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ARTICLE

Effect of water deprivation for three consecutive days on the proportions of androgen receptor-immunolabeled supraoptic nucleus magnoneurons in Wistar rats

Samir Chetoui*, Hanane Touati, Soumia Remana, Nadir Benhaferi, Nadia Hanniche, Amina Talmatamar, Souhila Benarab, Abdelkader Bougrid

USTHB, Faculty of Biological Sciences, Laboratory of Biology and Physiology of Organisms, Neurobiology Team, BP 32, El Alia, 16111 Bab Ezzouar, Algiers, Algeria

ABSTRACT The androgen receptor is an androgen-dependent transcription factor that belongs to the nuclear receptor superfamily. When the androgen receptor is not bound to its ligand, it is mainly localized in the cytoplasm, bound to chaperone proteins, which stabilizes its inactive conformation and confers a high affinity for the ligand. The conformational change of the androgen receptor begins when the androgen molecule binds to the receptor, which subsequently homodimerizes and it is actively translocated to the nucleus. In the nucleus, the receptor homodimers bind to androgen target genes, resulting in responses such as growth and survival. In this study, we used the immunohistochemistry technique to look for the proportions of androgen receptor-immunolabeled magnoneurons categories according to the sub-cellular localization of the receptor, in the supraoptic nucleus of control (tap water + *ad-libitum* food) and dehydrated male rats by tap water deprivation for three consecutive days. Our results show that the proportions of androgen receptor-immunolabeled magnoneurons would depend on the hydration status of the rats (Chi-square test of independence, $P < 0.001$), but this dependence relationship is weak (Cramer's v value is equal to 0.30). Indeed, based on the results of our study, we hypothesized that dehydration by water deprivation for three consecutive days in adult male rats acts mainly on both of nuclear and cytoplasmic magnoneurons categories and has very little influence on the nucleocytoplasmic magnoneurons category. The effect could be due to activation of the entry of cytoplasmic non-androgen bound androgen receptors into the nucleus, activation of degradation of nuclear non-androgen bound androgen receptors, and inhibition of the binding of androgen molecules to androgen receptors. **Acta Biol Szeged 66(2):162-168 (2022)**

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***Corresponding author**
E-mail: chetouisamir3@yahoo.fr

Introduction

The NR3C4 gene encodes the androgen receptor (AR) (Jamroze et al. 2021), it comprises 8 exons on chromosome Xq 11-12 (Santos et al. 2004). This gene plays an important role in the growth and differentiation of prostate cells, as well as in the development and maintenance of male reproductive organs (Mellado et al. 2009).

Androgen receptor is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily (Mellado et al. 2009). This family includes receptors for steroid hormones, thyroid hormones, all-trans and 9-cis retinoic acid, 1,25dihydroxy-vitamin D, ecdysone, and peroxisome proliferator-activated receptors (Mellado et al. 2009). The AR protein comprises four domains: an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) (Jahan et al. 2021) that contains the COOH-terminal end of the protein. Two zinc finger motifs compose the LBD domain and determine the DNA sequences recognized by the AR. The LBD domain is composed of a region called activation function 2 (AF-2), it plays an important role in the transcriptional activity of the receptor. The DBD and LBD are linked by an amino acid sequence that forms the nuclear localization signal (NLS). The NTD domain houses activation function 1(AF-1), which appears to be the major transactivation domain of the receptor (Mellado et al. 2009).

The typical function of AR is to bind with its ligand and interact with its co-activators and chaperone proteins (Kumar et al. 2021). In the absence of its hormone (testosterone or its active metabolite, dihydrotestosterone (DHT), the AR remains in the cytoplasm, bound to

heat shock protein (HSP) complexes, which keep the AR in a conformation that allows it to bind to its hormone and protect itself from proteolysis (Mellado et al. 2009). Binding of the receptor to its hormone results in conformational changes and allows it to phosphorylate (Kumar et al. 2021). In the classical, or genomic, or canonical androgen signaling pathway, the AR binds to its natural ligands, dissociate from the HSP protein complex and dimerize. Then, the AR dimer translocates to the nucleus, where it binds to androgen response elements (AREs) in the regulatory regions of androgen target genes and subsequently modulates the expression of downstream genes (Jahan et al. 2021), resulting in responses such as growth and survival (Kumar et al. 2021). AR can also regulate gene transcription by interacting with other transcription factors without directly binding to DNA (Jahan et al. 2021). In addition, it can also repress gene expression through relatively poorly understood mechanisms (Jahan et al. 2021).

Non-classical, or non-genomic, or non-canonical androgen signaling pathways mediated by membrane-associated androgen receptors (mARs) have been described (Jahan et al. 2021). The mARs are ARs that are localized in the cell membrane or attached within the cell membrane or at other extranuclear locations. They can induce rapid intracellular changes when bound to their ligands (Jahan et al. 2021). Classical effects of androgen signaling take hours or days to materialize, whereas non-classical effects are within seconds or minutes (Jahan et al. 2021).

In this study, we wanted to investigate the proportions of AR-immunolabeled magnoneurons (SON), according to the sub-cellular localization of AR, in control male rats and in male rats deprived of tap water for three consecutive days, and to find out if these proportions of AR-immunolabeled magnoneurons would depend on the hydration status of the rats. To carry out this work, we chose as the brain nucleus to be used in the rat brain the SON, because it is involved in the hydro-mineral balance, and the magnoneurons that make up them are large, as well as their cellular nuclei, which helps to detect, with optical microscope, the sub-cellular distribution of AR after using the immunohistochemistry technique.

Materials and methods

In this study, we used eight male *Wistar* rats, nine weeks old, weighing 179-217 g, purchased from the Pasteur Institute (Kouba, Algiers). The animals were housed in the animal house of the Institute of Biological Sciences of the University of Bab Ezzouar (Algiers), under constant conditions of temperature (23.4 ± 0.2 °C: mean temperature ± SEM, respectively; SEM: Standard Error of Mean), humidity (38 ± 1%: mean humidity ± SEM, respectively) and light (10 h - 14 h: light-dark cycle, light on at 8 h). Each rat was placed alone in a conventional cage. The acclimation period lasted seventeen days, during which the rats had access to food (standard laboratory rat pellets, Bouzareah, Algiers) and tap water. After this period, the animals were subdivided into a control group (n = 5, 192.9 ± 6.0 g: mean weight ± SEM, respectively), with access to food and tap water *ad libitum*, and a dehydrated group (n = 3, 192.5 ± 9.6 g: mean weight ± SEM, respectively), for which only tap water was deprived for three consecutive days prior to the day of sacrifice.

At the end of the experimental period, the rats were anesthetized with *i.p.* injections of 25% urethane (1 ml injected/100 g rat body weight). We perfused intracardially them with isotonic sodium chloride solution (0.9%, 5 ml), followed by 10% formalin in 1 x Phosphate Buffer Saline (PBS; Concentration of PBS ready to be used after dilution to a physiological concentration, which does not alter the cells; 200 ml perfused / rat). We removed the fixed rat brains from their skulls, and we placed them in the same fixative fluid overnight post-fixation. The fixed brains underwent a first rinse in 1 x PBS for 30 min, followed by a second rinse overnight. Subsequently, the brains were dehydrated by ethanol of increasing percentage (70% ethanol / 1 h; twice 90% ethanol / 1 h; and three times 100% ethanol / 1 h). The clearing step was performed by three butanol baths, 1 h, overnight, and 1 h, respectively. We impregnated the brains twice in paraffin at 60 °C for 2 h each. We made paraffin blocks of the fixed rat brains using Leuckart bars. Histological sections of the supraoptic nucleus (SON) of fifteen micrometers thickness were accomplished with a manual rotary microtome of Minot type (American optical). We spread them on super-frost ++ glass slides and dried in the oven at 37 °C for two days.

The structures were dewaxed with xylene (20 min, twice), then hydrated with ethanol at a decreasing percentage (100%, 96%, and 70%, 10 min each), followed by rinsing in distilled water. We removed the endogenous peroxidase by placing the slides in 3% H_2O_2 in methanol for 30 min, followed by rinsing in 0.05% Tween in 1 x PBS (three times, 10 min each). We surrounded the structures with Dakopen (Dako, Glostup, Denmark) and we incubated them in 1:200 horse serum diluted in 0.05% Tween in PBS x1 for 1 h at room temperature to block aspecific sites. Then, the structures were incubated with the primary anti-androgen receptor antibody (ab 74272, Abcamplc, Cambridge, UK) at 1:100 overnight at 4 °C in a humid environment. The slides were rinsed with 0.05% Tween in PBS x1, three times for 5 min each. The structures were incubated with the biotinylated secondary antibody (Anti-Mouse IgG/Rabbit IgG, Cat.

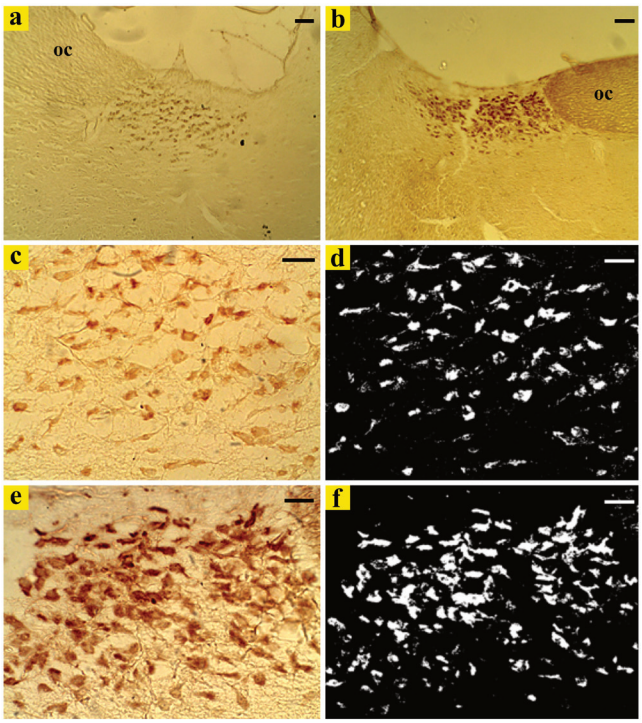

Figure 1. Photomicrographs of AR-immunolabeled magnoneurons from the supraoptic nucleus of control and dehydrated male rats. AR-immunolabeled supraoptic nucleus at x100 magnification from the control (a) and the dehydrated (b) rats. AR-immunolabeled magnoneurons at x400 magnification from the control rat (c) and micrograph of the same AR-immunolabeled magnoneurons from the control rat at the same magnification, processed with the H-DAB option of the imagej software, where we notice the presence of DAB deposits in the magnoneurons at the cytoplasmic, nucleocytoplasmic, and with a very low frequency at the nuclear level (d) AR-immunolabeled magnoneurons of the dehydrated rat at magnification x400 (e) and micrograph of the same AR-immunolabeled magnoneurons of the dehydrated rat, at the same magnification (x400), processed with the H-DAB option of the imagej software, where we notice, as in the control rat, the presence of DAB deposits in the magnoneurons at the cytoplasmic and nucleocytoplasmic level (f). Scale bar: (a-b) = 50 µm, (c-f) = 20 µm. OC: optic chiasma.

No. PK-6200, Vectastain® Universal Elit® ABC Kit, Vector Laboratories, Burlingame, CA, USA), diluted in 1:100 in 0.05% Tween in PBS x1, for 2 h at 4 °C in a humid environment. After rinsing with 0.05% Tween in PBS x1 (three times 5 min each), we incubated the structures in 1:100 Avidin-Biotin-Peroxidase complex for 1 h at room temperature. It was followed by another rinse with 0.05% Tween in PBS x1 (three times 5 min each). The revelation of the antigen-antibody complex takes place in the dark as a consequence of the formation of the diaminobenzidine complex (DAB). Once the brown deposits were observed under the light microscope, the chemical reaction was stopped in control and dehydrated groups by rinsing with PBS x1, dehydrated with ethanol at an increasing percentage (70%, 96%, and 100%; 3 min each), followed by xylene for 5 min. The slides were covered with coverslips by PDX (Polystyrene Dibutylphtalate Xylene). The experimental procedure was carried out according to the recommendations of "Association Algérienne des sciences en expérimentation animale" (AASEA) (http://aasea.asso.dz/).

In this study, we collected three hundred and seventy magnoneurons from nine supraoptic nuclei, selected from the five control rats. Similarly, two hundred and forty-two magnoneurons were collected from five supraoptic nuclei, selected from the three dehydrated rats. For each selected SON sample, RGB (Red, Green, and Blue) images in JPEG (Joint Photographic Experts Group) format, with a resolution of 2592 x 1944 pixels, were made by the Zeiss Axioplan optical microscope, equipped with a MA88-500 camera, at a magnification of x400. The JPEG images were transformed into TIFF format (Tagged Image File format; this, unlike JPEG, supports image compression without pixel loss) by the open-source software ImageJ version 2.1.0/1.53c (Schindelin et al. 2012) (http://imagej.net/software/fiji/downloads). After numbering the individual magnoneurons, the colors of the Tiff images were deconvoluted, and then the DAB deposits were located on the magnoneurons using the "H-DAB" option of ImageJ software. The magnoneurons were subdivided into three categories according to the localization of the DAB deposit in the cell: the nuclear magnoneurons category (where AR immunolabeling is localized only in the nucleus); the cytoplasmic magnoneurons category (where AR immunolabeling is localized only in the cytoplasm); and the nucleocytoplasmic magnoneurons category (where AR immunolabeling is localized in the nucleus and in the cytoplasm).

All statistical tests used are performed using R software version 4.1.3 (R Core Team, 2022) (https://www.R-project.org/). At $p < 0.05$, we considered all differences statistically significant.

Results

In our study, we observed that magnoneurons in the supraoptic nuclei of the control and dehydrated groups express AR. Our results show that AR localizes either in the nucleus or in the cytoplasm and either in the nucleus and cytoplasm at the same time (Fig. 1).

In the control group, out of the 370 magnoneurons analyzed, we found 64 nuclear- immunolabeled AR magnoneurons, 127 cytoplasmic-immunolabeled AR magnoneurons and 179 nucleocytoplasmic-immunolabeled AR magnoneurons (Table 1). Analysis of the control group shows that the proportions of AR-immunolabeled

Table 1. Proportions of AR-immunolabeled magnoneurons in the control and dehydrated groups, with their respective percentage.

AR-immunolabeled sub-cellular localization	Control		Dehydrated	
	Proportions	%	Proportions	%
Nuclear	64	17.30	5	2.07
Cytoplasmic	127	34.32	146	60.33
Nucleocytoplasmic	179	48.38	91	37.60
Sum	370		242	

magnoneurons differ according to their category types. The proportion of the nucleocytoplasmic magnoneurons category is significantly higher than the other two cytoplasmic and nuclear magnoneurons categories proportions (proportion test, $p < 0.001$) (Fig. 2; control group). The proportion of the nuclear magnoneurons category is significantly lower than the other two proportions of the cytoplasmic and nucleocytoplasmic magnoneurons categories (proportion test, $p < 0.001$) (Fig. 2; control group).

In the dehydrated group, of the 242 magnoneurons analyzed, 5 magnoneurons were in the nuclear category, 146 magnoneurons were in the cytoplasmic category, and 91 magnoneurons were in the nucleocytoplasmic category (Table 1). In this group, the proportion of the cytoplasmic magnoneurons category is significantly higher than in the nuclear and nucleocytoplasmic magnoneurons categories (proportion test, $p < 0.001$) (Fig. 2; dehydrated group). The proportion of the nuclear magnoneurons category, as in the control group, proved to be the lowest among the proportions of all three AR-magnoneurons categories (proportion test, $p < 0.001$) (Fig. 2; dehydrated group).

Analysis of the proportions of the AR-magnoneurons categories between the control and dehydrated groups shows that the proportion of magnoneurons in the cytoplasmic category does not change between the two groups (proportion test, $p > 0.05$) (Fig. 2), in contrast to the proportions of magnoneurons in the other nuclear and nucleocytoplasmic categories, which change significantly between the control and dehydrated groups (proportion test, $p < 0.001$) (Fig. 2).

Using Chi-squared test for independence, we concluded that the proportions of AR-immunolabeled magnoneurons categories in the supraoptic nuclei would depend on the hydration status of the experimental male rats (Chi-squared test for independence, $p < 0.001$).

Discussion

Our aim was to investigate whether the subcellular distribution of AR in magnoneurons of supraoptic nuclei of adult male Wistar rats would depend on their hydration state. As this topic has not been studied before, the discussion of our results remains essentially speculative because of the lack of bibliographic data.

Androgen receptors have been found in the brains of fish, amphibians, reptiles, birds, and mammals (Clancy et al. 1992). In the male rat brain, AR has been localized in neurons of the amygdala, hippocampus, nucleus of the stria terminalis, septum, preoptic area, several hypothalamic nuclei including supraoptic and paraventricular nuclei, several motor nuclei of the brainstem, and the cerebral cortex (Clancy et al. 1992). We found AR immunolabeling in the cytoplasm and cell nucleus of the supraoptic nucleus magnoneurons of the male rat in both the control and dehydrated groups. This result seems to agree with Roselli's work (1991), where AR was localized in the nucleus and cytoplasm of male rat brain neurons.

In the control group, we quantified a proportion of 48.38% and 34.32% for the nucleocytoplasmic and cytoplasmic magnoneurons categories, respectively; this would mean that AR non-androgen bound would be localized mainly in the cytoplasm (Gao et al. 2005), and that magnoneurons would rapidly re-enter a quantity of this cytoplasmic AR stock inside cell nuclei, which could explain the achievement in the control group of a significantly higher proportion of the nucleocytoplasmic magnoneurons category (48.38%) than the proportion of the cytoplasmic magnoneurons category (34.32%) (proportion test, $p < 0.001$) (Table 1). The proportion of the cytoplasmic magnoneurons category remains relatively high, because it would make up the reservoir of cytoplasmic AR non-androgen bound in case the cell nucleus will need more of this molecule, which could save the cell the step of receptor synthesis that can take some time to the magnoneuron. Also, we found a relatively very low proportion of the nuclear magnoneurons category (17.30%) compared with the two proportions found of the cytoplasmic and nucleocytoplasmic magnoneurons categories, which may tell us something about the significantly lower rate of the proportion of magnoneurons in the

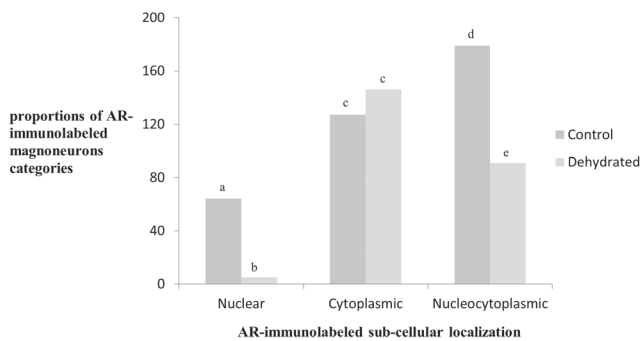

Figure 2. Comparison between the proportions of AR-immunolabeled magnoneurons according to the sub-cellular localization of AR, between control and dehydrated groups. (See text for more details).

nucleocytoplasmic category that completely empty their cytoplasmic AR pool, moving it to the nucleus. Since in the new model of AR trafficking that was updated by Cole and coworkers in 2021, there is no recycling of AR from the nucleus to the cytoplasm, as claimed in the classical model (see Bennett et al. 2010); thus, there is no turnover of the cytoplasmic AR pool, which left the cytoplasm in these magnoneurons devoid of ARs (Cole et al. 2021). In the nucleus, non-androgen-bound ARs can originate from two different sources: either they originate directly from the cytoplasmic non-androgen-bound ARs, where they have undergone direct translocation to the nucleus; or they can originate from the androgen-bound ARs, once transcription of the receptor target genes is complete (Cole et al. 2021). In either case, these non-androgen-bound nuclear ARs undergo degradation in the nucleus (Cole et al. 2021) (Fig. 3).

In the dehydrated group, a proportion of 60.33% and 37.60% were found for magnoneurons in the cytoplasmic and nucleocytoplasmic categories (Table 1.), respectively. This would show that non-androgen-bound AR would be localized primarily, as in the control group, in the cytoplasm (Gao et al. 2005), and that dehydration would not affect the predominantly cytoplasmic localization of AR. Magnoneurons in the dehydrated group bring less and less AR from their cytoplasmic stores into the interior of their cell nuclei, which influenced the proportion of the nucleocytoplasmic magnoneurons category (37.60%) to be significantly smaller than the proportion of the cytoplasmic magnoneurons category (60.33%) (proportion test, $p < 0.001$) (Table 1), which is still the highest among the three categories (proportion test, $p < 0.001$). The proportion of magnoneurons in the nuclear category of the dehydrated group is significantly smaller than the proportion of cytoplasmic and nucleocytoplasmic magnoneurons categories (60.33% and 37.60%, respectively) (proportion test $p < 0.001$) (Table 1), this would show that, in this group, very small proportion of nucleocytoplasmic magnoneurons category have their cytoplasmic AR stocks completely emptied into their cell nuclei (Fig. 2, dehydrated group; Table 1). This conclusion that was reached for the dehydrated group would be like that found in the control group.

When Chi-square test of independence (Pearson Chi-square test) was used, it was concluded that there is a dependent relationship between the hydration status of experimental male rats with the proportions of the magnoneurons categories (Chi-square test of independence, $p < 0.001$) (Table 2). To know whether this correlation was strong enough, the Cramer's v value (V) was also calculated. The Cramer's v value (V), which is derived from the Chi-square function, takes values between 0 and 1; if it is close to 1, we have a strong correlation, and

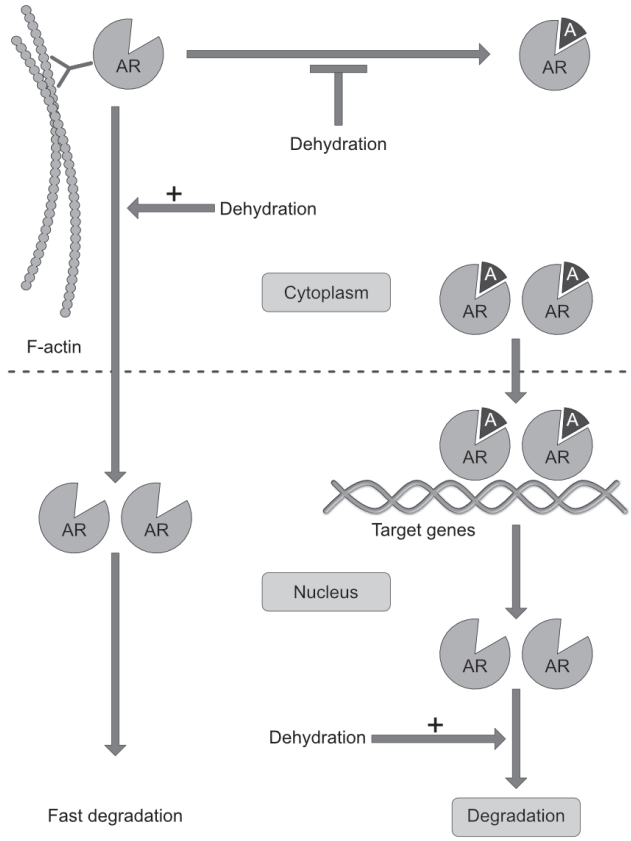

Figure 3. Hypothetical diagram summarizing our proposed model as a possible hypothesis of the effect of dehydration by water deprivation for three consecutive days on the sub-cellular distribution of ARs in male rat supraoptic nucleus magnoneurons. A: androgen; AR: androgen receptor; F-actin: filamentous actin (See text for more details).

if it is far from 1, it is a weak correlation. Our experiments resulted a V value equal to 0.30 (Table 2), so the dependence relationship between rat hydration status and the proportions of magnoneurons categories is weak.

We wanted to investigate the categories of magnoneurons in the control and dehydrated groups that strongly participated in this dependence. The $(\text{residual})^2$ value was calculated, which is a formula that calculates the influence of each magnoneurons category in establishing the correlation. Table 2 shows the $(\text{residuals})^2$ of the magnoneurons categories that participated in this correlation, whose values are in the following decreasing order: nuclear dehydrated magnoneurons category (18.20), cytoplasmic dehydrated magnoneurons category (13.41), nuclear control magnoneurons category (11.90), cytoplasmic control magnoneurons category (8.77), nucleocytoplasmic dehydrated magnoneurons category (2.32), and nucleocytoplasmic control magnoneurons category (1.52) (Table 2). These results suggest that the

Table 2. Results from the R statistical software scripts of the experimental data.

AR-immunolabeled sub-cellular localization		
Observed values	Control proportion	Dehydrated proportion
Nuclear	64	5
Cytoplasmic	127	146
Nucleocytoplasmic	179	91
Expected values	Control proportion	Dehydrated proportion
Nuclear	41.71569	27.28431
Cytoplasmic	165.049	107.951
Nucleocytoplasmic	163.2353	106.7647
Pearson's Chi-squared test: $\chi 2 = 56.138$; df = 2; p- value = 6.455e-13		
Cramer's value = 0.3028663		
Residual values	Control	Dehydrated
Nuclear	3.450242	4.266215
Cytoplasmic	-2.961672	3.662100
Nucleocytoplasmic	1.233897	-1.525710
$(\text{Residual})^2$ values	Control	Dehydrated
Nuclear	11.90417	18.20059
Cytoplasmic	8.771503	13.410975
Nucleocytoplasmic	1.522501	2.327791

proportion of the nuclear magnoneurons category in the dehydrated group is the category that participates most in this correlation (Table 2). When the proportion of this nuclear magnoneurons category between the control and dehydrated groups was compared, a significant decrease was found in the dehydrated group (Fig. 2). This result would seem to be a consequence of a possible activation of the degradation of non-androgen bound ARs in the nucleus of the nuclear magnoneurons category, when the ARs are detached from their genes after the transcription is completed. The immunohistochemistry technique used in these experiments does not detect the nuclear non-androgen bound ARs that originate from the cytoplasmic non-androgen bound ARs, because their degradation is rapid (Cole et al. 2021) (Fig. 3). Table 2 shoes that the $(\text{residual})^2$ of the cytoplasmic magnoneurons category of the dehydrated group is equal to 13.41, which allowed us to deduce that this category is strongly involved in establishing this correlation. We found that the proportion of the cytoplasmic magnoneurons category does not change between the control and dehydrated groups. This would seem to be the consequence of a double action of dehydration on the stock of cytoplasmic non-androgen bound ARs, there would be an activation of the recycling of this cytoplasmic stock towards the nucleus, and this activation would be counterbalanced by an inhibition of the binding of androgen molecules to cytoplasmic non-androgen bound ARs. This action of dehydration on cytoplasmic non-androgen bound ARs could also explain the decrease in the proportion of the nucleocytoplasmic magnoneurons category in the dehydrated group compared with the control group, following the decrease in the amount of cytoplasmic androgen-bound ARs, which can enter the cell nuclei (Fig. 3).

The nucleocytoplasmic magnoneurons category seems to have a very low participation in this correlation since $(\text{residual})^2$ values of 1.52 and 2.32 were found for the control and dehydrated groups, respectively (Table 2). This suggests that dehydration by water deprivation would act strongly in the nuclear and cytoplasmic magnoneurons categories, and weakly on the nucleocytoplasmic magnoneuron category, which is why Cramer's v of our experimental data is far from 1 (V = 0.30). Dehydration by water deprivation seems to involve only the two nuclear and cytoplasmic magnoneurons categories, and to spare almost totally the nucleocytoplasmic magnoneurons category, which represents 48.38% in the control group and 37.60% in the dehydrated group (Table 1). This weakens the strength of this dependence, although it exists, between dehydration and the proportions of magnoneurons categories, because it does not influence the total sample of magnoneurons in the control and dehydrated groups.

Conclusion

Presented experimental data displays that the water deprivation for three consecutive days has influence mainly on the two nuclear and cytoplasmic magnoneurons categories and it has little influence on the nucleocytoplasmic magnoneurons category. The hypothesis based on this suggests that this effect could be through activation of the penetration of cytoplasmic non-androgen bound ARs to the nucleus, degradation of nuclear non-androgen bound ARs, and inhibition of the binding of androgen molecules to ARs. Further work will be needed to consolidate this hypothesis, which gains importance if it can be shown that dehydration can inhibit the binding of the androgen molecule to the cytoplasmic AR in cells in tissues of organs that are places of cancerous processes (e.g., brain, prostate, and breast).

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