

GO term	Description	Corrected p-value
GO:0001228	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific DNA binding	4.93E-4
GO:0001077	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding	4.88E-4
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	4.51E-4
GO:0001012	RNA polymerase II regulatory region DNA binding	4.1E-4
GO:0000987	proximal promoter sequence-specific DNA binding	5.27E-4
GO:0003690	double-stranded DNA binding	8.07E-4
GO:0019904	protein domain specific binding	1.15E-3
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	2.27E-3
GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	2.63E-3
GO:0008134	transcription factor binding	1.07E-2
GO:0005488	binding	1.59E-2
GO:0046982	protein heterodimerization activity	2.93E-2

Components

GO term	Description	Corrected p-value
GO:0005634	nucleus	3.38E-3
GO:0044422	organelle part	3.57E-3
GO:0044446	intracellular organelle part	4.19E-3

DHT-KCl 6h vs Vehicle-KCl 6h

Processes

GO term	Description	Corrected p-value
GO:0042221	response to chemical	1.68E-3
GO:0031323	regulation of cellular metabolic process	2.02E-3
GO:0060255	regulation of macromolecule metabolic process	1.38E-3
GO:0048519	negative regulation of biological process	2.05E-3
GO:0048523	negative regulation of cellular process	1.65E-3
GO:0051128	regulation of cellular component organization	2.05E-3
GO:0006357	regulation of transcription from RNA polymerase II promoter	1.93E-3
GO:0051171	regulation of nitrogen compound metabolic process	1.77E-3

GO term	Description	Corrected p-value
GO:0032501	multicellular organismal process	1.87E-3
GO:0031326	regulation of cellular biosynthetic process	2.52E-3
GO:0080090	regulation of primary metabolic process	2.37E-3
GO:0044093	positive regulation of molecular function	3.1E-3
GO:0007610	behavior	2.89E-3
GO:0010468	regulation of gene expression	2.94E-3
GO:0019222	regulation of metabolic process	2.92E-3
GO:0043085	positive regulation of catalytic activity	2.94E-3
GO:0032268	regulation of cellular protein metabolic process	2.79E-3
GO:0009893	positive regulation of metabolic process	2.63E-3
GO:0009889	regulation of biosynthetic process	2.72E-3
GO:0019219	regulation of nucleobase-containing compound metabolic process	3.06E-3
GO:0070887	cellular response to chemical stimulus	3.31E-3
GO:0010556	regulation of macromolecule biosynthetic process	3.36E-3
GO:0008284	positive regulation of cell proliferation	3.52E-3
GO:0010604	positive regulation of macromolecule metabolic process	3.95E-3
GO:0051254	positive regulation of RNA metabolic process	4.53E-3
GO:0006355	regulation of transcription, DNA-templated	4.88E-3
GO:2001141	regulation of RNA biosynthetic process	4.7E-3
GO:1903506	regulation of nucleic acid-templated transcription	4.54E-3
GO:2000112	regulation of cellular macromolecule biosynthetic process	4.42E-3
GO:0031325	positive regulation of cellular metabolic process	4.31E-3
GO:1901362	organic cyclic compound biosynthetic process	7.38E-3
GO:0031399	regulation of protein modification process	7.15E-3
GO:0051246	regulation of protein metabolic process	7.15E-3
GO:0051173	positive regulation of nitrogen compound metabolic process	7.16E-3
GO:0048518	positive regulation of biological process	7.48E-3
GO:0001932	regulation of protein phosphorylation	8.51E-3
GO:0009991	response to extracellular stimulus	8.38E-3
GO:0031667	response to nutrient levels	8.16E-3
GO:1902680	positive regulation of RNA biosynthetic process	8.14E-3
GO:0045893	positive regulation of transcription, DNA-templated	7.93E-3
GO:1903508	positive regulation of nucleic acid-templated transcription	7.74E-3
GO:0034645	cellular macromolecule biosynthetic process	7.76E-3
GO:0032502	developmental process	7.59E-3
GO:0051960	regulation of nervous system development	7.58E-3
GO:0051252	regulation of RNA metabolic process	7.91E-3
GO:0009059	macromolecule biosynthetic process	7.91E-3
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	7.82E-3

GO term	Description	Corrected p-value
GO:0050794	regulation of cellular process	8.39E-3
GO:0048856	anatomical structure development	8.42E-3
GO:0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	8.65E-3
GO:0031324	negative regulation of cellular metabolic process	9,00E-03
GO:0007626	locomotory behavior	9.39E-3
GO:1902679	negative regulation of RNA biosynthetic process	9.3E-3
GO:0045892	negative regulation of transcription, DNA-templated	9.13E-3
GO:1903507	negative regulation of nucleic acid-templated transcription	8.96E-3
GO:0065009	regulation of molecular function	8.86E-3
GO:0050767	regulation of neurogenesis	9.21E-3
GO:0060284	regulation of cell development	9.05E-3
GO:0009892	negative regulation of metabolic process	9.54E-3
GO:0019220	regulation of phosphate metabolic process	9.7E-3
GO:0051174	regulation of phosphorus metabolic process	9.54E-3
GO:0042325	regulation of phosphorylation	1,00E-02
GO:0050789	regulation of biological process	1.08E-2
GO:0042127	regulation of cell proliferation	1.07E-2
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	1.09E-2
GO:0071900	regulation of protein serine/threonine kinase activity	1.09E-2
GO:0045859	regulation of protein kinase activity	1.1E-2
GO:0045595	regulation of cell differentiation	1.09E-2
GO:0006928	movement of cell or subcellular component	1.15E-2
GO:0010562	positive regulation of phosphorus metabolic process	1.15E-2
GO:0045937	positive regulation of phosphate metabolic process	1.13E-2
GO:0010033	response to organic substance	1.19E-2
GO:0010629	negative regulation of gene expression	1.27E-2
GO:0010038	response to metal ion	1.26E-2
GO:0071310	cellular response to organic substance	1.28E-2
GO:0043069	negative regulation of programmed cell death	1.3E-2
GO:0043066	negative regulation of apoptotic process	1.29E-2
GO:0001934	positive regulation of protein phosphorylation	1.27E-2
GO:0042327	positive regulation of phosphorylation	1.25E-2
GO:0009653	anatomical structure morphogenesis	1.36E-2
GO:0051172	negative regulation of nitrogen compound metabolic process	1.37E-2
GO:0051253	negative regulation of RNA metabolic process	1.38E-2
GO:0007154	cell communication	1.49E-2
GO:0048511	rhythmic process	1.49E-2
GO:0071496	cellular response to external stimulus	1.47E-2
GO:0042594	response to starvation	1.45E-2
GO:0009267	cellular response to starvation	1.44E-2

GO term	Description	Corrected p-value
GO:0031668	cellular response to extracellular stimulus	1.42E-2
GO:0031669	cellular response to nutrient levels	1.4E-2
GO:0071850	mitotic cell cycle arrest	1.53E-2
GO:0060548	negative regulation of cell death	1.53E-2
GO:0010605	negative regulation of macromolecule metabolic process	1.52E-2
GO:0045664	regulation of neuron differentiation	1.57E-2
GO:0050793	regulation of developmental process	1.6E-2
GO:0019438	aromatic compound biosynthetic process	1.66E-2
GO:0050790	regulation of catalytic activity	1.69E-2
GO:0043408	regulation of MAPK cascade	1.69E-2
GO:0071248	cellular response to metal ion	1.68E-2
GO:0065007	biological regulation	1.69E-2
GO:1904029	regulation of cyclin-dependent protein kinase activity	1.69E-2
GO:1901215	negative regulation of neuron death	1.87E-2
GO:0009890	negative regulation of biosynthetic process	2.01E-2
GO:0051239	regulation of multicellular organismal process	1.99E-2
GO:0043549	regulation of kinase activity	2.07E-2
GO:0051347	positive regulation of transferase activity	2.06E-2
GO:0051271	negative regulation of cellular component movement	2.22E-2
GO:0044249	cellular biosynthetic process	2.23E-2
GO:0051338	regulation of transferase activity	2.22E-2
GO:0009891	positive regulation of biosynthetic process	2.3E-2
GO:0031328	positive regulation of cellular biosynthetic process	2.28E-2
GO:0010628	positive regulation of gene expression	2.43E-2
GO:1901576	organic substance biosynthetic process	2.43E-2
GO:0009058	biosynthetic process	2.64E-2
GO:0010224	response to UV-B	2.82E-2
GO:0048522	positive regulation of cellular process	2.81E-2
GO:0051353	positive regulation of oxidoreductase activity	2.79E-2
GO:0051341	regulation of oxidoreductase activity	2.77E-2
GO:0043523	regulation of neuron apoptotic process	2.81E-2
GO:0031401	positive regulation of protein modification process	2.83E-2
GO:0043271	negative regulation of ion transport	2.89E-2
GO:0032270	positive regulation of cellular protein metabolic process	3,00E-02
GO:0051247	positive regulation of protein metabolic process	2.97E-2
GO:0010035	response to inorganic substance	3.03E-2

Functions

GO term	Description	Corrected p-value
GO:0005515	protein binding	1.72E-3
GO:0043565	sequence-specific DNA binding	1.56E-2
GO:1990837	sequence-specific double-stranded DNA binding	3.49E-2
GO:0001067	regulatory region nucleic acid binding	3.79E-2
GO:0044212	transcription regulatory region DNA binding	3.03E-2
GO:0000976	transcription regulatory region sequence-specific DNA binding	4.51E-2
GO:0003690	double-stranded DNA binding	5.54E-2
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	5.08E-2
GO:0001012	RNA polymerase II regulatory region DNA binding	4.51E-2
GO:0008134	transcription factor binding	4.55E-2
GO:0030332	cyclin binding	5.53E-2
GO:0000982	transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding	5.49E-2

Components

GO term	Description	Corrected p-value
GO:0044459	plasma membrane part	2.03E-1
GO:0005886	plasma membrane	1.27E-1
GO:0005667	transcription factor complex	1.33E-1
GO:0120025	plasma membrane bounded cell projection	1.18E-1

***6.2. Table 3*. List of the phosphatase and kinase inhibitors used in the high-throughput screening.**

PHOSPHATASE INHIBITORS	**KINASE INHIBITORS**
Cantharidic acid	Linifanib (ABT-869)
Cantharidin	Axitinib
Endothall	Saracatinib (AZD0530)
Benzylphosphonic acid	AZD6244 (Selumetinib)
L-p-Bromotetramisole oxalate	BEZ235 (NVP-BEZ235)
RK-682	BIBF1120 (Vargatef)
RWJ-60475	Afatinib (BIBW2992)
RWJ-60475 (AM)3	Bosutinib (SKI-606)
Levamisole HCl	Cediranib (AZD2171)
Tetramisole HCl	Dovitinib (TKI-258)
Cypermethrin	CI-1033 (Canertinib)
Deltamethrin	CI-1040 (PD184352)
Fenvalerate	Dasatinib (BMS-354825)
Tyrphostin 8	Deforolimus (Ridaforolimus)
CinnGel	Erlotinib HCl
NSC-95397	Gefitinib (Iressa)
BN-82002	Imatinib Mesylate
Shikonin	Lapatinib Ditosylate (Tykerb)
NSC-663284	Motesanib Diphosphate
Cyclosporin A	Nilotinib (AMN-107)
Pentamidine	Pazopanib HCl
BVT-948	PD0325901
B4-Rhodanine	PI-103
Alexidine·2HCl	Rapamycin (Sirolimus)
9,10-Phenanthrenequinone	Sorafenib (Nexavar)
BML-260	Sunitinib Malate (Sutent)
Sanguinarine chloride	Tandutinib (MLN518)
BML-267	Temsirolimus (Torisel)
BML-267 Ester	Vandetanib (Zactima)
OBA	VX-680 (MK-0457, Tozasertib)
OBA Ester	Y-27632 2HCl
Gossypol	Enzastaurin (LY317615)
Alendronate	BMS-599626 (AC480)
	Masitinib (AB1010)
	GDC-0941
	SB 431542
	Crizotinib (PF-02341066)
	PHA-665752
	ZSTK474
	SB 216763

	SB 203580
	SB 202190
	MK-2206 dihydrochloride
	PD153035 HCl
	SU11274
	Brivanib (BMS-540215)
	NVP-ADW742
	Linsitinib (OSI-906)
	KU-55933
	GSK1904529A
	PF-04217903
	MLN8054
	Vatalanib dihydrochloride (PTK787)
	U0126-EtOH
	ZM-447439
	GDC-0879
	LY294002
	OSU-03012
	Danusertib (PHA-739358)
	TAE684 (NVP-TAE684)
	BI 2536
	Foretinib (GSK1363089, XL880)
	SGX-523
	GSK690693
	JNJ-38877605
	PD 0332991 (Palbociclib) HCl
	Triciribine (Triciribine phosphate)
	XL147
	XL-184 free base (Cabozantinib)
	Everolimus (RAD001)
	MLN8237 (Alisertib)
	AT9283
	Brivanib alaninate (BMS-582664)
	AG-490
	SNS-032 (BMS-387032)
	Barasertib (AZD1152-HQPA)
	PLX-4720
	Roscovitine (Seliciclib, CYC202)
	SNS-314
	E7080 (Lenvatinib)
	CP-724714
	TGX-221
	WZ3146
	CYC116
	WZ4002

	PD98059
	Regorafenib (BAY 73-4506)
	WZ8040
	ENMD-2076
	PIK-90
	PIK-75
	Tivozanib (AV-951)
	YM201636
	OSI-930
	Ku-0063794
	AG-1024
	Amuvatinib (MP-470)
	JNJ-7706621
	PD173074
	WYE-354
	Vemurafenib (PLX4032)
	IC-87114
	BX-795
	BX-912
	AMG-208
	TG100-115
	GSK1059615
	MGCD-265
	ON-01910
	Ki8751
	Ruxolitinib (INCB018424)
	Pelitinib (EKB-569)
	AS-605240
	Staurosporine
	Aurora A Inhibitor I
	PHA-680632
	Thiazovivin
	SP600125
	TSU-68
	AS703026
	SB 525334
	HMN-214
	AEE788 (NVP-AEE788)
	PHA-793887
	PIK-93
	Ponatinib (AP24534)
	LY2228820
	CCT129202
	XL765
	AT7519

	Quizartinib (AC220)
	Hesperadin
	BIX 02188
	BIX 02189
	AZD7762
	R406(free base)
	CP 673451
	AZD8055
	PHT-427
	KRN 633
	AT7867
	BMS 777607
	PD318088
	KU-60019
	BS-181 HCl
	BIRB 796 (Doramapimod)
	Tie2 kinase inhibitor
	TWS119
	BMS-265246
	AZD8330
	Neratinib (HKI-272)
	KW 2449
	LY2784544
	BGJ398 (NVP-BGJ398)
	AST-1306
	AZD8931
	GSK461364
	R406
	Raf265 derivative
	BMS 794833
	NVP-BHG712
	OSI-420 (Desmethyl Erlotinib)
	R935788 (Fostamatinib disodium, R788 disodium)
	PIK-293
	AZ 960
	Mubritinib (TAK 165)
	PP242
	Cyt387
	SB590885
	Apatinib (YN968D1)
	CAL-101 (GS-1101)
	PIK-294
	Telatinib (BAY 57-9352)
	BI6727 (Volasertib)
	Palomid 529

	WP1130
	BKM120 (NVP-BKM120)
	cx-4945 (Silmitasertib)
	Indirubin
	Quercetin (Sophoretin)
	Imatinib (Gleevec)
	Phenformin hydrochloride
	TAK-733
	LDN193189
	AZD5438
	PP-121
	OSI-027
	R788 (Fostamatinib)
	LY2603618 (IC-83)
	PF-05212384 (PKI-587)
	DCC-2036 (Rebastinib)
	CCT128930
	A66
	NU7441(KU-57788)
	GSK2126458
	WYE-125132
	WYE-687
	A-674563
	AS-252424
	PF-00562271
	GSK1120212 (Trametinib)
	Flavopiridol hydrochloride
	PCI-32765 (Ibrutinib)
	AS-604850
	CAY10505
	CHIR-124
	NVP-BSK805
	WAY-600
	TG101209
	GDC-0980 (RG7422)
	A-769662
	KX2-391
	GSK1838705A
	TAK-901
	AMG 900
	ZM 336372
	PF-03814735
	PH-797804
	Dacomitinib (PF299804,PF-00299804)
	AG-1478 (Tyrphostin AG-1478)

	SB 415286
	Crenolanib (CP-868596)
	TG101348 (SAR302503)
	PKI-402
	GSK1070916
	PHA-767491
	PF-04691502
	CCT137690
	CHIR-98014
	AZ628
	AMG458
	NVP-BGT226
	PHA-848125
	Arry-380
	ARQ 197 (Tivantinib)
	ARRY334543
	Wortmannin
	NVP-BVU972
	CH5424802
	3-Methyladenine
	Dinaciclib (SCH727965)
	Dovitinib Dilactic acid (TKI258 Dilactic acid)
	MK-5108 (VX-689)
	MK-2461
	AZD2014
	TAK-285
	INCB28060
	Tofacitinib (CP-690550, Tasocitinib)
	Sotrastaurin (AEB071)
	WP1066
	AZD4547
	CEP33779
	Dabrafenib (GSK2118436)
	GDC-0068
	INK 128
	BYL719
	Tyrphostin AG 879 (AG 879)
	Torin 2
	NVP-TAE226
	Tideglusib
	TPCA-1
	Desmethyl Erlotinib (CP-473420)
	Torin 1
	SAR131675
	Semaxanib (SU5416)

	Baricitinib (LY3009104)
	Golvatinib (E7050)
	IMD 0354
	WHI-P154
	TG 100713
	Piceatannol
	Tofacitinib citrate (CP-690550 citrate)
	VX-702

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