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The effects of bisphenol-A, zearalenone, arsenic and 4-methylbenzylidene camphor alone and in combination on the estrogen- and thyroid hormone receptor expression in developing rat cerebellum

PhD thesis

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0. LIST OF ABBREVIATIONS

As	arsenic
AraC	cytosine β-D-arabinofuranoside
BBB	blood brain barrier
BCA	bicinchoninic acid
BMR	basal metabolic rate
BPA	bisphenol-A
CNS	central nervous system
D2	type 2 iodothyronine deiodinase
DDT	dichlorodiphenyltrichloroethane
E1	estrone
E2	17β-estradiol
E3	estriol
ED	endocrine disruptor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	estrogen receptor
ERE	estrogen response elements
FSH	follicle-stimulating hormone
GFAP	glial fibrillary acidic protein
Glia+	glia containing
Glia-	glia reduced
GPR30	G-protein connected 7-transmembrane receptor
GnRH	gonadotropin-releasing hormone
HRE	hormone response element
HPG	hypothalamic – pituitary - gonadal axis
HPT	hypothalamic – pituitary - thyroid axis
HSD	hydroxysteroid dehydrogenase
IGF-I	insulin-like growth factor 1
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MBC	4-methylbenzylidene camphor

ntC	non-treated control
P0	postnatal day 0
PCB	polychlorinated biphenyl
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PVDF	polyvinylidene fluoride
qPCR	quantitative Polymerase chain reaction
ROS	reactive oxygen species
RXR	retinoid X receptor
T3	triiodothyronine
T4	thyroxine
TBT	tributyltin
TH	thyroid hormone
TR	thyroid receptor
TRE	thyroid response elements
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
WB	Western blot
ZEA	zearalenone

1. SUMMARY

The term “endocrine disruptor” (ED) refers to a group of substances, which – even in small doses – alter the physiological regulatory pathways of endogenous hormones, and thus, disorganize the normal neuroendocrine functions of the body. EDs are found almost everywhere. Animals, and human beings are continuously surrounded by a multitude of EDs from different origin. Consequence of ED incorporation depends on the time of exposure. Prenatal exposure mostly leads to serious developmental alterations inducing life-long complications, while incorporation during postnatal periods mainly lead to reversible changes of the physiological functions. The hormonal imbalance caused by these foreign substances is a result of dysregulated feedback loops and/or disturbed cellular signaling pathways. ED induced disturbance of the physiological synchrony between estrogen receptors (ERs) and thyroid receptors (TRs) during the central nervous system development could be more pervasive and far-reaching than currently appreciated, and merits investigation.

In this thesis the effect of four well-known and widespread EDs of different origins were tested, by examining the effect of individual and co-administered substances on the TR and ER mRNA and protein expression in cultures of neuronal cells. Postnatal rat cerebella were used as primers for the cell cultures. Proliferation of glia cells were inhibited by cytosine β-D-arabinofuranoside in half of the cell cultures. Following the ED and/or hormone treatments qPCR and Western blot methods were used to determine changes in mRNA and protein levels.

Interpreting the results of the conducted experiments the following conclusions can be drawn: 1.) All of the used EDs alone or in combination disrupt the TRα, TRβ and ERβ transcription and translation; 2.) Thyroid hormones and estrogens affect the interference caused by endocrine disruption. Combined ED effects are exerted when E2 and T3 levels are low; 3.) Glia modulates ED effects on the mRNA and protein level of receptors in the cultured cell populations, and most probably the neuronal receptor transcription and translation as well.

Disrupting the physiology of the neurons and the glia by exposure to the test substances may lead to yet unknown – either beneficial or adverse – biological consequences in the CNS, and in the neuroendocrine organs of the organism. By shedding light on the significance of EDs, we can eliminate or at least minimize the unfavorable effects of the disruptors present in our environment thus decrease the harmful consequences of the exposure.

2. INTRODUCTION AND LITERATURE OVERVIEW

2.1. Endocrine disruptors

The term “endocrine disruptor” (ED) refers to a group of substances, which – even in small doses – alter the physiological regulatory pathways of endogenous hormones, and thus, disorganize the normal neuroendocrine functions of the body. The hormonal imbalance caused by these foreign substances is a result of dysregulated feedback loops and/or disturbed cellular signaling pathways. The term “endocrine disruptor” (ED) refers to a group of substances, which – even in small doses – alter the physiological regulatory pathways of endogenous hormones, and thus, disorganize the normal neuroendocrine functions of the body. The hormonal imbalance caused by these foreign substances is a result of dysregulated feedback loops and/or disturbed cellular signaling pathways.

(1) Hypothalamus is the main integrator of the neuroendocrine feedback mechanisms directly regulating the hormone levels of the body. Direct or indirect interruptions of hypothalamic functions lead to serious hormonal alterations (Fliers et al., 2014; Zhou et al., 2014).

(2) Endocrine imbalance might also be caused either by influencing the hormone synthesis, release, storage, transport and degradation, or by altering the function of specific hormone carrier proteins (Bretveld *et al.*, 2006).

(3) The intracellular signaling cascades (e.g.: mitogen-activated protein kinase [MAPK], phosphoinositide 3-kinase [PI3K] pathways) that transfer endocrine signals within the cells towards the nucleus, might also be targets of EDs. Disruption of these pathways alters the cellular responses, thus indirectly altering the endocrine system (Pistol et al., 2015).

EDs are found almost everywhere. The animals and human beings are continuously surrounded by a multitude of EDs from different origin (Figure 1) (Zsarnovszky *et al*., 2007; Jocsak *et al*., 2016). Many of the EDs are able to breach the physical barriers through different mechanisms. For example, absorption through the skin or through wounds (or sometimes by simultaneous exposures: one of the EDs weakens the protective mechanisms of the organism helping the other substance to enter the body (Boonen et al., 2012; Nicolopoulou-Stamati *et al*., 2015), some substances may get into the bloodstream through mucous membranes (oral, nasal, eye,

respiratory tract, etc.) (Mandich et al., 2005; Suen et al., 2012), while orally incorporated EDs might be absorbed through the intestines.

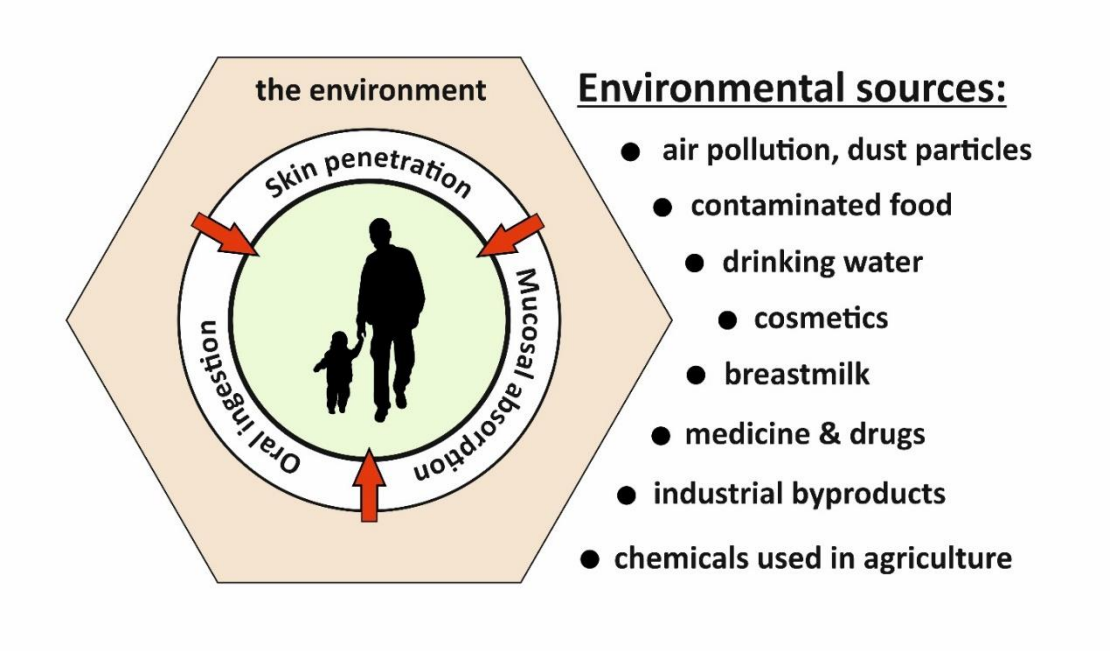

Figure 1: The possible sources of endocrine disruptor (ED) exposure. Humans and animals are continuously exposed to a number of environmental substances that act as endocrine disruptors (EDs).

After entering the organism, the effects of the toxic materials are usually described by a sigmoid dose-response curve: the chance of toxicosis is increased by elevated doses. Regarding the EDs, according to recent literature, this is not the case. Of course, EDs have a well-defined effect in the higher dose-ranges correlating with the actual dosage. On the other hand, an interesting phenomenon has been described in case of many EDs. Namely that the lower ranges (10^{-10} M) also have a potent effect causing a “U-shaped” dose-response curve (Calabrese and Baldwin, 2003).

Consequence of ED exposure depends on the developmental stage of the target organism. Prenatal exposure, for example, mostly leads to serious developmental alterations inducing life-long complications (activational effects), while incorporation during postnatal periods mainly lead to reversible changes of the physiological functions (modificational effects). An interesting phenomenon concerning ED exposure is that the substances may be “transmitted” to the consumer by food (e.g.: meat, or milk of contaminated animals), also from the mother to the fetus through the placenta or after birth with breast milk (Hagler *et al.*, 1980; Völkel *et al*., 2011;

Azzouz *et al*., 2016; Filippou *et al*., 2017), while in case of birds, even the egg might contain EDs (Zhu *et al*., 2015).

Altering the balance of the neuroendocrine regulation will lead to serious developmental, medical and even agricultural consequences. Depending on the point of disruption in the organism, EDs have a serious impact on the cellular components of the blood; the quality, and cellular quantity of the immune response (Jócsák *et al*., 2017); the homeostasis and the functions during detoxification of the liver and kidneys; the neuroendocrine organ functions (disrupting the regulative characteristics of specific parts of the hormonal milieu in the animals) (Chevalier and Fénichel, 2015; Palioura and Diamanti-Kandarakis, 2015) and even on the central nervous system (Weidner *et al*., 2013). Addition to the adverse health effects, EDs interfere with the reproductive physiology of animals, thus lower the possible productivity of the livestock causing major economic losses (Price *et al*., 1993; Jócsák *et al*., 2017).

From an economic point of view, the most dangerous EDs are the ones having estrogen-like effects, and therefore affecting the reproductive processes of the animals. These EDs – synthesized by the industry, or found as a natural compound – are called “xenoestrogens” (estrogen-like compounds originating from the environment). The natural xenoestrogens are found in plants (as fitoestrogens), and in specific fungus species (mycoestrogens). On the other hand, xenoestrogens are also produced by industrial activities. Often, they are by-products of plastic creations (polychlorinated biphenyl [PCB], bisphenol-A [BPA], phtalates), but industrial xenoestrogens may also originate from the agriculture (as pesticides or fungicides; dichlorodiphenyltrichloroethane [DDT], vinclozolin), or they can be found in household products (e.g.: paint – tributyltin [TBT]). In the experiments in this thesis we used four well-known and widespread EDs of different origins (Figure 2):

1. Bisphenol-A (BPA), an industrial byproduct during plastic synthesis, banned in the European Union since 2011, currently under re-evaluation;
2. Zearalenone (ZEA), an exogenous mycotoxin well-known for its adverse effects in livestock animals, causing major losses in both animal counts and agricultural profit in Europe;
3. Camphor (4-methylbenzylidene camphor, MBC) a natural phytoestrogen found as a component of most cosmetic and medical product worldwide, and
4. Arsenic (As), a simple but especially strong substance with an ED characteristic, occurring naturally or after contamination in groundwater and drinking water.

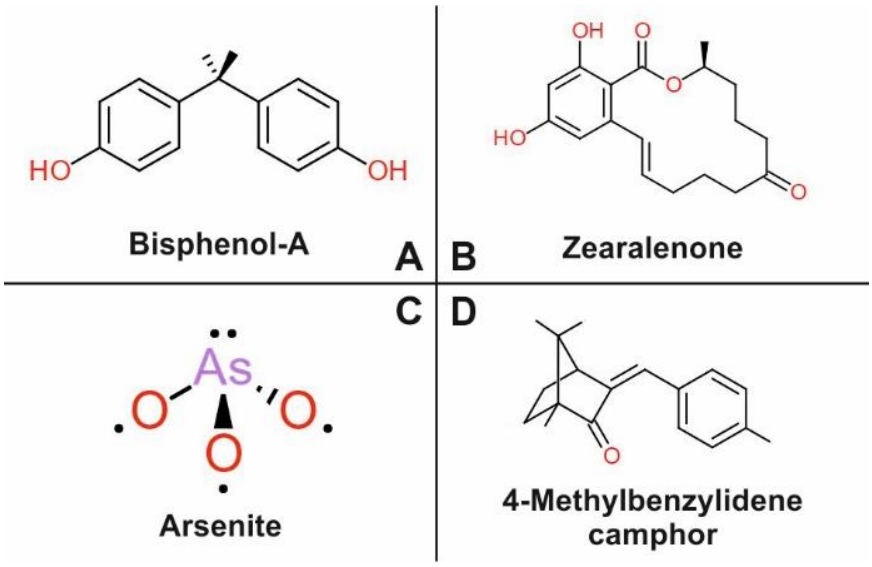

Figure 2: The endocrine disruptor (EDs) substances used in our experiments.

2.1.1. Bisphenol-A

Bisphenol-A (BPA; Figure 2/A) is a well-known and widely researched organic compound synthetized by the plastic industry. The molecule is used in the manufacture of certain plastics and epoxy resins, including a variety of common consumer goods, such as water bottles and feeding bottles, food containers, sports equipment, re-usable plastic tableware, CDs, and DVDs. BPA can also be found in dental sealants and other medical devices (Olea *et al*., 1996). BPA containing epoxy resins are used to line water pipes, as coatings on the inside of many food and beverage cans, from which potential contamination of the food is very likely. BPA is also found in thermal paper such as that used as sales receipts.

The main route of BPA exposure is oral ingestion as food contaminant, but it also absorbs through the skin. In the body, BPA is able to mimic the natural hormone estrogen (17-beta-estradiol [E2]) (Dodds and Lawson, 1936), therefore, since 1997, it has been considered as an ED. Firstly, it was considered as a weak estrogen-mimicking substance with minimal significance on the organism, but later studies on the molecular mechanisms revealed that even in small doses, receptor binding (to estrogen receptor α, β; [ERα, ERβ]) stimulates a huge variety of different intracellular pathways and cell responses (Wozniak *et al*., 2005; Welshons *et al*., 2006; Alonso-Magdalena *et al*., 2012). Recent literature suggest that excessive BPA exposure can be linked to several reproductive disorders: reduced fetal survival, advancement of puberty, ovarian malformations and reductions in maternal and fetal body weight (Ranjit *et al*., 2010; Adewale *et*

al., 2011). Besides the negative impact on reproductive functions, other adverse effects have also been indicated. For example, the quality and quantity of the immune response (the T cell count, B cell functions, and dendritic cell and macrophage physiology) is altered by the disruptor as well (Rogers *et al*., 2013). Based on the diverse mechanisms of endogenous estrogen, the estrogen-mimicking BPA might be responsible for a huge number of other pathological conditions. Furthermore, studies indicated that BPA also affects the thyroid functions of the organism by binding to thyroid hormone receptors (α, β; [TRα, TRβ]) (Moriyama *et al*., 2002; Zoeller, 2007). The physiological process of thyroid and estrogen signaling within the brain is essential for healthy orchestration of CNS in infants. Therefore, manifestation of serious developmental disorders in neonates is quite possible, if pregnant women are exposed to BPA during gestation (Boas *et al*., 2009; Vandenberg *et al*., 2009).

BPA is poorly soluble in water, but it dissolves in organic solvents. Derived from consumer products, it can significantly contribute to the pool of estrogenic substances in the environment (Krishnan *et al*., 1993).

2.1.2. Zearalenone

Zearalenone (ZEA; Figure 2/B), also known as F-2 mycotoxin, is an exceptionally strong mycoestrogen, a mixed agonist-antagonist of specific receptors (e.g.: ERα and ERβ) (Kuiper *et al*., 1998). The toxin is synthetized as a secondary metabolite by *Fusarium* and *Gibberella* molds (Kuiper-Goodman *et al*., 1987). The fungal infection of agricultural grain products (for example maize, barley, oats, wheat, rice, and sorghum) may start at the cultivation area, before or after harvest (in the silage). Improper storage favors the proliferation of the fungi, so they may reproduce in a short time on the forage kept in a dark and damp environment (Escrivá *et al*., 2015). A European study examined 5010 grain samples in 2004, and found F-2 mycotoxin contamination in 32% of samples (Schothorst and van Egmond, 2004). In Hungary, Fazekas *et al*. (1996) found ZEA in 17% of the analyzed probes. These studies prove that ZEA exposure is a serious problem in agriculture implying a notable risk on animals and humans as well.

Ingestion of contaminated grain product (as forage or processed food) transfers the toxin into the body of the livestock or the human beings. In some organs (mostly liver, kidneys, testes, prostate, ovaries, intestines and the hypothalamus), a specific enzyme group called 3α- and 3β-hydroxysteroid dehydrogenase (HSD) break ZEA down into metabolites (α- and β-zearalenol; zearalanon; α- and β-zearalenon). Some of the metabolites possess a stronger estrogen-like

effect than the original molecule (for example, estrogen-like effect of α-zearalenol is 3-100 times higher than ZEA) (Kiessling *et al*., 1984; Dong *et al*., 2010).

After entering the circulation, ZEA and its metabolites reach the ERs all over the body. The chemical bond between the ER and the substances is 20 times lower as in case of E2 (Kuiper-Goodman *et al*., 1987). The estrogen-like molecular structure allows the mycotoxins to exert an anabolic effect on the target cells. The most important and well researched targets of ZEA are the neuroendocrine and reproductive organs. In those organs, the mycotoxin causes severe anatomical and physiological disorders leading to anoestrus, pseudopregnancy, elevated chance of stillbirth and developmental disorders in the developing fetus of the pregnant animal. The effect of ZEA is not restricted on reproduction however, it affects the immune system, the physiology of the bones, liver, kidneys and the CNS, as well (Kuiper-Goodman *et al*., 1987; Price *et al*., 1993; Seeling *et al*., 2006; Hueza *et al*., 2014; Escrivá *et al*., 2015; Jócsák *et al*., 2017). The mycotoxin (and its metabolites) is able to penetrate the blood brain barrier (BBB), therefore they easily reach the cells of the CNS (Weidner *et al*., 2013). Furthermore, the mycotoxin may also disintegrate the BBB, possibly weakening the animal against other toxins and diseases (Wang *et al*., 1998; Chaudhary and Lakshmana Rao, 2010; Ravindran *et al*., 2011).

2.1.3. Arsenic

Arsenic (As) is a metalloid, a chemical element with the atomic number 33. Usually it can be found in many minerals (in combination with sulfur and metals) or in pure form as a crystal, and it can be found naturally in the groundwater. Some of the naturally occurring common oxidation states of arsenic are As^{III} (arsenite; +3; Figure 2/C) and As^{V} (arsenate; +5), and they have the potency to alter the neuroendocrine system. Due to industrial use of As in semiconductors, and in alloys of lead (ammunition, car batteries), and improper waste disposal, As contaminates the soil, groundwater and food. Also, the agricultural use as a component in herbicides, pesticides, and insecticides, and wood preservative agent contributes to the chance of exposure in environments (Watson and Yager, 2007). Contamination of drinking water occurs globally, and – as the anthropogenic activity is increasing – the amount of As is continuously accumulating in the environment. In Hungary arsenic can be found in the groundwater in the Southern Great Plain, exposing many individuals to As via drinking water (Varsányi *et al*., 1991; Lindberg *et al*., 2006) This problem affects millions of people across the world, even in First World countries (Vromman *et al*., 2014).

Arsenic is associated with different cancer types, mostly due its estrogenous activity. Arsenic was indicated as a causing agent in skin, lung, breast and bladder cancers. It is also involved in

cardiovascular diseases, and – as a potent ED – it disrupts the gonadal, adrenal and thyroid functions, as well (Muñoz *et al*., 2015; Huff *et al*., 2016; Sun *et al*., 2016). The role of arsenic has been indicated in many reproductive disorders (Reilly *et al*., 2014; Sengupta *et al*., 2015).

As^{III} has a potent E2-like activity, and can connect to ERs altering the expression levels or E2 responsive genes. On the other hand, As^{V} does not have any direct effect on the endocrine systems, but it can be enzymatically converted to As^{III}, therefore it may provide an arsenite „reservoir" (Romagnolo *et al*., 2016). Besides the estrogenic effects, As^{III} affects the thyroid functions, and it lowers the TRβ and deiodinase type 2 mRNA levels (Gibson *et al*., 2016).

2.1.4. 4-methylbenzylidene camphor

Camphor (4-methylbenzylidene camphor, MBC; Figure 2/D) is a naturally occurring fitoestrogen found in some tree species like camphor laurel (*Cinnamomum camphora*), kapur trees (*Dryobalanops aromatica*), East African camphorwood (*Ocotea usambarensis*), but rosemary oil also contains ~20% of MBC. In the industry, MBC is synthetically produced from turpentine oil. MBC is used for its scent, as an ingredient in cooking, an embalming fluid, and in religious ceremonies. As an ED, Camphor affects both the E2 and TH levels in the organism. It has E2-like agonistic effects on cells with ERs, and elevates serum luteinizing hormone (LH), and lowers serum T4 levels (reduced serum T4 leads to increased thyroid-stimulating hormone [TSH] secretion) (Schlumpf *et al*., 2004; Seidlová-Wuttke *et al*., 2006).

Although it can be absorbed through mucous membranes, and gastrointestinal tract, the main route of MBC exposure is through the skin. It might penetrate the epidermis and accumulate in the dermis (Sasson *et al*., 2009). This is the cause of the wide usage of MBC in medicine, and as a cosmetic ingredient. Camphor acts as a local anesthetic and antimicrobial agent (Chen *et al*., 2013; Tran *et al*., 2015). It modulates the physiology of the thermoreceptors in the skin, causing a local „coolness sensation" (Green, 1990). It protects the skin as an UV filter, has potent wound healing properties and acts as an anti-wrinkle agent (Tran *et al*., 2015). As result of these effects, MBC is widely used for medicinal purposes as balms or ointments, treating burns. Also, MBC is widely used by the cosmetic industry as a vehicle to assist other substances in penetration to the outer skin layers. Due to this attribute of the substance, many cosmetics contain it marked as „UV filters", even those that are not sunscreens or sun lotions (Nicolopoulou-Stamati *et al*., 2015). Most of these cosmetics contain a mixture of substances – possibly other EDs as well (for example BPA, Parabenes, Phtalates, Aluminium salts). If the barrier function of the skin is weakened, or the entry is made easier by MBC, there is an

elevated risk of exposure simultaneously to any substance in contact of the organism. The possible toxin combinations may strengthen the effect of endocrine disruption.

Camphor is highly lipid soluble and easily crosses the blood brain barrier. In the CNS the substance act as a stimulant with effects that range from mild CNS excitation to generalized seizures (Manoguerra *et al*., 2006; MacKinney *et al*., 2015).

2.2. The estrogen and thyroid hormone system

The endocrine disrupting potency of the above mentioned EDs is a result mostly of their ability to bind to ERs and TRs. Available data suggest that E2 and THs are equally important regulators of cerebellar development (Vasudevan *et al*., 2001; Zhao *et al*., 2005). Based on the previous studies by our research group (Somogyi *et al*., 2011) and the existing literature it is strongly possible that the disturbances caused by EDs will alter the physiological synchrony between the hormones of the neuroendocrine system (in the activational and modificational level).

2.2.1. Thyroid hormones and receptors

Thyroid hormones (TH) triiodothyronine (T3) and its prohormone, thyroxine (T4) are mainly metabolic regulator hormones produced by the thyroid gland. In the circulation mainly T4 can be found, in the tissues specific enzymes called deiodinases convert it to T3 (the active form of the THs). T3 acts as a metabolic stimulator, although this effect might be tissue specific. TH production is regulated by the neuroendocrine system: the hypothalamus in the hypothalamic–pituitary–thyroid axis (HPT) actively monitors the T4 and T3 serum levels. As a negative feedback thyrotropin-releasing hormone (TRH) is released as a response to the change in blood TH concentration. TRH stimulates the thyroid-stimulating hormone (TSH) production of the pituitary gland, which in turn stimulates the TH production of the thyroid gland until hormone levels in the blood return to normal. Due to the feedback system a given substance – e.g. an ED – can alter the TH balance by affecting the HPT axis on a number of points.

In the tissues TH levels are regulated by deiodinases. T4 and T3 are partially composed of iodine. Deiodination is a process where the passive form of the THs (T4) are converted by the enzyme to the bioactive form (T3) by reducing the iodine content of the substance by 1. Specific cell types in the targeted tissues have the ability to locally produce T3 from T4, resulting in a tissue specific local response depending on the types of deiodinases and the receptors in the target organs (St. Germain *et al*., 2009).

The majority of TH actions are mediated through the binding of T3 to TRs located in the cell nucleus. The receptors act as ligand-modulated transcriptional factors, after activation they modify the targeted gene expression through thyroid response elements (TRE) located on specific DNA segments. Thyroid hormone receptors have distinct functional roles during development of the mammalian CNS. Three TR subtypes (TRα1, TRα2 and TRβ1) can be found in mammals (Bradley *et al*., 1994). The TRα subtypes are localized mainly in the heart, bones,

gastrointestinal tract and in specific neurons while TRβ isoforms can be found in the liver and in the brain (pituitary, hypothalamus, cerebellum) (Schoenmakers *et al*., 2013; Mendoza and Hollenberg, 2017).

Both isoforms modulate the majority of the same target genes, however, some TR isoform specific signaling pathways were observed on a few target genes in specific cell cultures (Lin *et al*., 2013), furthermore specific genes can have a receptor selective response to THs (Chatonnet *et al*., 2012). It is currently still unclear where the TR subtypes are localized in the cerebellum. TRα can be found mainly in the granule cells, TRβ is a main receptor during the maturation of the Purkinje cells. Recent literature presents contradictory statements about TRβ localization, it is still not clear whether it can be found in the granule cells or it is absent from them (Strait *et al*., 1991; Fauquier *et al*., 2014). Although the TH effect is greater during cerebellar development, interestingly the levels of TRs are greater in the adult cerebellum (Bradley *et al*., 1992).

THs mainly act through the stimulation of basal metabolic rate (BMR), and stimulates catabolic processes in the cells of the organism. T3 – the active TH form – stimulates the heart by increasing the cardiac output, and the heart rate. The hormone elevates the ventilation rate in the lungs, and it thickens the endometrium in the females. An adequate T3 level is necessary for normal bone and cartilage development. In the neuroendocrine system T3 increases sympathetic activity by potentiating the catecholamine effects.

One of the main functions of the THs are the coordination of brain development. The altered balance of the THs (hormone deficiency or overproduction) during pre- and postnatal growth might lead to many developmental disorders in the CNS (Ng *et al*., 2009; Ibhazehiebo *et al*., 2011; Stenzel and Huttner, 2013). The active compound of the THs is T3, it is converted locally from T4 with the help of the type 2 iodothyronine deiodinase (D2) found in astrocytes. Without the glial support the nerve cells lack of T3, however, T4 regulates the development of specific cell types in the CNS. The arborization of Purkinje cell dendrites were induced after T4 treatment (Koibuchi, 2013), and it caused an increase in neurite extension in purified cerebellar granule cell culture as well (Ibhazehiebo *et al*., 2011). These experiments indicate that T4 acts directly on the development of the CNS through a non-genomic pathway. One possible method of activation is found by Bergh *et al*. (2005), integrin αVβ3 acts as a cell surface receptor for T4. The receptor is linked to MAPK activation, thus T4 can influence specific intracellular pathways without nuclear receptor activation. Inadequate T3 amount in the brain is associated with neurological diseases and interestingly a longer life span. Furthermore, lower T3 amount leads to elevated circulating TSH levels due to the negative feedback. Excessive amounts of TSH may

be associated with mental disorders like schizophrenia or bipolar disorder (Wysokiński and Kłoszewska, 2014; Jansen *et al*., 2015).

2.2.2. Estrogen hormones and receptors

The Estrogen hormones estrone (E1), estradiol (E2), and estriol (E3) are steroid hormones, and part of the sex hormones in animals (the others being androgens and progesterons), playing a critical role as the female sex hormone. Estrogens can be found in the males as well, although in a significantly lower level. Mainly, estrogens are responsible for the development of the female reproductive system and the secondary sex characteristic, and they have a regulatory role on the developed systems as well. Similarly to the HPT axis the estrogen levels are maintained and regulated by the hypothalamic–pituitary–gonadal axis (HPG). The Gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus regulates the LH and follicle-stimulating hormone (FSH) secretion of the pituitary gland. The gonads produce estrogens (and testosterone) reacting to the levels of LH and TSH, finally the levels of estrogens regulate the functions of the hypothalamus and the pituitary gland by a feedback mechanism.

Due to the steroid nature of estrogens they can diffuse through the cell membrane, and bind to estrogen receptors (ERs). Reports by Kirby *et al*. (2004) support the idea that E2 stimulates cell migration through ERs. The receptors are part of the nuclear receptor family; they modulate the expression of specific target genes after activation (thus enabling the developmental role of estrogens). The nuclear ERs can be divided to two subtypes, ERα and ERβ. It is accepted that expression of ERα is related to reproductive function (Ogawa *et al*., 2011) and its expression level steadily changes throughout early postnatal life showing sexual dimorphism (Camacho-Arroyo *et al*., 2003). ERα and ERβ play crucial roles in neural tissue development, with ERα being expressed in the fetal brain and during early postnatal days and ERβ persisting life-long in most cerebellar cell types (Belcher, 1999; Wang *et al*., 2001). ERβ is necessary for neuronal survival and the developmental formation of the cerebellar cytoarchitectonics. Knock-out of ERβ leads to several morphological alterations in the cerebellar tissue, most likely due to the lack of estrogenic effects in the regulation of cell migration (Wang *et al*., 2001). Parallel to the “classic receptor pathway” through ERα,β receptors (activated estrogen response elements [ERE] of the target genes) there is a rapid, non-genomic pathway through a G-protein connected 7-transmembrane receptor (GPR30) (Prossnitz *et al*., 2007; Micevych and Kelly, 2012; Soltysik and Czekaj, 2013) as well.

Estrogens are present in both sexes, although they are present at significantly higher levels in women of reproductive age. Almost every cell has ERs but the distribution of the receptors are different in the target tissues. A vasoprotective action and anti-inflammatory properties were observed by estrogens. Due to a strong metabolic effect, they increase the storage in the fatty tissue, thus having an important role in the development of female secondary sex characteristics during puberty. Estrogens maintain the estrus cycle and they are essential during pregnancy. In males estrogens have a role in the maturation of the sperm. In both sexes the sexual behavior is regulated by estrogen, and it has a positive effect on the libido.

Estrogens play a critical role during the development of the CNS, and they are important regulators of the neuron and glial cell physiology. In the CNS ERα-level is present in the neonatal rat cerebellum, however, in a significantly lower level than ERβ (Guo *et al*., 2001). In the first 4 days of the postnatal development the ERα mRNA and protein level is low, between 4 and 10 days the expression level steadily grows, and declines between days 10–15. The ERß mRNA and protein expression shows a complementary pattern, as seen it in the case of ERα. It dominates in the first 4 days, declines between 4–10, and grows between 10–15 days of development (Wang *et al*., 2001; Fan *et al*., 2010; Belcher, 1999).

The main functions of estrogens are the development and regulation of almost all of the mammalian tissues. If the balance of the regulatory hormones becomes disturbed (by endogen or exogen factors) the outcome may lead to serious anatomical and physiological alterations. Disturbances in estrogen regulation might lead to tumor development, mostly in the primary and secondary sexual organs, e.g.: breast cancer and prostate cancer (Withanage *et al*., 2001; Chen *et al*., 2002; Pillay *et al*., 2002). Hyperestrogenism is linked with many physiological alterations like an increased risk of thrombosis, myocardial infarction, and stroke (Wang *et al*., 2001; Artero *et al*., 2012).

Estrogen insufficiency alters the CNS as well. Inadequate hormone levels might lead to developmental problems in the fetus or in neonates (Wang *et al*., 2001; Maerkel *et al*., 2007; Fan *et al*., 2010). Insufficient estrogen levels are connected with disturbances in the higher cognitive functions of the brain by altering the physiology of the synapses (Hara *et al*., 2015; Korol and Pisani, 2015), a phenomenon which even leads to secondary behavioral or psychological problems. Optimally the HPG axis keeps the effect of estrogen within physiological levels, but due to the fact that the regulatory chain can be influenced on many points in the organism, the balance is strongly vulnerable against estrogen-like substances, like the EDs.

2.2.3. Signaling pathways

Most of the EDs act through different signaling pathways found in the cell. Due to the estrogen-like structure of the substances, after ED exposure a specific part of the intracellular signaling pathway will be activated or inactivated by the molecules. Natively, TH and E2 acts on the TRs and ERs trough at least four known signaling pathways in the affected cells (Figure 3):

1) Genomic estrogen signaling with an E2-ER dimer complex binding to ERE sequences in the nucleus.
2) ERE independent genomic actions on target genes.
3) Membrane initiated non-genomic E2 signaling (on ERs or the GPR30 receptor, through MAPK and PI3K pathways).
4) Genomic TH signaling by T3 binding on TR, TR-retinoid X receptor (RXR) complex binding to TRE sequences in the nucleus.

Additionally E2 may act on steroid receptors found in the mitochondria, thus influencing the cellular energy balance (Arnold *et al*., 2012; Rettberg *et al*., 2014). The cross-talk between the mitochondrial effects and the genomic and non-genomic pathways will lead to the final response in the cell (Zane et al., 2014). As an end-result of decreased ER-TR mediation of hormone effects, functional deficiencies evolve later in life (Andreescu et al., 2007). Thus, some of the developmental roles of TRs and ERs are well established; however, little is known about the potential influential effects of the different EDs on cerebellar TR and ER expression. To the best of our knowledge, no report thus far is available on the effect(s) of more EDs combined on cerebellar, or for that matter, any other cell type's TR & ER expression.

Endocrine disruptors may alter the fine balance between the listed pathways. The method of disruption depends on the type of ED, hypothetically all biochemical processes may be altered between the receptor activation and the gene transcription. EDs might act as an agonist, or an antagonist of the target receptor, depending on the structure and biochemical traits of the substance. Considering the lack of experiments on the intracellular mechanisms of specific EDs, and the absence of specified antagonistic/agonistic roles of the substances, it is necessary to conduct an experiment with an "ED cocktail" – a mixture of different EDs. This may shed light on the possibly cumulative physiological effects of the adverse substances in our environment. It is important to note, however, that the additive characteristic of the possible adverse final effect is just a hypothesis. It is still a possibility that the different ED effects will somehow counterbalance

themselves and as a result of the cumulative outcome the physiology of the target cells will be disturbed but only to a lesser extent than we anticipated (if we will detect any change at all).

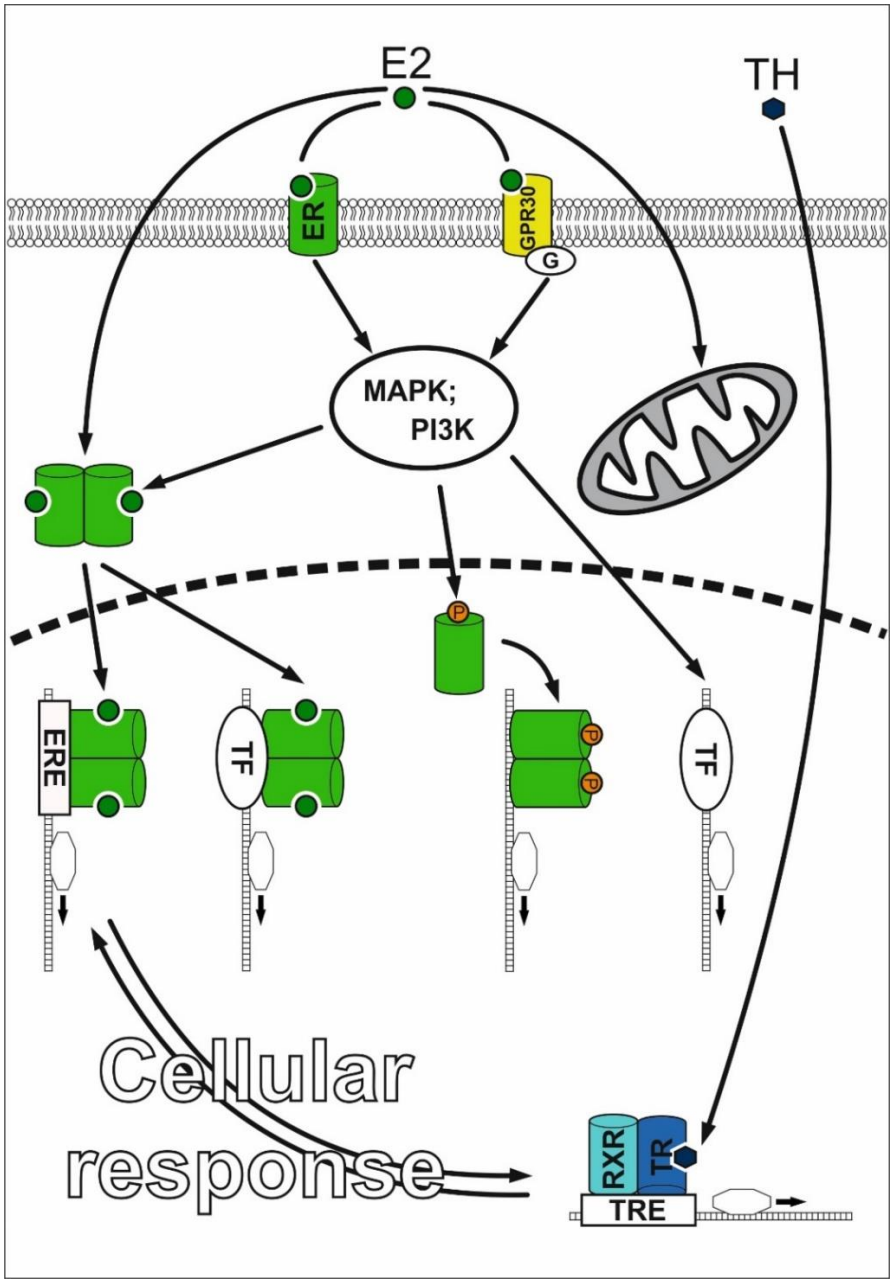

Figure 3: The cellular response mediated by estrogen (E2) and thyroid hormones (THs). E2 and THs regulate cellular homeostasis, proliferation, development, motility, differentiation and even cellular death – apoptosis. Activated estrogen receptors (ERs) form a dimer. The ER dimer – estrogen response element (ERE) combination elicit gene transcription. E2 on membrane receptors, and stimulated ERs may activate other transcription factors (TFs) as well. After TH activation on the thyroid receptor (TR), a TR – RXR dimer is formed. TR-RXR activate the thyroid response element (TRE) on the target genes. The "final cellular response" is a result of a cross talk between E2 and TH effects on genomic and non-genomic cellular pathways.

2.2.4. Using primary cerebellar cell culture as a model

Contact can be made with bisphenol-A (BPA), zearalenone (Zea), arsenic (As) and 4-methylbenzylidene camphor (MBC) in our environment almost daily. The mentioned substances can be found in our food, in the water, on our clothes, in the cosmetics we use during the day and even in the objects made from plastic or wood around us. Due to the numerous adverse effects associated with those substances, but considering the lack of studies in the topic of combinatory effects, it is necessary to observe what happens in the cells of the CNS after a simultaneous ED exposure. To test the neuronal effects of the four EDs, individually or combined, on the mRNA and protein expression levels of TRα, TRβ and ERβ, we needed a primary neuronal cell culture containing the target receptors, supporting glial cells and a steroid free environment.

In the developing CNS, there is a highly complex interplay between E2 and THs in the maintenance of normal levels of each-other's cognate receptors, and that the hormone effects are most probably mediated by the glia. Our observations implicate that abnormalities in glial and/or thyroid functions or in tissue E2/TH levels impact, on multiple levels (e.g.: neural development, cerebellar functions later in life, and the regenerative capability of the cerebellar tissue in case of injury). All of which should be considered in the diagnostics and treatment of relevant clinical conditions (Scalise *et al*., 2012). Developmental abnormalities may also lie on the grounds of altered glia differentiation or due to insufficient TR-ligand interactions (Mendes-de-Aguiar *et al*., 2010; Martinez *et al*., 2011; Picou *et al*., 2012). In addition to single or combined hormone effects, based on the relevant literature and on our previous research, we hypothesized that the astroglia cells have a major role in maintaining and controlling the hormonal environment during the CNS development in young animals (Guadaño-Ferraz *et al*., 1997; Pruvost *et al*., 1999; Scalise *et al*., 2012). Due to the supporting nature of the glial cells, it is a strong possibility that they might mitigate the (presumably) negative effect of the endocrine disruption on the neuron cells.

In our previous experiments by Somogyi *et al*. (2011) we compared the TR and ER expression levels from glia containing cell cultures (Glia+) with samples containing glial cells with inhibited proliferational ability (Glia-). The experiment showed that the absence of glial cells resulted in a non-physiological receptor expression. Glial cell count and astrocyte density in specific brain areas may decrease due to many factors. Foreign substances can cause cell cytotoxicity in the glial tissue (Seo *et al*., 2017), even emotional disorders may affect the physiological cell count

(Frintrop *et al*., 2017). Due to the cumulative effects of possible EDs, toxins, neurological and emotional disorders that damage the physiological state of the neuronal tissue, it is not negligible to discuss the results from Glia- cultures (a possible non-physiological „state" of the organ).

Due to the receptor availability on the cerebellar granule cells, we decided to use the postnatal rat cerebellum as a source of primary cells and as a cell culture model. Estrogens and THs play critical roles in the regulation of neural development (Jakab *et al*., 2001; Ikeda, 2008; Fan *et al*., 2010). Estrogen receptors are expressed in the cerebellum from early ages (postnatal period in rats) in a developmentally regulated fashion (Belcher, 1999; Price and Handa, 2000; Jakab *et al*., 2001). During postnatal life, the ERβ receptor can be found on the Purkinje cells, stellar cells, basket cells and Golgi cells. The migrating glial cells, and the developing neurites also express the ERβ receptor protein (Price and Handa, 2000; Jakab *et al*., 2001). Likewise, THs also target just about all cell types in the cerebellum (Wallis *et al*., 2010; Fauquier *et al*., 2014). Abnormalities in the hormonal milieu or in the expression and function of ERs and TRs result in deviations in the normal development of this brain area. For example, in individuals with hypothyroidism the migration and terminal differentiation of granule cell precursors could be impaired (Fauquier *et al*., 2014). Both the *in vitro* experimental model (primary cerebellar cell culture) applied in this study (Jakab *et al*., 2001) and the expression characteristics of ERβ (Belcher, 1999) have been described previously in details. In addition, we recently demonstrated that the expression of ERβ (as well as that of TRs α and β) are regulated by both E2 and the TH triiodothyronine (T3).

2.3. Aims of the Thesis

Animals, and human beings make contact with endocrine disruptors frequently. EDs can be found almost everywhere in our environment, and after incorporation it is a strong possibility that the different substances can strengthen the effect of each other. The exact method of disruption on the neuroendocrine system is not known due to the complexity of the signalization pathways between the hypothalamus, hypophysis, thyroid gland, gonads and the target cells. One of the route of distruption happens through the disruption of the hormone–receptor complex.

The hormonal imbalance caused by EDs is a result mostly of their ability to bind to estrogen receptors (ERs) and TH receptors (TRs) causing dysregulated feedback loops and/or disturbed cellular signaling pathways. Based on the existing literature and our studies, ED induced disturbance of the physiological synchrony between E2 and THs during CNS development could be more pervasive and far-reaching than currently appreciated, and merits investigation considering that the possible outcome of prenatal exposure to a diverse selection of EDs is not known.

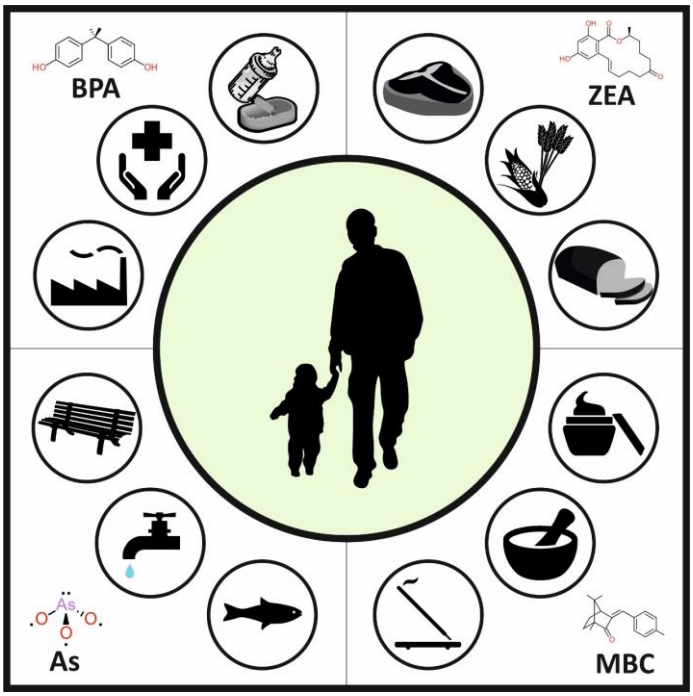

Figure 4: Possible sources of the endocrine disruptor (EDs) substances used in our experiments (Bisphenol-A (BPA); Zearalenone (ZEA); Arsenic (As); 4-methylbenzylidene camphor (MBC)).

The aim of this thesis is **to prove additive adverse effects of different xenoestrogen on the differentiating neuron cells**. Therefore, in the experiments, we used four well-known and widespread EDs of different origins (Figure 4), individually and simultaneously. To shed some light on this scientific puzzle the following aims were established:

A. Determination of the potential effects of the different endocrine disruptors (BPA, ZEA, As, MBC) alone and in combination on the expression of TRα, TRβ and ERβ;

B. Determination of the individual and combined effects of 17-beta-estradiol (E2) and thyroid hormones (THs) parallel with the applied ED substances on the expression of TRα, TRβ and ERβ;

C. Characterization of the effect of presence or absence of glial cells on the applied ED agents on the expression of TRα, TRβ and ERβ.

3. MATERIALS AND METHODS

3.1. Animals

Since our previous studies does not indicated gender differences in the developing rat cerebellum, both male and female Wistar rat pups (body weight: 18–20 g; vendor: TOXI-COOP Zrt. Budapest, Hungary) were used in the experiments of this thesis. Timed pregnant Wistar rats were obtained from the vendor at least four days before pre-partum. Animals were kept under standard laboratory conditions, with tap water and regular rat chow *ad libitum* in a 12-h light, 12-h dark cycle. The date of the pup's birth was considered as postnatal day 0 (p0). Animals were used for granule cell preparation on their postnatal day 7. Attention has been paid to sort the siblings into different treatment groups.

Following the guidelines established by the National Institutes of Health, the use of animals was approved by the Animal Welfare Board at Szent Istvan University Faculty of Veterinary Science and were approved by the regional animal welfare authority (registry No: XIV-I-001/2201-4/2012).

3.2. Antibodies, reagents and materials

TRα (PAB11276) and TRβ (PAB11277) primary antibodies were purchased from Abnova (Hungary). ERβ (E-1276) antibodies were purchased from Sigma Aldrich (Hungary). Secondary antibodies were from Vector Laboratories.

Protease inhibitor cocktail, β-D-arabinofuranoside, 17β-estradiol, 3,3',5-triiodo-L-thyronine, L-thyroxine, bisphenol-A, zearalenone, 4-methylbenzylidene camphor and arsenic were purchased from Sigma-Aldrich (Hungary).

Culture media and TRI reagent were purchased from Invitrogen, penicillin/streptomycin and heat-inactivated fetal bovine serum was purchased from GIBCO (Hungary). BCA kit was ordered from Pierce (Hungary), while Immobilon Western chemiluminescent HRP Substrate was purchased from Millipore.

3.3. Preparation of primary granule cell cultures

In the present study a frequently used in vitro rat model was deployed that is widely accepted for experiments on neuronal development, neurogenesis, neurotoxicity and neuronal survivor and apoptosis (S M Belcher, 1999; Contestabile, 2002; Anderson, 2008; Patel and Reynolds, 2013).

It is evident since long ago that brain functions largely depend on intimate and bidirectional signaling ways between neuron and glia. This bidirectional signaling happens at least in two ways: 1.) through direct contact between the cellular elements or 2.) in paracrine way. It is to note here, however, that the *in vitro* model applied in this study is set up to represent only paracrine effects between glia and neuron, since physical contacts are minimized due to the experimentally regulated low distribution of cellular elements in the culture dishes. The used culture medium in the primary rat cerebellar cell culture is steroid free. The cells in the model does not have aromatase activity (Stanić et al., 2014), this way the amount of E2 or THs – along with the EDs – in the experimental model can be strictly controlled.

Primary cerebellar cultures were prepared as described (and patented) earlier by Wong *et al*. (2001) with modifications, as follows. Animals were sacrificed by quick decapitation and the cerebella were removed.

The removed cerebella were dissociated without enzymatic treatment applying gentle trituration. Triturated cells were filtered through a nylon mesh by gravity (pore size 40 µm) to remove large non-dissociated and non-neuronal cells. Cells were plated in poly-L-lysine pre-coated Petri dishes at densities of approximately 2300–2700 cells/mm^2 and maintained in culture for 7 days (37°C, 5% CO_2) in serum and steroid free Dulbecco's modified eagle medium supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium and 20 mM KCl for mild depolarization and survival of the cells (Gallo *et al*., 1987; Campbell, 2010; Thangnipon *et al*., 1983). The resulting cultures consisted mostly of non-clustered granule cell type neurons (~ 95%). Cerebella of rat pups were seeded into separate culture dishes (1 dish from 1 pup, 6 dishes per treatment, n=6). One dish contained around 21.3×10^6 granule cells.

3.4. Treatments

For analysis of primary cerebellar granule cells in a glia reduced environment, a final concentration of 10 µM cytosine β-D-arabinofuranoside (AraC; Sigma Aldrich Ltd., Hungary) was added 24 hr after seeding to inhibit the proliferation of non-neuronal cells (Glia- experimental groups). In contrast, no AraC was added to the media for analysis of neurons grown in a regular glia containing environment (Glia+ experimental groups). Cultures were simultaneously treated

with the following hormones (at physiologically relevant concentrations) and/or endocrine disruptors 7 days after seeding and 6 hours before harvesting: E2 (1.16×10^{-10} M); T3, (9.2×10^{-10} M); T4, (6.5^{-8} M); BPA (10^{-10} M); ZEA (10^{-10} M); As (10^{-7} M); MBC (6.3×10^{-8} M). In the case of BPA and ZEA the carrier solvent was 0,1% dimethyl sulfoxide (DMSO).

The applied concentration of the EDs was chosen based on our previous experiments (Wong *et al*., 2003), in which we tested BPA effects *in vitro*, as follows: The biphasic effects of E2 and BPA at the low concentration range (10^{-12} to 10^{-9} M) were assumed to act at two binding sites, one stimulatory with a high affinity and one with a lower affinity that inhibits the effect of the first site. Based on the observed similarity in effect at each concentration it was also assumed that neither of the two sites distinguishes between E2 and BPA, and as of the present experiments, in the absence of relevant information, the same was assumed to all other EDs as well. Thus, data points were fit with the Hill-type equation:

$$\text{Effect=maximal stimulation}\ \frac{1}{1+\left(\frac{EC50_{Stim}}{[C]}\right)^{H1}}\ \frac{1}{1+\left(\frac{[C]}{EC50_{Inhib}}\right)^{H2}}$$

where [C] is the concentration of the ligand. A best fit of the equation to the data resulted from setting the maximum effect of the stimulatory site (maximal stimulation) at 110%, with resulting values of 0.4 and 2 obtained for the stimulatory and the inhibitory Hill coefficients (H1 and H2), respectively. Under these conditions, EC50Stim and EC50Inhib were determined as 8×10^{-12} M and 4×10^{-9} M, respectively. We used the same method in comparison with data from the available literature in determining the concentration of ZEA (Bannert *et al*., [2017], Gajecka *et al*., [2016]), As (Sah *et al*., [2013], Li *et al*., [2015]) and MBC (Shahabi *et al*., [2012]) in our treatments.

3.5. Western blot studies

Cerebellar cells grown on Petri dishes were harvested by washing with ice-cold phosphate buffered saline (PBS) and collected by centrifugation as previously described by Wong et al (Wong *et al*., 2001). Total cell lysates were prepared by homogenization and sonication for 10 seconds a total of 3 times in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM Ethylenediaminetetraacetic acid (EDTA), 2.5 mM sodium pyrophosphate, 1 mM beta-glycerol phosphate, and 1 mM Na_3VO_4 plus 1 mg/mL Pefabloc, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 1 µg/mL aprotinin, and 1% Triton X-100, 0.05% sodium

deoxycholate). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts (10 µg/lane) of protein were loaded onto 8–12.5% (w/v) SDS-polyacrylamide gels. Separated proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore AB, Hungary). Membranes were blocked in 5% (w/v) nonfat milk for 1 h diluted in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST). Samples were incubated with primary antibodies [anti-thyroid hormone receptor alpha1 (c-erbA-1, PAB11276), dilution: 1:1000; anti-thyroid hormone receptor beta1 (c-erbA-2, PAB11277) dilution: 1:500; anti-estrogen receptor beta (ESR2, E-1276) dilution 1:1000] at 4°C overnight. Incubation with secondary peroxidase coupled anti-rabbit or anti-mouse antibodies (1:2000, Vector Laboratories, Hungary) were made at room temperature for 1 h. After exposure to Hyperfilm MP (Amersham Biosciences) intensities were analyzed using Image J software.

Multiple exposures of each blot were collected, and used for densitometric analysis. All data that have been presented are representative of at least three independent experiments (n = 6 per treatment). Optical densities were calculated as arbitrary units, normalized to the protein concentrations measured from the control group (non-treated control [ntC]). The measured ntC value was arbitrarily set to 1 and results from other groups were expressed as fold changes relative to the ntC protein expression level.

3.6. Quantitative real-time PCR measurements

The total RNA was isolated from the cell samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA,) according to the manufacturer's instructions, and the concentration were quantified by NanoDrop spectrophotometer ND-1000 (Thermo scientific, Wilmington, USA). Reverse transcription polymerase chain reaction was carried out as described by Sayed-Ahmed *et al*. (2004): 3 µg of total RNA was reverse transcribed by RT-PCR in a final volume of 30 µL using Red Taq Polymerase (Sigma) and oligo(dt) primers. cDNA was stored at -80°C and 2 µL was analyzed in triplicate by quantitative Polymerase chain reaction (qPCR) (Master SYBRGreen, Hoffmann-La Roche, Basel, Switzerland) in a LightCycler 2.0, F. device (Hoffmann-La Roche, Basel, Switzerland) using beta-actin gene as endogenous control for normalization of the data.

ERβ Primer sequences were as published by Vaillant *et al*. (2002). The primer sequences used for amplification of TRα and TRβ were as previously published by Billon *et al*. (2002) and Kariv *et al*. (2003), respectively. qPCR cycles and controls were planned according to the manufacturer's instructions and were optimized for the primer pair.

Cycling parameters were as follows: one cycle at 95°C for 30 sec, 45 cycles of 72°C for 15 sec, annealing temperature for 1 min, 70°C and one cycle of 95°C for 30 sec, and 40°C for 30 sec.

Amplified products were identified by agarose gel electrophoresis, melting point and sequence analysis (Applied Biosystems ABI 3100 Genetic Analyzer, Agricultural Biotechnology Center, Gödöllő, Hungary).

Real-time fluorescent measurements were taken at every cycle and change in threshold cycle (ΔCt) was calculated. Real-time PCR threshold cycle (Ct) data were analyzed using the REST-XL software version 2.0 (Pfaffl *et al*., 2002). Cycle threshold values were normalized to those of beta-actin. The relative expression ratios of mRNA (fold changes) were calculated using the $2^{-\Delta\Delta Ct}$ method. In the control group (ntC) mRNA value was arbitrarily set to 1 and results from other groups were expressed as fold changes relative to the control group.

3.7. Immunohistochemical labeling of glial fibrillary acidic protein (GFAP)

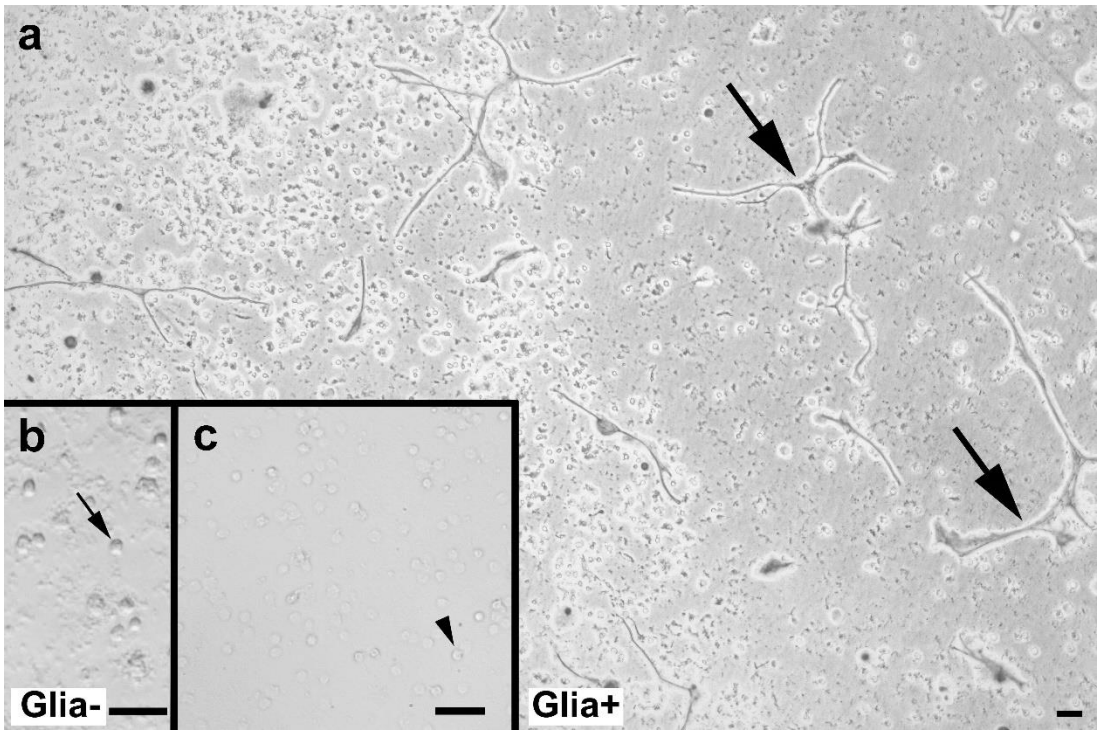

Figure 5. Immunohistochemical glial fibrillary acidic protein (GFAP) labeling on our primary cerebellar cell cultures. a: In cell cultures without cytosine β-D-arabinofuranoside (AraC) treatment (Glia+ cultures) numerous glial fibrillary acidic protein (GFAP) labeled glial cells can be found (arrows). The cells possess strongly developed cell processes. **b:** Negative control. During the process of immunostaining the primary antibody for GFAP was omitted from the experiment. The arrow points to an unpainted granule neuron. **c:** GFAP labeling of AraC treated (Glia-) cultures. A small amount of GFAP positive large cell bodies were labeled. The cells are undeveloped, without any visible cell processes. Bars represent 50 μm.

Astroglia in the cultures was identified by Scalise *et al*. (2012) in one of a previous publication by our research team (Figure 5). A standard immunohistochemical labeling protocol for glial fibrillary acidic protein (GFAP) was followed as described earlier by Wong *et al*. (2003), with the exception of the visualization method.

3.8. Statistical analyses

All data that have been presented are representative of at least three independent measurements. Statistical analyses were conducted using Excel (Microsoft, Microsoft Co., Redmond, WA, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA), by means of one-way ANOVA with Tukey's multiple comparison test.

The levels of statistical differences were noted in text in the Results and Discussion chapters in the corresponding paragraphs. All of the differences between treatment groups were shown in the figures in the Appendix chapter. The level of shown statistical significance in the Appendix is $p<0.05$.

4. RESULTS

4.1. Guidance to the figures

It can be generally stated that the figures are consisted of data from three different receptors – TRα on the upper part of every picture, TRβ in the middle and ERβ in the bottom. On the Y axis the fold difference vs. ntC (Glia+) is plotted. All of the results are compared to the Glia+ ntC value, which we set arbitrarily to „1". The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. The specific treatments can be found below the ERβ graphs. Clean bars are representing the "untreated" cell cultures, colored bars are illustrating the different ED effects.

The left half of the groups contain results from glial cells containing cultures (Glia+), all of the treatments containing AraC are presented on the right side as glia reduced (Glia-) group. For the purpose of easier understanding of the results, Glia+ and Glia- ntC values are represented with a dotted line on the corresponding half of the figures.

Due to the large amount of measured data in this chapter only the results which are essential to understand the discussion will be presented. All results with the raw data produced during our experiments can be found in the appendix. The differences between the untreated receptors are already published in Scalise *et al*. (2012), they are not part of this thesis. This chapter will only list the numeric data from the ED experiments, however, the ED untreated results are shown on the diagrams to ease the interpretation of the disruptor effects on the receptor expression in the neuron cells.

4.2. Effects of BPA on TRα, TRβ, ERβ

4.2.1. Effect on receptor mRNA expression (Figure 6)

TRα mRNA (Figure A1 in Appendix)**:** The measured TRα mRNA values in non-treated controls of Glia- were 6.4 ± 2.7 times higher (p=0.0326) than ntC Glia+. A notable reduction (p<0.0001) was found between Glia+ BPA (0.08 ± 0.04) and Glia- BPA (2.79 ± 1.06). **In Glia+** cultures BPA lowered (0.08 ± 0.04) the fold difference of the mRNA amount compared to Glia+ ntC and a strong upregulation was found between every other BPA containing and their BPA untreated counterparts, respectively. Notable differences were measured between BPA (0.08 ± 0.04) vs. E2+BPA (35.93 ± 21.81; p<0.0001); vs. T4+BPA (9.27 ± 6.87; p<0.0001); vs. E2+T3+BPA (13.71 ± 10.06; p<0.0001) and vs. E2+T4+BPA (21.72 ± 4.91; p<0.0001). The trend **in the Glia-** cultures were similar, BPA alone (2.79 ± 1.06) lowered (p=0.046) the fold difference compared to Glia- ntC, but in every other treatment pair we found a strong upregulation. The differences between BPA vs. E2+BPA (18.29 ± 5.36; p=0.0005), vs. T3+BPA (3.92 ± 2.48; p<0.0001); T3+BPA vs. T4+BPA (1.61 ± 0.91; p<0.0001); E2+T3+BPA (4.63 ± 1.45) vs. E2+BPA (p=0.0012), and vs. T3+BPA (p<0.0001) were noteworthy.

TRβ mRNA (Figure A3 in Appendix)**:** Significantly strong differences were found between Glia+ and Glia- in three cases: Glia+ BPA (0.07 ± 0.02) was significantly lower (p=0.0046) than Glia- BPA (5.02 ± 2.54;); the upregulation found in Glia+ E2+T3+BPA (107.56 ± 9.49) was a tenfold (p<0.0001) compared to Glia- E2+T3+BPA (11.19 ± 5.71); and Glia+ T3+BPA (5.37 ± 1.46) was almost 12-times lower (p<0.0001) than Glia- T3+BPA (69.41 ± 6.6). Our results in TRβ resemble, in many respects, our TRα-related findings. Compared to ntC (both in Glia+ and Glia-), BPA showed a downregulation, however, in every other pair a strong upregulation was found after BPA treatment.

Notable differences **in Glia+** were found between BPA (0.07 ± 0.02) vs. E2+BPA (63.98 ± 9.87; p<0.0001); vs. T3+BPA (5.37 ± 1.46; p<0.0001); vs. T4+BPA (39.22 ± 27.92; p=0.023); vs. E2+T3+BPA (107.56 ± 9.49; p<0.0001); and vs. E2+T4+BPA (44.36 ± 14.06; p=0.0002). A tenfold increase (p<0.0001) was found in E2+T3+BPA compared to T3+BPA; and the increased mRNA measured from E2+T3+BPA compared to E2+T4+BPA was also significantly strong (p<0.0001). **In Glia-** E2+BPA (75.89 ± 9.2; p<0.0001) and T3+BPA (69.41 ± 6.6; p<0.0001) caused a strong upregulation compared to BPA (5.02 ± 2.54). The mRNA expression found in T4+BPA (9.28 ± 2.75) was significantly less (p<0.0001) than in T3+BPA. The results from E2+T3+BPA (11.19 ± 5.71) were one seventh (p<0.0001) of the expression level in E2+BPA.

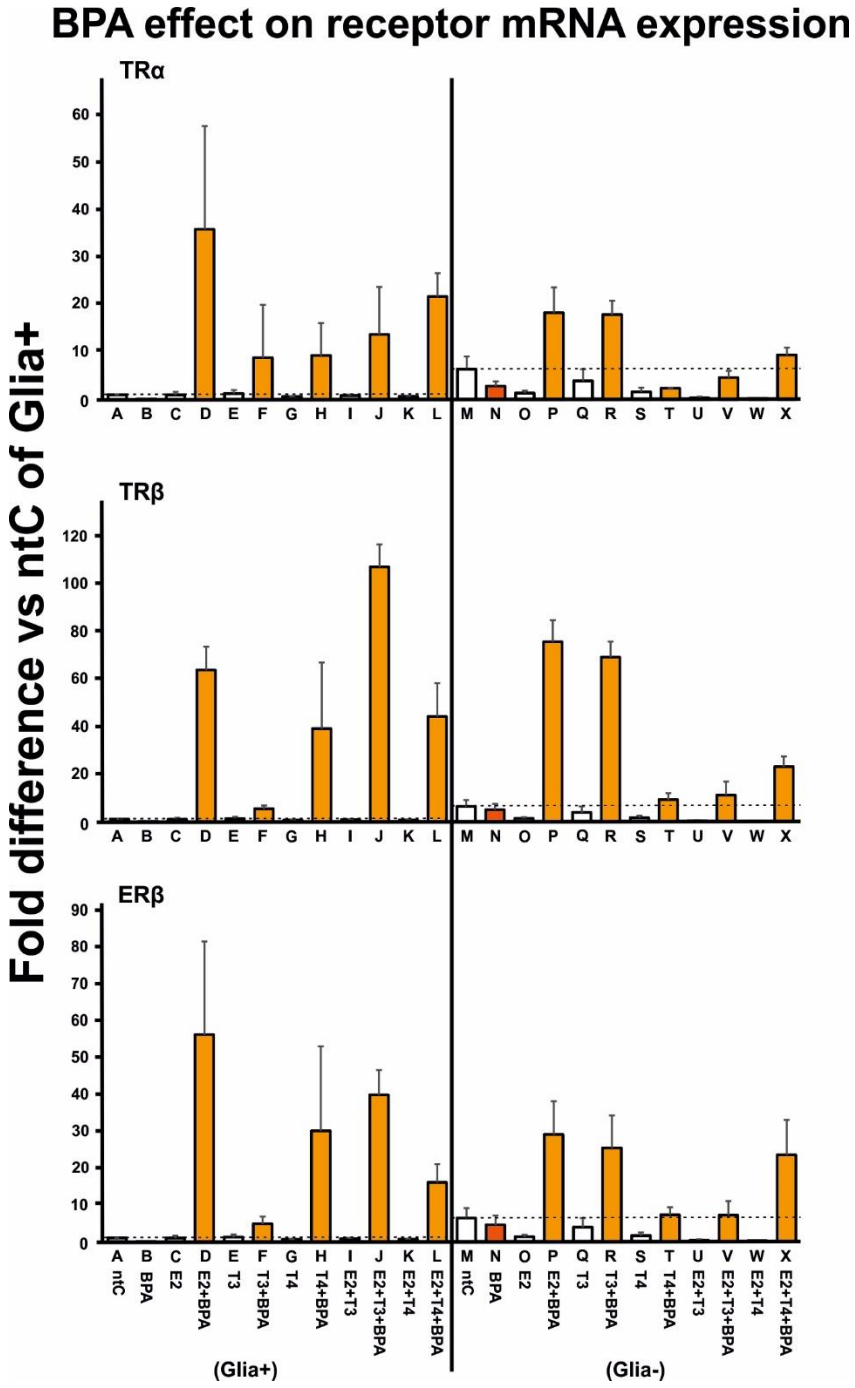

Figure 6: The effect of bisphenol-A on the TRα, TRβ and ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

ERβ mRNA (Figure A5 in Appendix)**:** We found notable differences between the glial effects between Glia+ T3+BPA (4.85 ± 1.95) vs. Glia- T3+BPA (25.43 ± 8.85; p=0.0019) and between Glia+ E2+T3+BPA (39.82 ± 6.75) vs. Glia- E2+T3+BPA (7.18 ± 3.87; p<0.0001). ERβ values follow the trend seen in case of TRα and TRβ, we found an upregulation in every treatment pair except in Glia+ BPA vs. ntC, and in Glia- BPA vs. ntC. All of the BPA treated groups in **Glia+** showed a significant upregulation compared to BPA (0.03 ± 0.01): the mRNA expression in E2+BPA (56.19 ± 25.31; p=0.0022); T3+BPA (4.85 ± 1.95; p=0.0011); T4+BPA (30.06 ± 22.95; p=0.0308); E2+T3+BPA (39.82 ± 6.75; p<0.0001); and E2+T4+BPA (16.08 ± 4.96 p=0.0002) was in some cases 2-fold higher than the results from BPA. A notable difference was found between T3+BPA and E2+T3+BPA (p=0.0002), the latter was one ninth of the former. **In Glia-** compared to BPA (4.53 ± 2.56) only E2+BPA (29.11 ± 8.98; p=0.0008); T3+BPA (25.43 ± 8.85; p=0.0019) and E2+T4+BPA (23.53 ± 9.48; p=0.0047) was higher. T4+BPA (7.25 ± 2.1) showed a downregulation compared to T3+BPA (p=0.004). Less mRNA was produced – approximately one third – in E2+T3+BPA (7.18 ± 3.87) than E2+BPA (p=0.002) or T3+BPA (p=0.0054). Interestingly E2+T4+BPA was nearly equal to E2+BPA and T3+BPA but showed an upregulation compared to E2+T3+BPA (0.0127).

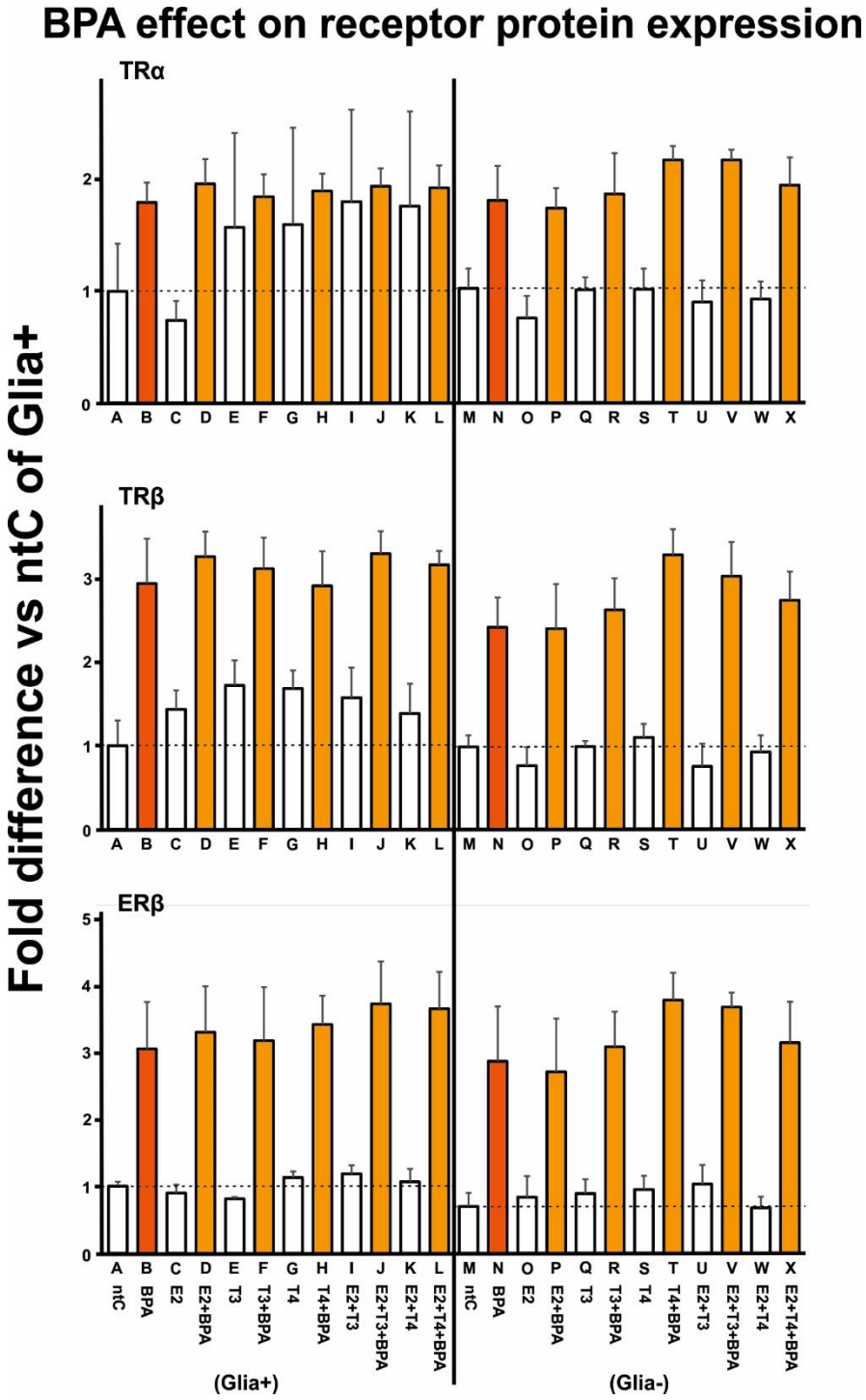

Figure 7: The effect of bisphenol-A on the TRα, TRβ and ERβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.2.2. Effect on receptor protein expression (Figure 7)

TRα protein (Figure A2 in Appendix)**:** In **Glia+** BPA alone nearly doubled the protein expression (1.79 ± 0.18) compared to ntC (p=0.0069), there was a strong elevation (p<0.0001) between E2 (0.74 ± 0.18) vs. E2+BPA (1.96 ± 0.22) as well. The effect were negligible in other treatments. Compared to the ntC, upregulation was measured in T3+BPA (1.85 ± 0.2; p=0.0155); T4+BPA (1.9 ± 0.16; p=0.0031); E2+T3+BPA (1.94 ± 0.16; p=0.0024); and E2+T4+BPA (1.93 ± 0.2; p=0.0034). We found noteworthy differences **in Glia-** cultures between BPA (1.81 ± 0.31) vs. T4+BPA (2.18 ± 0.13; p=0.036); BPA vs. E2+T3+BPA (2.18 ± 0.09; p=0.0318); and in E2+BPA (1.74 ± 0.18) vs. E2+T3+BPA (p=0.0007). The results were similar to BPA in T3+BPA (1.87 ± 0.37) and E2+T4+BPA (1.95 ± 0.25).

TRβ protein (Figure A4 in Appendix)**:** A strong upregulation was found in Glia+ E2+BPA (3.26 ± 0.3) compared to Glia- E2+BPA (2.4 ± 0.54; p=0.0105); and in Glia+ E2+T4+BPA (3.16 ± 0.17) vs. Glia- E2+T4+BPA (2.74 ± 0.34; p=0.0322). In **Glia+** BPA nearly tripled the protein expression in every treatment pair. The upregulation was 2.94 ± 0.53 in the BPA-treated group; 3.26 ± 0.3 in E2+BPA; 3.11 ± 0.37 in T3+BPA; 2.91 ± 0.41 in T4+BPA; 3.29 ± 0.27 in E2+T3+BPA; and 3.16 ± 0.17 in E2+T4+BPA. Similarly to TRα, the difference was significantly strong **in Glia-** between BPA (2.41 ± 0.36) vs. T4+BPA (3.28 ± 0.31; p=0.0021) and BPA vs. E2+T3+BPA (3.02 ± 0.41; p=0.0313). The upregulation was more intense in T4+BPA compared to T3+BPA (2.62 ± 0.38; p=0.0129). E2 lowered the mRNA expression in the E2+T4+BPA (2.74 ± 0.34) compared to T4+BPA (p=0.0248). E2+BPA (2.4 ± 0.54) was similar to BPA, statistical difference was not found between the results.

ERβ protein (Figure A6 in Appendix)**:** Significantly strong differences were not found between Glia+ and Glia-. BPA elevated the mRNA expression in every treatment pair, a three- or fourfold upregulation was observed. In **Glia+** compared to the ntC, upregulation was measured in the BPA treated group (3.05 ± 0.7); in E2+BPA (3.3 ± 0.69); in T3+BPA (3.17 ± 0.8); in T4+BPA (3.42 ± 0.43); in E2+T3+BPA (3.72 ± 0.63); and in E2+T4+BPA (3.65 ± 0.55). Noticeable differences between treatments were found **in Glia-** between T3+BPA (3.08 ± 0.52) vs. T4+BPA (3.78 ± 0.41; p=0.0407); and in T3+BPA (3.08 ± 0.52) vs. E2+T3+BPA (3.67 ± 0.22; p=0.0417). The other groups were identical to BPA (2.87 ± 0.82): protein fold difference was 2.71 ± 0.79 in E2+BPA; 3.14 ± 0.61 in E2+T4+BPA.

4.3. Effects of ZEA on TRα, TRβ, ERβ

4.3.1. Effect on receptor mRNA expression (Figure 8)

TRα mRNA (Figure A7 in Appendix)**:** We found evidence of glial modulation in TRα regulation**, In Glia+** a downregulation was observed in all of ZEA treated cultures. The following fold difference leves were measured compared to ntC: in ZEA a value of 0.4 ± 0.21 (p=0,0058); E2+ZEA 0.52 ± 0.41 (p=0,0149); T3+ZEA 0.64 ± 0.62 (p=0,0342); T4+ZEA 0.21 ± 0.19 (p=0,0474); the changes measured in E2+T3+ZEA 0.09 ± 0.08 and in E2+T4+ZEA a 0.22 ± 0.24 was non-significant. In contrast **in Glia-** compared to the corresponding ZEA untreated groups the downregulation was only detectable in ZEA (0.7 ± 0.47; p=0,0046) and in T3+ZEA (0.53 ± 0.33; p=0,0405) treated cultures. There was a lack of change in T4+ZEA (0.37 ± 0.35); E2+ZEA (1.38 ± 1.66); E2+T3+ZEA (0.32 ± 0.39) and E2+T4+ZEA (0.82 ± 0.54) cultures.

TRβ mRNA (Figure A9 in Appendix)**:** In both Glia+ and Glia- a downregulation was found in every pair after ZEA treatment compared to the corresponding ZEA untreated groups. The changes were non-significant except in T4+ZEA (0.3 ± 0.25; p= 0,0023). The fold difference values **in Glia+** were the following: ZEA (0.67 ± 0.66); E2+ZEA (0.69 ± 0.59); T3+ZEA (0.47 ± 0.53);; E2+T3+ZEA (0.13 ± 0.1); E2+T4+ZEA (0.28 ± 0.28). **In Glia-** the values are: ZEA (0.72 ± 0.29); E2+ZEA (2.55 ± 3.72); T3+ZEA (0.77 ± 0.38); T4+ZEA (0.36 ± 0.27); E2+T3+ZEA (0.43 ± 0.46); E2+T4+ZEA (0.99 ± 0.7). Change compared to the ED untreated groups were not found.

ERβ mRNA (Figure A11 in Appendix)**: In Glia+** ZEA lowered the expressed mRNA levels in the following treatment pairs: in ZEA (0.39 ± 0.15; p= 0,0008); in T4+ZEA (0.15 ± 0.13; p= 0,0477) and in E2+T3+ZEA (0.1 ± 0.07; p= 0,0007). The changes were non-significant in E2+ZEA (0.39 ± 0.26); in T3+ZEA (0.43 ± 0.37) and in E2+T4+ZEA (0.3 ± 0.42). **In Glia-** ZEA lowered the expressed mRNA levels in the following treatment pairs: in ZEA (0.66 ± 0.36; p<0.0001); in T3+ZEA (0.62 ± 0.51; p=0,0212); in T4+ZEA (0.38 ± 0.42; p=0,0307); and in E2+T4+ZEA (0.63 ± 0.35; p=0,0062). The changes were non-significant in E2+ZEA (1.33 ± 1.48) and in E2+T3+ZEA (0.42 ± 0.38)

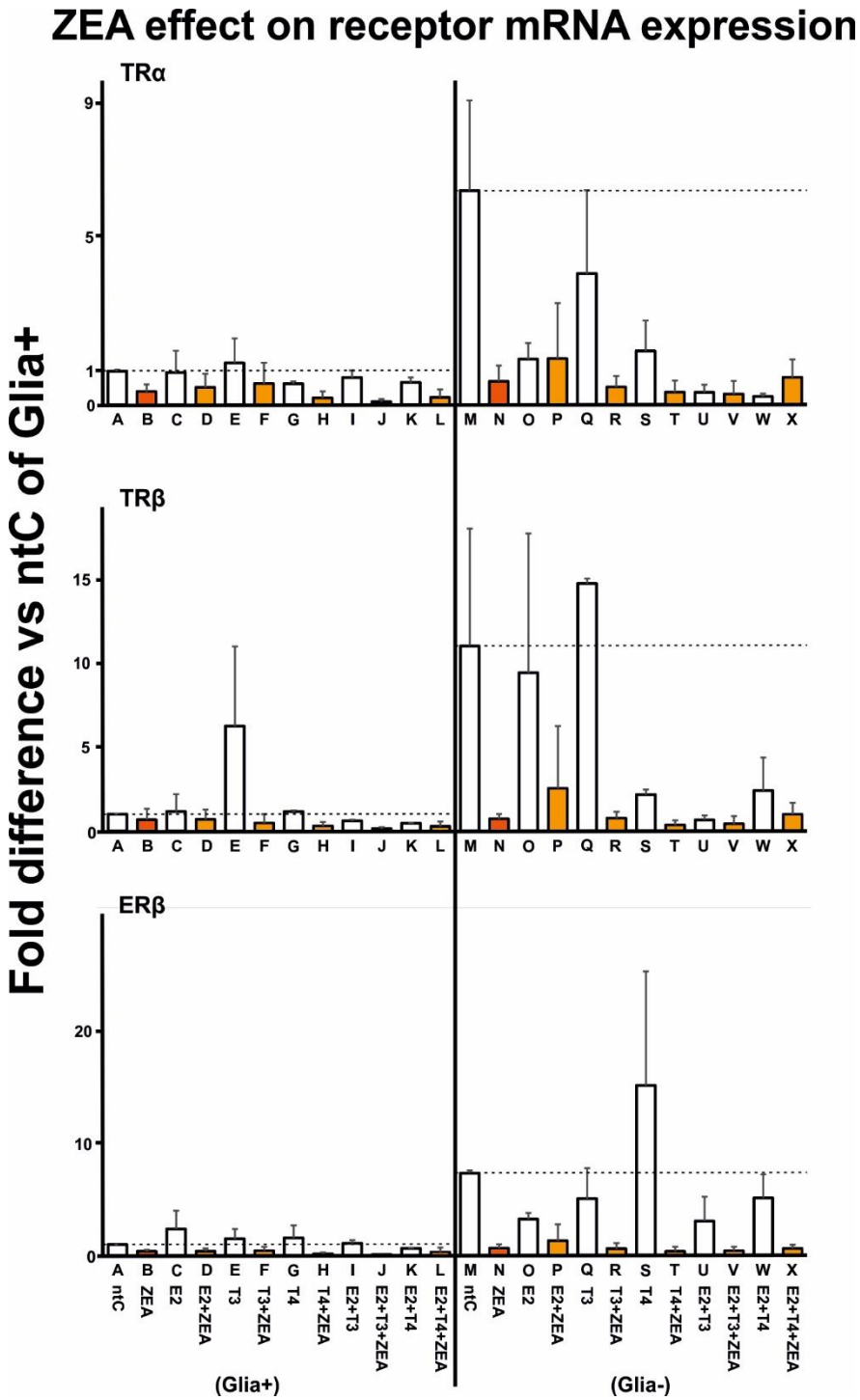

Figure 8: The effect of zearalenone on the TRα, TRβ and ERβ mRNA protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.3.2. Effect on receptor protein expression (Figure 9)

TRα protein (Figure A8 in Appendix)**:** Significantly strong differences between Glia+ and Glia- were not found. **In Glia+** was a strong upregulation in E2+ZEA (1.21 ± 0.27; p=0,0230) compared to E2. The effect in other treatments were negligible. Compared to the ntC, the upregulation measured in the T3+ZEA was 1.38 ± 0.31; in T4+ZEA 1.84 ± 0.12; in E2+T3+ZEA 1.76 ± 0.19; and in E2+T4+ZEA 1.79 ± 0.25. We found noteworthy differences in **Glia-** cultures between T3+ZEA (1.39 ± 0.21) and T4+ZEA (1.83 ± 0.16; p=0,0039); and between E2+T3+ZEA (1.77 ± 0.11) vs. E2+T4+ZEA (1.96 ± 0.12; p=0,0238). ZEA (1.49 ± 0.35) and E2+ZEA (1.42 ± 0.48) produced approximately the same levels of protein.

TRβ protein (Figure A10 in Appendix)**:** A two- or treefold upregulation was observed in all of ZEA treated groups compared to the ZEA untreated ones. No significant difference was found **In Glia+** between ZEA treated groups. In ZEA the fold difference of protein was 3.12 ± 0.24; in E2+ZEA was 2.9 ± 0.33; in T3+ZEA 2.72 ± 0.23; in T4+ZEA 2.98 ± 0.35; in E2+T3+ZEA 3.05 ± 0.2 and in E2+T4+ZEA 2.8 ± 0.34. **Glia-** groups were similar to ZEA alone (3.26 ± 1.77): protein fold difference in E2+ZEA 2.63 ± 0.38; in T3+ZEA 2.64 ± 0.55; in T4+ZEA 2.66 ± 0.61; in E2+T3+ZEA 3.02 ± 0.39; and in E2+T4+ZEA 2.75 ± 0.46.

ERβ protein (Figure A12 in Appendix)**:** ZEA elevated the protein expression in every treatment pair, a treefold upregulation was observed both in all of Glia+ and in Glia- groups. Differences between Glia+ and Glia-; and between ZEA treated samples were not found. **In Glia+** compared to the ntC, the upregulation measured in the ZEA treated group was 3.13 ± 0.98; in E2+ZEA 2.86 ± 0.59; in T3+ZEA 3 ± 0.51; in T4+ZEA 3.18 ± 0.46; in E2+T3+ZEA 2.89 ± 0.18; and in E2+T4+ZEA 3.18 ± 0.58. **Glia-** groups were similar to ZEA (3.09 ± 0.51): protein fold difference in E2+ZEA 3.08 ± 0.43; in T3+ZEA 2.7 ± 0.53; in T4+ZEA 3.14 ± 0.35; in E2+T3+ZEA 2.42 ± 1.03; and in E2+T4+ZEA 3.1 ± 0.4.

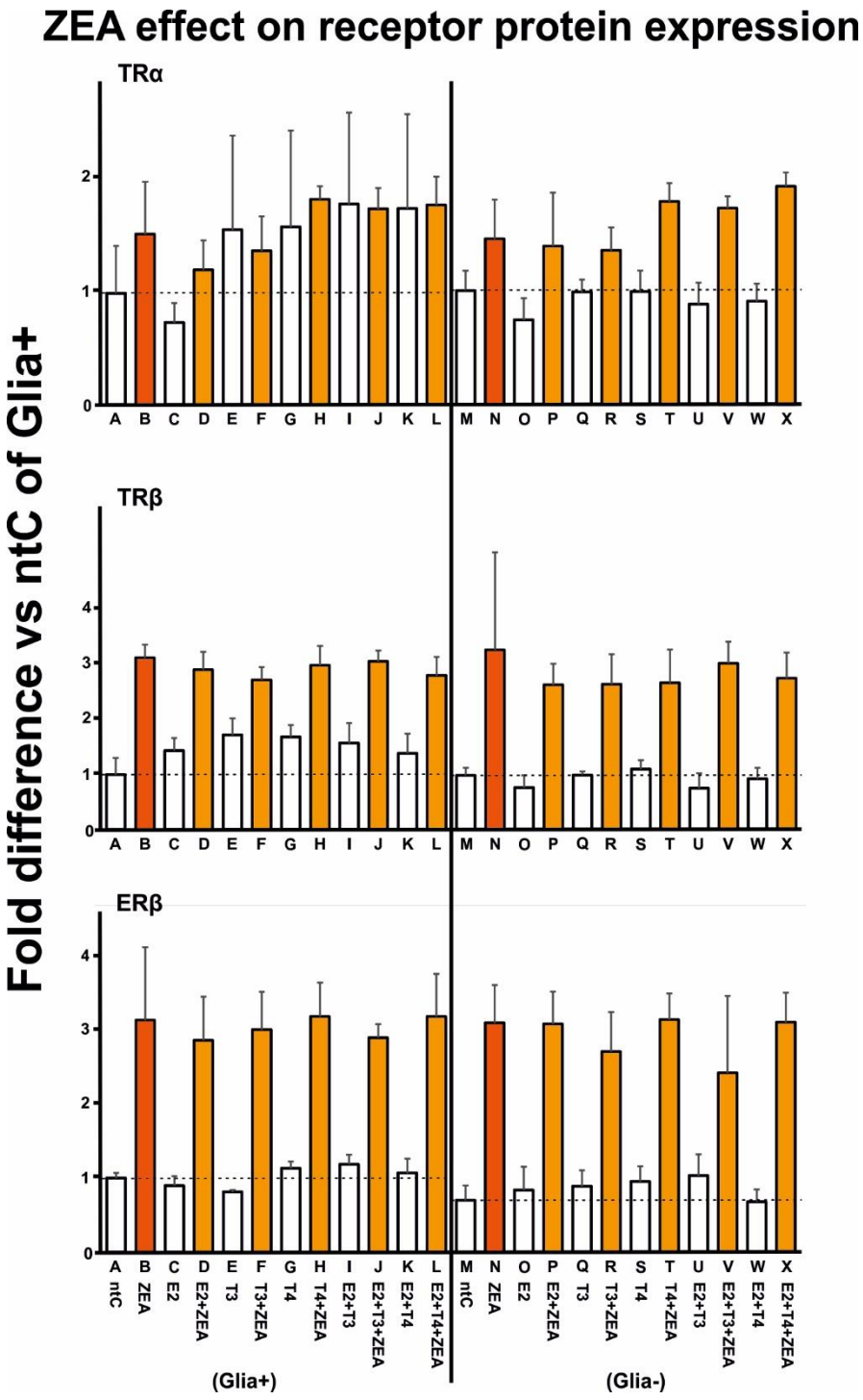

Figure 9: The effect of zearalenone on the TRα, TRβ and ERβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.4. Effects of As on TRα, TRβ, ERβ

4.4.1. Effect on receptor mRNA expression (Figure 10)

TRα mRNA (Figure A13 in Appendix)**:** Significantly strong differences in TRα mRNA levels between Glia+ and Glia- were not found. Comparing to ntC a similar level of mRNA was found in As (0.94 ± 0.45); in E2+As (2.17 ± 2.29); in T3+As (0.88 ± 0.39); in T4+As (1.33 ± 0.64); in E2+T3+As (0.99 ± 0.95) and in E2+T4+As (0.83 ± 0.51) as well. Fold difference levels after As exposure were similar **in Glia-** as well, but comparing to the As untreated cell cultures a strong downregulation was found in As (0.62 ± 0.2; p=0,0040) and a weak upregulation in T3+As (0.62 ± 0.59; p=0,0491). E2+T4+As (0.82 ± 0.3; p=0,0312) caused an upregulation compared to E2+T4. We found similar mRNA leves in E2+As (0.88 ± 0.38); in T4+As (1.08 ± 0.34). and in E2+T3+As (0.74 ± 0.3) compared to their respective untreated halfs.

TRβ mRNA (Figure A15 in Appendix)**:** Similarly to TRα a glial modulation on TRβ mRNA expression was not found. **In Glia+** the mRNA values from the As treated samples – As (0.77 ± 0.14); in E2+As (1.09 ± 0.66); in T3+As (0.88 ± 0.37); in T4+As (1.4 ± 0.95); in E2+T3+As (1.47 ± 1.07) and in E2+T4+As (0.99 ± 0.47) – had no difference in comparison to each other or to ntC. **In Glia-** cultures a significantly strong downregulation was found in T3+As (0.68 ± 0.24; p<0.0001) a weak upregulation in As (1 ± 0.43; p=0,0257); and T4+As (1.28 ± 0.37; p=0,0229) compared to their As untreated counterparts. The fold difference in T4+As was nearly double of the measured mRNA levels from T3+As (p=0,0275). On the contrary, a lack of change was detected from E2+As (1.08 ± 0.78); from E2+T3+As (0.72 ± 0.23) and from E2+T4+As (0.8 ± 0.33).

ERβ mRNA (Figure A17 in Appendix)**:** Evidence of a strong effect of glial modulation was found on ERβ. **In Glia+** cell cultures compared to ntC no change was observed. The mRNA levels measured were approximately equal in As (0.56 ± 0.26); in E2+As (0.88 ± 0.73); in T3+As (0.93 ± 0.23); in T4+As (0.7 ± 0.38); in E2+T3+As (1.43 ± 1.64); and in E2+T4+As (0.71 ± 0.54). On the contrary **in Glia-** the mRNA levels were decreased in every As treated samples compared to ntC or to their respective untreated half in the experimental pair. As alone (0.97 ± 0.38) caused a downregulation eight times less than ntC (7.39 ± 0.26; p<0.0001). A weak downregulation was observed in E2+As (0.82 ± 0.98; p=0,0136) and in E2+T4+As (0.74 ± 0.3; p=0,0068) as well. Compairing the effects of As treatments, a weak upregulation was found between T3+As (0.56 ± 0.24) vs. T4+As (1.12 ± 0.29; p=0,0183). In E2+T3+As (0.77 ± 0.08) the measured mRNA levels were nearly the same compared to E2+T3.

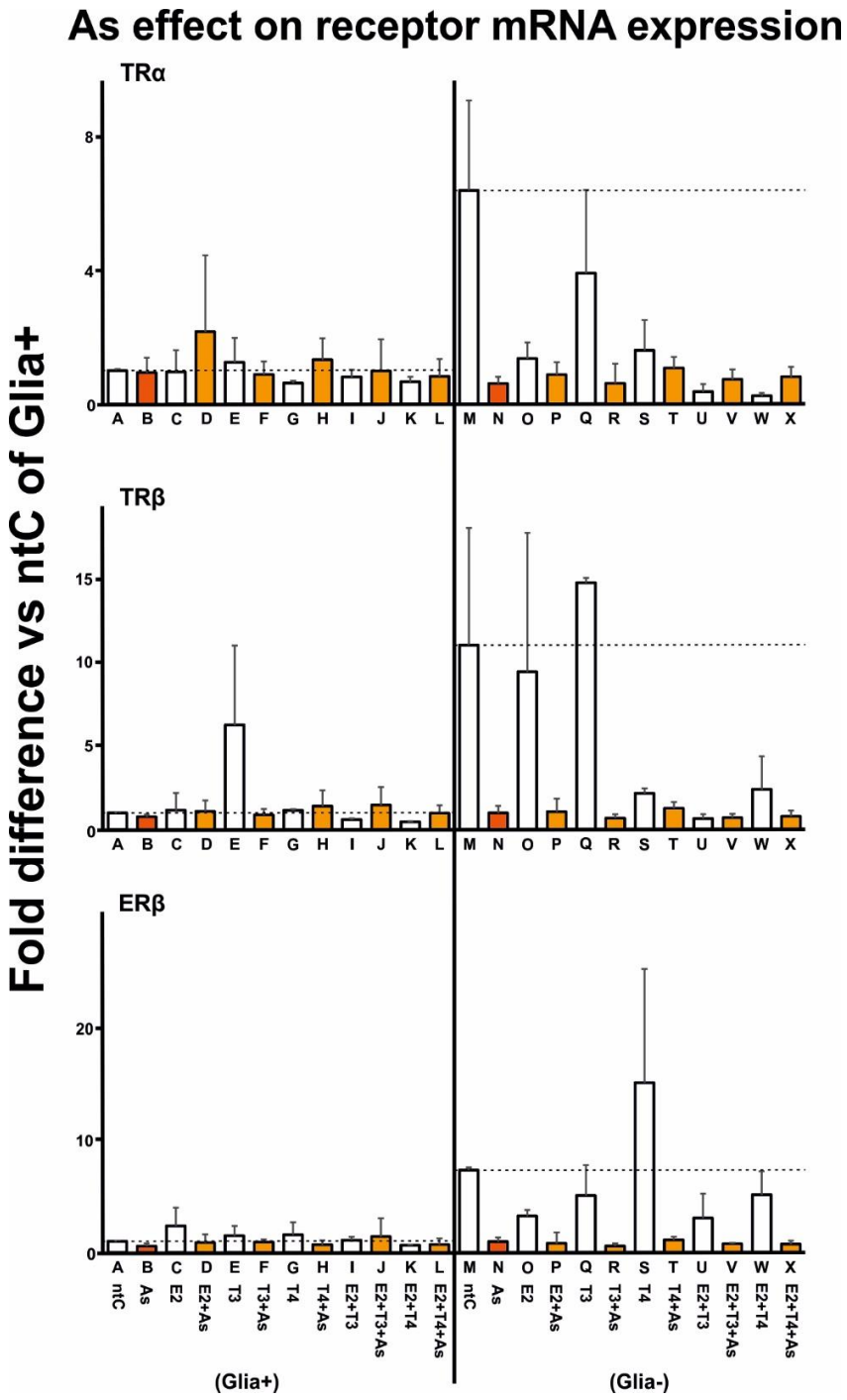

Figure 10: The effect of arsenic on the TRα, TRβ and ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.4.2. Effect on receptor protein expression (Figure 11)

TRα protein (Figure A14 in Appendix): Significantly strong differences were not found between Glia+ and Glia-. In **Glia+** As alone nearly doubled the protein expression (1.7 ± 0.24) compared to ntC (p=0,0181), there was a strong upregulation between E2 (0.74 ± 0.18) and. E2+As (1.77 ± 0.18; p<0.0001). The effect in other treatments were negligible. Compared to the ntC, the upregulation measured in the T3+As was 1.74 ± 0.15; in T4+As 1.97 ± 0.18; in E2+T3+As 2.12 ± 0.2; and in E2+T4+As 1.86 ± 0.24. **In Glia-** we observed a strong upregulation in all of the As treated cell cultures, compared to their respective ED untreated halfs the fold difference values in As were 1.71 ± 0.33 (p=0,0087); in E2+As 1.7 ± 0.4 (p=0,0045); in T3+As was 1.74 ± 0.31 (p=0,0041); in T4+As 1.83 ± 0.36 (p=0,0059); in E2+T3+As 1.84 ± 0.34 (p=0,0020); and in E2+T4+As 2.03 ± 0.13 (p<0.0001).

TRβ protein (Figure A16 in Appendix): Evidence of glial modulation was found in the TRβ protein expression. In the Glia+ treatment groups we found no significant As effect on the fold difference, however As elevated the protein levels in every As treated Glia- cell culture compared to the untreated samples. **In Glia+** the only notable change, a minor downregulation was found between T3 (1.72 ± 0.3) vs. T3+As (1.1 ± 0.29; p=0,0196). The other As treated groups – E2+As (1.19 ± 0.18); T4+As (1.31 ± 0.24); E2+T3+As (1.46 ± 0.19); E2+T4+As (1.33 ± 0.21) – were similar to As alone (1.23 ± 0.11). As elevated protein expression in every treatment **in the Glia-** groups: from As 1.39 ± 0.2 (p=0.0137); from E2+As 1.52 ± 0.16 (p=0.0006); from T3+As 1.42 ± 0.11 (p=0.0002); from T4+As 1.42 ± 0.19 (p=0.0373) and from E2+T3+As 1.44 ± 0.22 (p=0.0044) was measured in protein fold difference compared to ntC.

ERβ protein (Figure A18 in Appendix): Differences between Glia+ and Glia- were not found. **In Glia+** As alone nearly halved the protein expression (0.59 ± 0.05) compared to ntC (p<0.0001). We found an upregulation in T4+As (0.67 ± 0.06) compared to E2+As (0.56 ± 0.08; p=0,0246) and T3+As (0.55 ± 0.04; p=0,0033). The E2+T3+As (0.62 ± 0.06) and E2+T4+As (0.63 ± 0.04) treatments resulted in a similar protein expression level to As alone. After As treatment **Glia-** cultures showed no noteworthy differences compared to ntC and their respective untreated half although the mean values of the results often show an upregulation. Compared to the ntC, the upregulation measured in As was 0.56 ± 0.15; E2+As was 0.52 ± 0.13; T3+As was 0.53 ± 0.11; in T4+As 0.6 ± 0.08; in E2+T3+As 0.6 ± 0.08; and in E2+T4+As 0.63 ± 0.05.

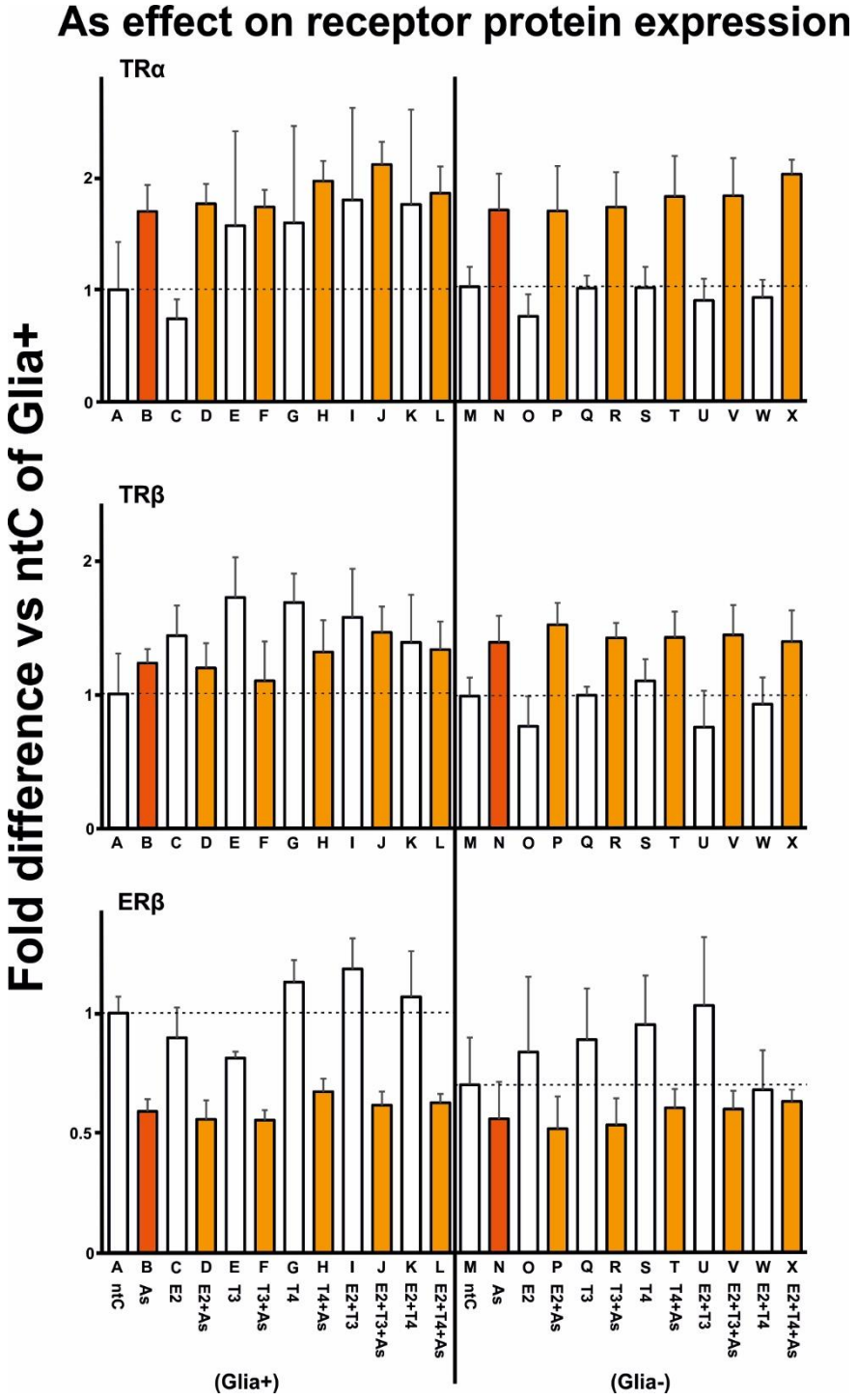

Figure 11: The effect of arsenic on the TRα, TRβ and ERβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.5. Effects of MBC on TRα, TRβ, ERβ

4.5.1. Effect on receptor mRNA expression (Figure 12)

TRα mRNA (Figure A19 in Appendix)**:** Effect of MBC was vague on the majority of cell cultures. Notable changes between Glia+ and Glia- were not found. An upregulation was observed as the overall direction of change compared to MBC untreated samples **in Glia+**. In E2+T3+MBC (2.54 ± 0.34) this effect was significantly strong compared to E2+T3 (0.81 ± 0.22; p= 0.0005). In MBC the measured fold difference vs. ntC was 1.35 ± 1.17; in E2+MBC 1.75 ± 0.68; in T3+MBC 3.07 ± 2.34; in T4+MBC 2.6 ± 1.88 and in E2+T4+MBC 3.17 ± 1.71. **In Glia-** MBC alone (1.44 ± 0.99; p= 0.0122) caused a downregulation and E2+T4+MBC (2.58 ± 1.18; p= 0.0252) caused an upregulation on the receptor mRNA levels compared to the transcriptional activity from their corresponding untreated half. The other treatments – E2+MBC (1.58 ± 1.12); T3+MBC (2.21 ± 1.16); T4+MBC (2.81 ± 1.13) and E2+T3+MBC (1.9 ± 1.08) – were similar to MBC and to their respective MBC untreated counterparts. A trend was not found in the mean values.

TRβ mRNA (Figure A21 in Appendix)**:** MBC effect on TRβ was vague, evidence of glial modulation was not found. MBC treatments **in Glia+** produced a similar level of mRNA compared to ntC: In MBC 1.03 ± 0.92; in E2+MBC 1.83 ± 1.29; in T3+MBC 3.67 ± 2.82; in T4+MBC 2.98 ± 2.04; in E2+T3+MBC 2.42 ± 1.95 and in E2+T4+MBC 2.51 ± 1.05 fold difference was found. **In Glia-** MBC (1.44 ± 0.93; p= 0.0319) caused a weak–, and T3+MBC (1.8 ± 1.23; p<0.0001) caused a strong downregulation compared to their respective MBC untreated counterpart. E2+MBC (1.54 ± 1.69); T4+MBC (1.93 ± 1.44); E2+T3+MBC (1.97 ± 1.96) and E2+T4+MBC (1.77 ± 0.85) only caused an insignificant change compared to MBC.

ERβ mRNA (Figure A23 in Appendix)**:** A modulatory glial effect (small upregulation) was observable in Glia+ E2+T3+MBC (3.1 ± 0.65) compared to Glia- E2+T3+MBC (1.13 ± 1.52; p=0.0446), although compared to ntC the mean values show an upregulation in Glia+ and a downregulation in Glia-. The fold difference of MBC treatment **in Glia+** was 2.11 ± 1.74. Compared to MBC alone, E2+T3 or E2+T4 co-administered with MBC caused a strong upregulation. The mRNA levels measured from E2+T3+MBC was 3.1 ± 0.65 fold higher than ntC, this value was 5.8 ± 2.94 in E2+T4+MBC. In E2+MBC (3.15 ± 1.32); T3+MBC (6.66 ± 6.11); T4+MBC (5.39 ± 3.46) a change compared to MBC alone was not observed. **In Glia-** all of the results were similar in all MBC treated groups: the following fold difference levels were measured compared to ntC: in MBC 3.62 ± 2.87; in E2+MBC 3.25 ± 2.95; in T3+MBC 3.6 ± 1.75; in T4+MBC 3.99 ± 2.72; in E2+T3+MBC 1.13 ± 1.52 and in E2+T4+MBC 4.23 ± 2.63.

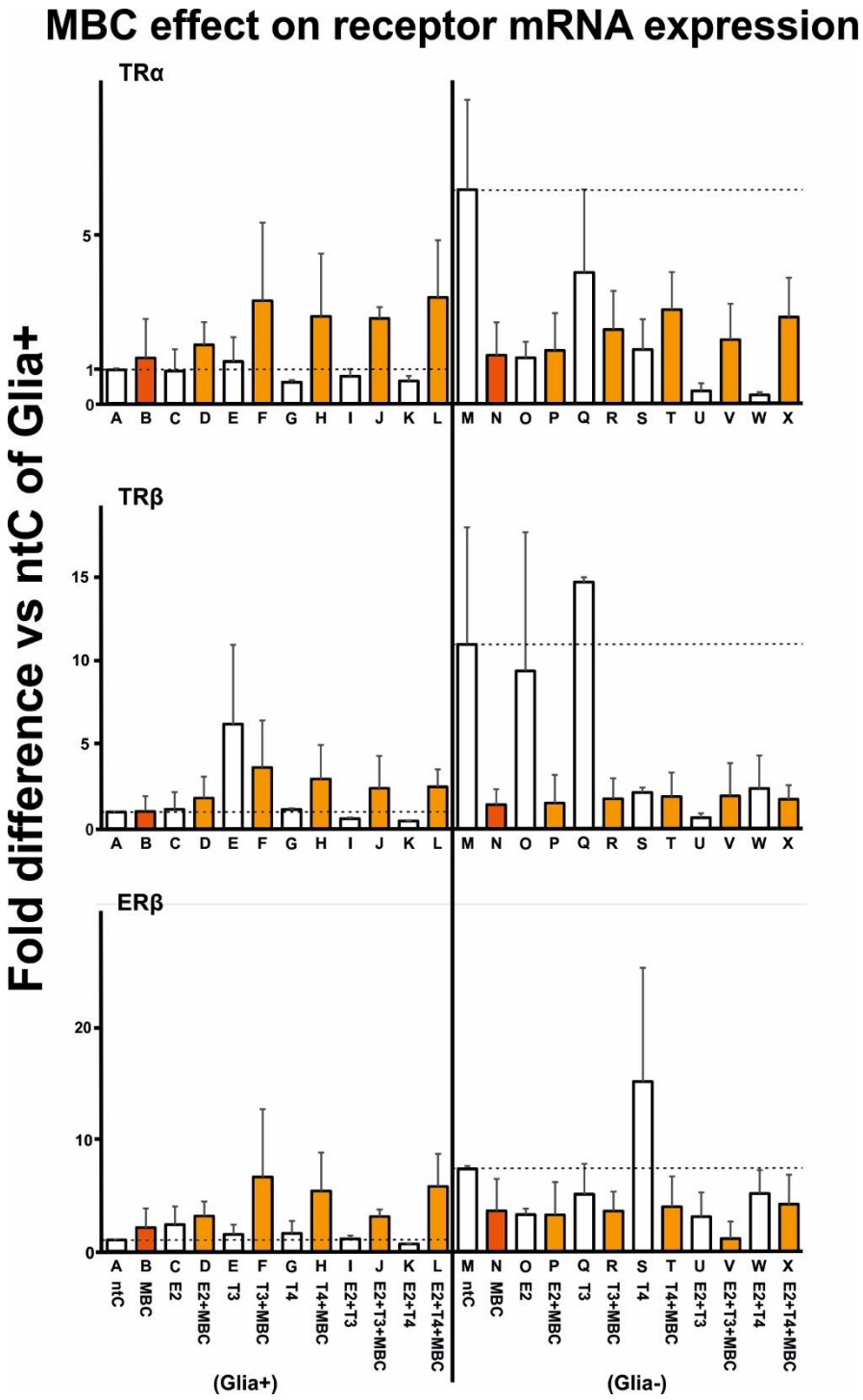

Figure 12: The effect of 4-methylbenzylidene camphor on the TRα, TRβ and ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.5.2. Effect on receptor protein expression (Figure 13)

TRα protein (Figure A20 in Appendix)**:** In Glia-T3+MBC (1.57 ± 0.99) we found a strong downregulation compared to Glia+T3+MBC (3.48 ± 0.38; p=0.0024). **In Glia+** compared to MBC (3.86 ± 0.24) a ligand depended downregulation was found in E2+MBC (3.41 ± 0.34; p=0.0354); in T4+MBC (3.3 ± 0.34; p=0.0121); in E2+T3+MBC (3.15 ± 0.23; p=0.0008) and in E2+T4+MBC (3.39 ± 0.26; p=0.0141). The effect of T3 in T3+MBC was negligible compared to MBC. **In Glia-** the protein levels measured in the aforementioned T3+MBC treatment group were significantly less than in MBC (3.16 ± 0.82; p=0.0201); T4+MBC (2.96 ± 0.9; p=0.0421); E2+T3+MBC (3.1 ± 0.53; p=0.0124) and in E2+T4+MBC (3.21 ± 0.32; p=0.0057). Results from E2+MBC (2.93 ± 0.2) were similar to MBC.

TRβ protein (Figure A22 in Appendix)**:** Differences between Glia+ and Glia- are not observeable. **In Glia+,** compared to MBC (1.6 ± 0.44), protein levels were similar in E2+MBC (1.71 ± 0.15); T3+MBC (1.49 ± 0.28); T4+MBC (1.76 ± 0.12); E2+T3+MBC (1.81 ± 0.18) and in E2+T4+MBC (1.61 ± 0.16). Comparing the results with the untreated cell cultures significant changes were not found. **In Glia-** E2+T3+MBC (1.67 ± 0.28; p=0.0019) and E2+T4+MBC (1.43 ± 0.25; p=0.0157) showed an upregulation compared to their respective untreated counterparts. In MBC (1.31 ± 0.44); E2+MBC (1.23 ± 0.5); T3+MBC (1.2 ± 0.37) and in T4+MBC (1.4 ± 0.56) a similar change was not observed. E2 caused an upregulation in E2+T3+MBC compared to T3+MBC (p=0.0485).

ERβ protein (Figure A24 in Appendix)**:** In Glia+ the MBC (7.57 ± 0.74) E2+MBC (6.18 ± 1) and T3+MBC (6.45 ± 0.55) treatment showed an upregulation compared to the respective Glia- MBC (5.91 ± 1.16; p=0.0229) E2+MBC (4.58 ± 0.53; p=0.0099) and T3+MBC (3.71 ± 0.73; p<0.0001) counterpart. The protein expression from Glia+ E2+T4+MBC (5.84 ± 0.72) cell culture was downregulated compared to Glia- E2+T4+MBC (7.29 ± 0.4; p=0.0028). **In Glia+** we found no statistical difference between MBC; E2+MBC; T3+MBC; T4+MBC (5.76 ± 1.04); E2+T3+MBC (5.81 ± 0.77) and E2+T4+MBC. **In Glia-** E2 lowered the ERβ protein expression in E2+MBC compared to MBC (p=0.0410), T3 had a similar effect in T3+MBC vs. MBC (p=0.0050). T4+MBC (5.6 ± 0.94) caused an upregulation in contrast of the protein levels measured in T3+MBC (p=0.0053). The measured protein levels in E2+T3+MBC (6.67 ± 0.7) were strongly upregulated compared to E2+MBC (p=0.0003) or T3+MBC (p<0.0001). In E2+T4+MBC an upregulation was found compared to MBC (p=0.0309); E2+MBC (p<0.0001); T3+MBC (p<0.0001) and T4+MBC (p=0.0041).

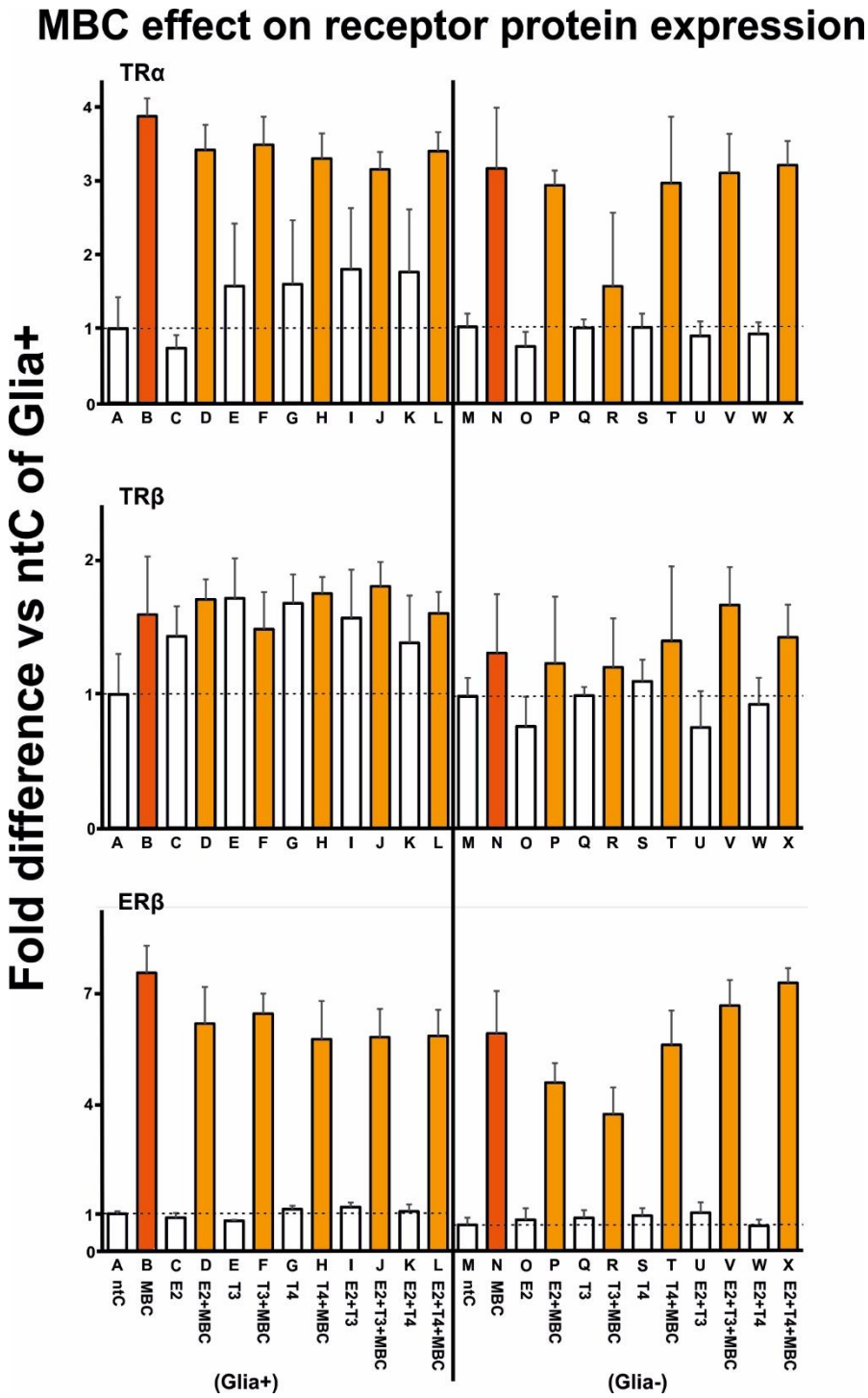

Figure 13: The effect of 4-methylbenzylidene camphor on the TRα, TRβ and ERβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.6. Effects of All-EDs on TRα, TRβ, ERβ

4.2.1. Effect on receptor mRNA expression (Figure 14)

TRα mRNA (Figure A25 in Appendix)**:** A strong difference was observed in the mean values between Glia+ and Glia- although the SEM values was high in the majority of the cell cultures. **In Glia+** ED treatment caused a non-significant tenfold upregulation in the All-ED (7.86 ± 7.64); E2+All-ED (8.56 ± 12.65) and T3+All-ED (8.99 ± 6.87) groups compared to their corresponding untreated groups. The fold difference measured in T4+All-ED (1.8 ± 1.82); E2+T3+All-ED (1.41 ± 0.52) and E2+T4+All-ED (3.03 ± 1.7) were similar to ntC. **In Glia-** significantly strong differences compared to All-ED (3.48 ± 2.27) or to the corresponding untreated samples were not observed. The fold difference values in E2+All-ED was 5.37 ± 4.97; in T3+All-ED was 4.38 ± 3.25; T4+All-ED was 2.51 ± 2.07; E2+T3+All-ED was 2.48 ± 1.56 and in E2+T4+All-ED was 1.11 ± 0.59.

TRβ mRNA (Figure A27 in Appendix)**:** The changes of TRβ mRNA measured from the cell cultures were similar to TRα. SEM values were high, statistically no difference was found between Glia+ and Glia-, and between ED treated samples. **In Glia+** in T3+All-ED (20.09 ± 13.05) an upregulation was observed compared to to E2+T3+All-ED (2.06 ± 0.65; p= 0.0429) and to E2+T4+All-ED (3.31 ± 1.9; p= 0.0214). T4+All-ED (3.66 ± 3.25) and E2+All-ED (10.15 ± 13.8) was similar to All-ED (10.13 ± 8.85). **In Glia-** compared to the untreated samples, or compared to All-ED (8.15 ± 7.75) no difference was observed in the E2+All-ED (7.74 ± 9.17); T3+ All-ED (7.96 ± 9.6); T4+ All-ED (4.56 ± 4.88); E2+T3+ All-ED (3.84 ± 3.53) and E2+T4+All-ED (1.44 ± 0.84) cell cultures.

ERβ mRNA (Figure A29 in Appendix)**:** Similarly to TRα and TRβ a major difference was found between Glia+ and Glia-, but only in the mean values. **In Glia+** All-ED caused a non-significant tenfold upregulation in the All-ED (12 ± 14.47); E2+All-ED (11.82 ± 16.51) and T3+All-ED (15.79 ± 10.29) groups. T4+All-ED (3.07 ± 3.17); E2+T3+All-ED (2.35 ± 1.22) and E2+T4+All-ED (3.05 ± 2.16) showed no difference compared to ntC. In T3+All-ED an upregulation was observed compared to E2+T3+All-ED (p= 0.0348) and to E2+T4+All-ED (p= 0.0227). **In Glia-** All-ED caused no notable difference compared to the untreated cell cultures, except in E2+T4+All-ED (1.26 ± 0.76) where a downregulation (p=0.0168) was found compared to E2+T4 (5.18 ± 2.09). Excluding the aforementioned difference, further deviations from All-ED (6.13 ± 4.94) was not observed in the E2+All-ED (6.31 ± 5.9); T3+ All-ED (6.05 ± 4.93); T4+ All-ED (4.33 ± 3.53) and E2+T3+ All-ED (2.52 ± 1.79) cell cultures.

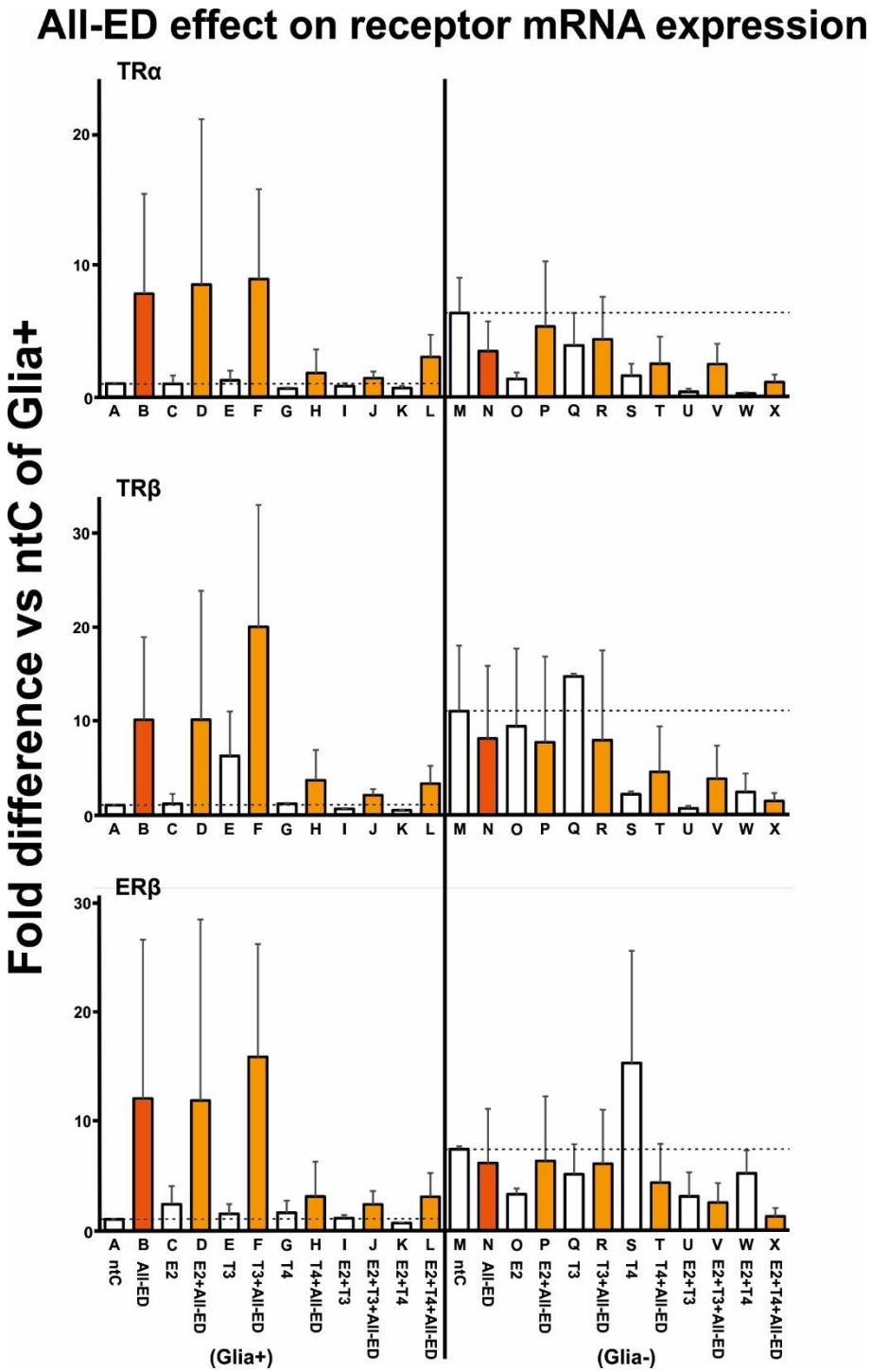

Figure 14: The effect of combined BPA, ZEA, As and MBC treatment on the TRα, TRβ and ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

4.2.2. Effect on receptor protein expression (Figure 15)

TRα protein (Figure A26 in Appendix)**:** A 2.5-fold upregulation was measured from every ED treated cell culture compared to the corresponding untreated samples. In Glia- E2+T3+All-ED (1.88 ± 1.04) a downregulation was found compared to Glia+ E2+T3+All-ED (3.01 ± 0.34; p= 0.0438). Changes in the protein levels were not found **in Glia+** E2+ All-ED (2.68 ± 0.49); T3+ All-ED (2.82 ± 0.26); T4+ All-ED (2.95 ± 0.5); E2+T3+ All-ED (3.01 ± 0.34) and in E2+T4+ All-ED (3.04 ± 0.21) compared to All-ED (2.63 ± 0.47). **In Glia-** results measured from All-ED (2.92 ± 0.31); E2+All-ED (2.67 ± 0.28); T3+All-ED (1.88 ± 1.19); T4+All-ED (2.71 ± 0.37); E2+T3+All-ED (1.88 ± 1.04) and E2+T4+All-ED (2.94 ± 0.43) were identical.

TRβ protein (Figure A28 in Appendix)**:** The fold difference measured from Glia+ T3+All-ED (1.12 ± 0.2) was significantly lower than the results from Glia- T3+All-ED (1.53 ± 0.34; p= 0.0412). **In Glia+** the ED treatment caused a minor downregulation compared to the corresponding untreated counterparts in T4+All-ED (1.23 ± 0.25; p= 0.0298). The All-ED (1.16 ± 0.29); E2+All-ED (1.38 ± 0.26); T3+All-ED (1.12 ± 0.2); E2+T3+All-ED (1.15 ± 0.2) and E2+T4+All-ED (1.1 ± 0.19) treated cell cultures produced similar protein levels compared to ntC and to their untreated counterparts. **In Glia-** an upregulation was found compared to the untreated samples in All-ED (1.35 ± 0.18; p=0.0172); in E2+All-ED (1.62 ± 0.22; p=0.0007); in T3+All-ED (1.53 ± 0.34; p=0.0216) and in E2+T3+All-ED (1.34 ± 0.14; p=0.0036). The results obtained from T4+All-ED (1.3 ± 0.18) and E2+T4+All-ED (0.77 ± 0.63) were similar to ntC. The protein levels measured from E2+T3+All-ED (p=0.0399) and from E2+T4+All-ED (p=0.0171) were lower than the results from E2+All-ED.

ERβ protein (Figure A30 in Appendix)**:** Significantly strong differences between Glia+ and Glia- were found only between Glia+ E2+T3+All-ED (2.64 ± 0.26) and Glia- E2+T3+All-ED (1.77 ± 0.48; p=0.0051). **in Glia+** the results from ED treated samples were similar to All-ED (2.55 ± 0.26): in E2+All-ED 2.33 ± 0.15; in T3+All-ED 2.47 ± 0.33; in T4+All-ED 2.34 ± 0.13; in E2+T3+All-ED 2.64 ± 0.26 and in E2+T4+All-ED 2.63 ± 0.27 was measured. We found a significantly strong upregulation in E2+T3+All-ED compared to T4+All-ED (p=0.0408). **In Glia-** a downregulation was measured in T3+All-ED (1.78 ± 0.7; p=0.0318); in T4+All-ED (2.14 ± 0.23; p=0.0127) and in E2+T3+All-ED (p=0.0065) compared to All-ED (2.61 ± 0.27). T3 caused a minor downregulation in E2+T3+All-ED compared to E2+All-ED (2.32 ± 0.23; p=0.0444). The fold difference measured in E2+T4+All-ED (2.73 ± 0.37) was significantly higher than the results from T3+All-ED (p=0.0225); T4+All-ED (p=0.0121) and E2+T3+All-ED (p=0.0053).

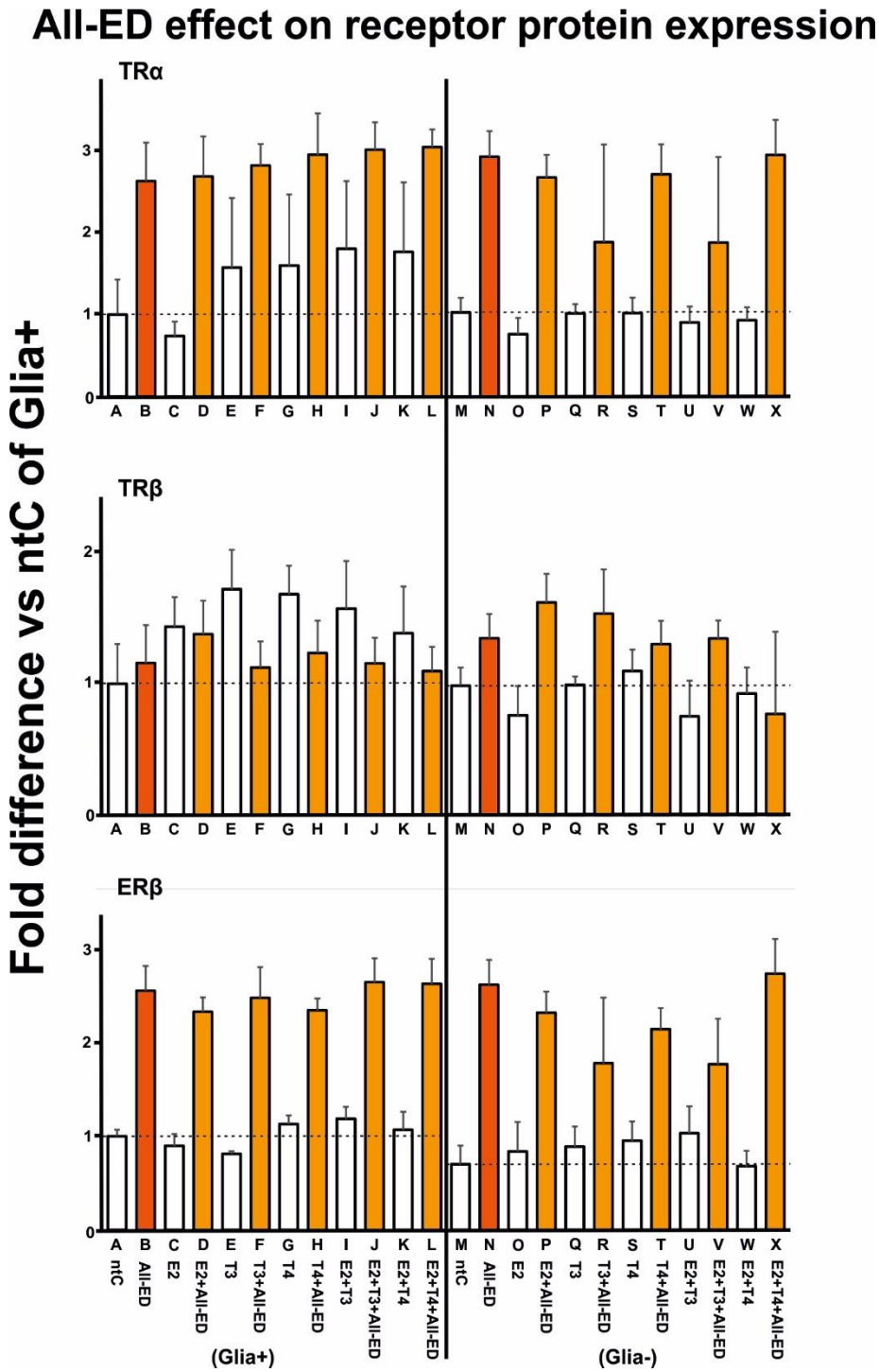

Figure 15: The effect of combined BPA, ZEA, As and MBC treatment on the TRα, TRβ and ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Clean bars are representing the "ED untreated" cell cultures, colored bars are illustrating different ED effects.

5. DISCUSSION

Exposure to EDs early in life – intrauterine or even postnatally – leads to developmental and physiological disorders later in adolescence. The disruption might be intracellular – changing the physiology of specific cells – or it may have a more generalized, tissue or organ physiology changing effect. EDs can increase reactive oxygen species (ROS) in the targeted cells (Huc *et al*., 2012; Babu *et al*., 2013). ROS induced oxidative stress in cell organelles (e.g. mitochondria and endoplasmic reticulum) results in an impaired state of energy in the target cell, possibly further enhancing the effect of the ED. Additionally, specific substances may disrupt specific intracellular pathways, resulting in defective gene transcription. The extracellular effect usually results in a global change in the neuroendocrine system of the animal, possibly influencing the physiology of the neural tissue. E.g. disrupting the hypothalamic–pituitary–thyroid axis may lead to hypothyroidism cause a variety of abnormalities – for example cretinism – in the CNS (Pasquini and Adamo, 1994; Martínez-Galán *et al*., 1997; Mathisen *et al*., 2013).

Numerous physiological functions are partly regulated by TRα and TRβ, alone or in combination. To a certain extent they might substitute for each other's function if co-expressed in the same tissues (Göthe *et al*., 1999), thus no clear roles are defined for the individual receptor subtypes. In animals where the TRα gene is modified (knock-out or knock-in), cardiac functions, thermogenesis, haematopoiesis, and the maturation of specific tissues (e.g. intestines and bones) show patological alterations (O'Shea *et al*., 2005; Plateroti *et al*., 2006). TRβ is required to maintain normal hepatic reactions to THs, it is crucial in regulating the hypothalamic–pituitary–thyroid axis by enabling the TH signals and balancing the hormonal feedback loop, and also essential to sustain the physiology of specific sensory functions (Amma *et al*., 2001; Flores-Morales *et al*., 2002; Abel *et al*., 2003; Esaki *et al*., 2003; Siesser *et al*., 2005; Ng *et al*., 2009).

ERβ plays a central role in the development of the CNS, neuronal network formation, and in establishing the neuroendocrine signaling between the hypothalamus and the reproductive organs. It is responsible for the healthy development and regulation of the female reproductive system, and maintenance a healthy spermatogenesis in males (Hess *et al*., 1997). Disturbances in the balance of the ERs may lead to cognitive deficiencies, developmental disorders and tumors in the CNS and/or the reproductive system (Horvath *et al*., 2001; Anderson *et al*., 2002; Woolley, 2007; Rocca *et al*., 2014; Au *et al*., 2016).

Due to the receptor availability on the cerebellar granule cells, we used the postnatal rat cerebellum as a primer for our cell culture model. This experimental model established from primary cerebellar cells has been extensively used over the past decades, both for testing of the

cellular effects of different experimental cues in neurons and for the testing of the potential mediating role of the glia in those cellular effects (Wong *et al*., 2003; Kirby *et al*., 2004; Zsarnovszky *et al*., 2007; Scalise *et al*., 2012). Additionally, in the present thesis I will compare endocrine effects in Glia+ (glia grown without growth-inhibitor, AraC-) versus Glia- (glia reduced AraC+) cultures. In the applied experimental setup the granule cells were well-dispersed without aggregates or cell clumps, thus the ED and hormonal treatments were more uniform within the culture as well as glia-neuron contacts were balanced. While difference between these groups are the result of the presence or absence of glia, differences between treatment groups in Glia+ cultures carry the enigma of the share of glial receptors in all cultured cell populations. While it has been proven that TRβ and ERβ is localized in the astroglia, the available literature is conflicted about the localization of TRα (Strait *et al*., 1991; Watson and Yager, 2007; Fauquier *et al*., 2014; Sarfi *et al*., 2017). Since, however, granule neurons extremely outnumber sporadic and rudimentary glial cells in Glia- cultures, it seems to be safe to interpret the treatment effects as if they were essentially exerted by neurons. One of the questions that we should address is with regard to the effect of glia in the adjustment of the mRNA and protein expression levels of the aforementioned receptors.

It has to be noted that a part of the following discussion is already published by Jocsak *et al*. (2016) and Somogyi *et al*. (2016). The thesis is a direct follow-up on the data published by Scalise *et al*. (2012), the experiments are based on the results of hormone treatments without EDs. The thesis will refer to those data when appropriate, but I am going to focus on the discussion of the ED effects.

Environmental chemicals change the steroid hormone- and TH-stimulated gene transcription and translation, but it is still not clear through which mechanisms these changes are induced. The aims of the experiments were to test whether the four different EDs (BPA, ZEA, As and MBC) could influence the expression of TRα, TRβ and ERβ mRNA and protein with special emphasis on the potential effects after the co-administration of them with or without treatment of E2, T3 and T4.

As it was already noted in the introduction, hypothetically all biochemical processes may be altered between the receptor activation and the gene transcription. EDs may act as an agonist, or an antagonist of the target receptor, depending on the structure and biochemical traits of the substance. Considering the lack of experiments on the intracellular mechanisms of specific EDs, the roles of the EDs as agonists or antagonists are not specified. Due to the possible stimulatory or inhibitory effects the additive characteristic of the possible adverse final effect is just a hypothesis. During the feedback mechanisms of the neuroendocrine regulation, the additive

effects might multiply or mitigate themselves. It is not specified which substance will cause an upregulation or a downregulation of the specific target receptors. It is still a possibility that the different ED effects will somehow counterbalance themselves and as a result of the cumulative outcome the physiology of the target cells will be disturbed but only to a lesser extent than we anticipated (if we will detect any change at all).

5.1. General observations

The results in this thesis clearly indicate that all of the EDs – alone or in combination – interferes with the physiological hormonal regulation of TRα, TRβ and ERβ mRNA and protein expression. The summarized "direction" of the effects can be seen on Figure 16 and 17. Every comparison was made between ED treated and untreated "sample pairs" (e.g. E2 vs. E2+BPA) if not noted otherwise. A detailed discussion of the individual EDs can be found in the next chapters, here only the trends are shown.

All of the EDs had a strong modulatory effect on the receptor mRNA and protein expression. BPA and ZEA mostly caused an upregulation in the receptor expression, As lowered the receptor mRNA in the cell cultures, and the effect of MBC was strongly ligand-dependent.

	mRNA						
	Glia+			Glia-			Glial effect
	TRα	TRβ	ERβ	TRα	TRβ	ERβ	
BPA	↕	↕	↕	↕	↕	↕	✓
ZEA	↓	↓	↓	↕	↓	↓	×
As	↕	↕	↕	↕	↓	↓	✓
MBC	↑	↕	↑	↕	↕	↓	✓
All-ED	↑	↑	↑	↕	↕	↑	?

Figure 16: **The summary of the effects of the EDs on the transcription of the receptors in our experiments.** Unidirectional arrows indicate an upregulation or downregulation found in every treatment on the tested receptor. Comparison was made between ED treated and untreated pairs. Bidirectional arrows show a ligand dependent direction of regulation. Positive glial effects suggest a modulation of ED effects by the presence of glial cells in the cell culture, the absence of the effects mean a glia independent ED effect.

When we treated the neurons with the EDs in combination (All-ED group), it caused a strong upregulation in every experimental group. Although the mean values were really high, the calculated SEM reached extreme levels in some of the hormone and ED treated groups. Nevertheless, the detected upregulation indicates an adverse effect on the neurons which is alarming due to the fact that a multitude of EDs are incorporated into our bodies daily.

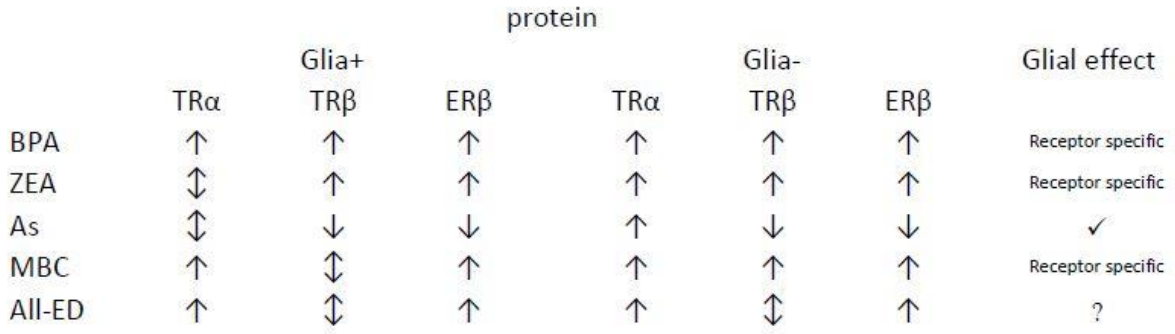

Figure 17: **The summary of the effects of the EDs on the translation of the receptors in our experiments.** One directional arrows indicate an upregulation or downregulation found in every treatment on the tested receptor. Comparison was made between ED treated and untreated pairs. Bidirectional arrows show a ligand dependent direction of regulation. Positive glial effects suggest a modulation of ED effects by the presence of glial cells in the cell culture, the absence of the effects mean a glia independent ED effect.

Due to different results measured in Glia+ and Glia- cultures, and the disparity of ED effects between the receptor transcription and translation indicate that not just a specific, but a complex group of regulatory processes might be disrupted by the tested substances. We suspect that in addition to the ligand-dependent mediating effect of the glia (type 2 deiodinase activity [conversion of T4 to T3], glial presentation of T3 to neurons or modulating the signals between the astroglia and neurons [Rao and Sikdar, 2006]), at least another modulatory method – possibly the microRNA regulation (Tilghman *et al*., 2012) – is targeted by EDs. The changes measured from the qPCR experiments (mRNA levels) were 2- or 3-magnitudes higher than the Western-blot results (protein levels). The mentioned mechanisms between the aforementioned processes apparently play a crucial role in directing ED effects towards the lowered level of transcription measured in our experiments. It is also possible that robust increases in transcription (caused by BPA, ZEA or MBC) may potentially exhaust cellular energy resources, thereby influencing other energy-dependent cellular processes, e.g. the unknown “interposed mechanisms” or the translation as well.

Nevertheless, disrupting the physiology of the neurons and the glia by the exposure of the test substances may lead to yet unknown – either beneficial or adverse – biological consequences in the CNS, and in the neuroendocrine organs of the organism.

5.2. Effects of BPA

It is long known that BPA acts as ED on specific tissues mostly due to its estrogenic or anti-estrogenic potencies. The quality and quantity of the immune response (the T cell count, B cell functions, and dendritic cell and macrophage physiology) can be altered by the ED (Rogers *et al*., 2013). Excessive BPA exposure is linked mostly with reproductive disorders: reduced fetal survival, advancement of puberty, reproductive development, ovarian malformations and reductions in maternal and fetal body weight (Ranjit *et al*., 2010; Adewale *et al*., 2011).

Many of the adverse effects caused by BPA is a direct effect of its ability to connect to specific receptors playing an essential role in signal transmission during neurodevelopment, and in the neuroendocrine functions of the target organism. If pregnant women are exposed to BPA during gestation, the effect will manifest later as a developmental disorder (Boas *et al*., 2009; Vandenberg *et al*., 2009).

According to recent literature, BPA targets not only the estrogen receptors (Wong *et al*., 2003; Zsarnovszky *et al*., 2005; Arambula *et al*., 2016) but as an ER-independent mechanism, it affects the thyroid hormone receptors as well (Delfosse *et al*., 2014; Somogyi *et al*., 2016), however, the mechanism of action is still unknown. A direct link between BPA and the changes in mRNA and protein expression levels of TRs has not been discovered yet. It is possible that another factor(s) may be involved during the intracellular signal transmission resulting an abnormal receptor composition (Xu *et al*., 2007), or it is possible that BPA may disrupt the deiodination pathways (Yang and Chan, 2015).

5.2.1. BPA effects on mRNA transcription

Introduction; Glia+ vs. Glia-

Regardless of the presence or absence of glial cells, all of the experimental groups showed suppressed receptor mRNA expression in BPA vs. ntC groups, but hormone treatment resulted in increased mRNA expression in every BPA treated culture. It should be noted, however, that such E2 and T3-deprived conditions cannot be considered as physiological. It is clear from our results that BPA alone suppresses TRα, TRβ and ERβ mRNA expression. This phenomenon is supported by the experiment of Sheng *et al*. (2012). However, BPA in combination with the hormones in this thesis will elevate the receptor expression. To our knowledge, currently there is no explanation for this massive upregulation, although it is likely that as an indirect BPA-linked

mechanism, the ED effect on TRs and ERs may potentiate the transcription (Zoeller, 2005). Such a robust ED effect has been reported earlier (Zhang *et al*., 2013), yet due to the lack of experiments in this topic this finding is still alarming.

TRα

In case of TRα mRNA, one of our most noteworthy finding is the cumulative effect of E2 and BPA on the mRNA expression in Glia+ cultures. Both E2 and THs added with BPA stimulated the expression compared to the result measured after BPA treatments only, but E2 is more potent than T3 and T4, and their effects are not cumulative. It is possible that BPA disturbs the E2 signaling pathways of the neurons, may act as an agonist on TRα which leads to a strong upregulation (Zoeller, 2007). T3 (direct or indirect from T4 with the help of the astroglia) decreases this ED effect on the TRα receptor because of the physiological downregulation of receptors after threating them with their respective ligands. This idea is supported by our results from the Glia- cultures. We found no significant change between E2+T3+BPA and E2+T4+BPA but the former was considerably lowered compared to its Glia+ counterpart. This means that the glia may mediate BPA effects on the level of transcription in a T3-dependent manner, which is likely due to the ability of astroglia to convert T4 to T3 with the type 2 deiodinase enzyme (Leonard, 1988).

TRβ

The same principle can be seen in case of the TRβ mRNA expression as well. In both the Glia+ and Glia- groups, every BPA+hormone threated group expressed more of the investigated mRNA than the group threated with only BPA. However, the dominance of the E2 effect can no longer be seen. In the Glia+ cultures T3+BPA and T4+BPA produced less mRNA than the E2+BPA, but in the E2+T3+BPA cultures, the effects were cumulative, possibly because BPA has an ED effect on the TRβ receptor (but not on the TRα) thus inducing a stronger upregulation due to the elevated TH need of the cells in the culture. According to Moriyama *et al*. (2002) BPA antagonizes T3 action at the transcriptional level. This effect was absent in our Glia- cultures indicating a strong hormone-dependent mediating effect of the glia in the regulation of TRβ mRNA, but the targeted mechanisms behind these changes are not known yet. In addition to the type 2 deiodinase activity (conversion of T4 to T3) and glial presentation of T3 to neurons, another modulatory method needs to exist, whose identification warrants further experiments.

ERβ

BPA's effect to induce ERβ transcription in neurons appears to be downregulated by the glia. Indeed, when BPA was co-administered with physiological concentrations of E2 and T3, results were significantly different from those found after BPA treatment alone, and even more so in glia containing cultures. In Glia+, there was about three magnitudes higher ERβ mRNA expression after E2+T3+BPA treatment than after BPA treatment alone. Such combined treatment did not change ERβ mRNA expression significantly in glia reduced cultures. This observation suggests that BPA has an additive effect to E2 and T3 not just on the TRβ but ERβ receptor as well, and that the glia plays a role in the mediation of this effect. Without this mediating role neither BPA nor BPA+E2+T3 can induce a change in ERβ mRNA levels compared to the respective ntCs.
When combined treatment contained T4 instead of T3, higher ERβ mRNA expression was detected in neuronal cultures without glia when compared to those found in glia containing samples. Considering our previous results that E2 and THs regulate the expression of each other's receptors, this finding may suggest that BPA is able to directly affect ERβ mRNA expression, in neurons when the mediating effect of glia is limited or absent.

5.2.2. BPA effects on protein translation

mRNA vs. protein expression

It was generally observed that the effects of BPA was less prominent on the receptor protein expression, than those found with regard to mRNA expression. The downregulation in the mRNA levels between ntC and BPA disappeared in both of the TRα, TRβ and ERβ receptors. BPA caused an upregulation in all of the expression levels of the measured receptors in every cell culture and treatment. Our results – the differences between BPA effects on the receptor transcription and translation – indicate that some of the regulatory mechanisms interposed between the mentioned processes (e.g. microRNA regulation) may also be affected by BPA (Avissar-Whiting *et al*., 2010; Tilghman *et al*., 2012).

Glia+ vs. Glia-

TRβ and ERβ showed an elevated receptor protein expression in BPA vs ntC groups. We found a notable difference between Glia+ vs. Glia- in case of TRα receptor protein expression. Interpreting our results it is safe to say that under physiological conditions (if the glia is also present in the cell culture) BPA does not alter the TRα protein expression. Zoeller *et al*. (2005)

found the same result in rats, BPA only influenced the TRβ receptor expression, TRα was mainly influenced by the serum T4 levels (Zoeller *et al*., 2005).

TRα

Compared to the native expression levels of TRα, in the Glia+ cultures BPA had a distinct effect only in the BPA and E2+BPA treatment groups. If T3 or T4 was added in addition to BPA to the cell cultures, the difference between the BPA treated and the BPA untreated cultures disappeared. In Glia- TRα BPA doubled the level of protein expression in every cell culture compared to their untreated counterparts, although compared to the same BPA treatments in Glia+, the expression level stagnated. It seems that without the supporting function of the glial tissue, T3 has a synergistic effect with BPA on the expression level of the measured receptor protein, and this effect also persists with T4 with a higher magnitude. E2 alone does not alter this value but in co-treatment with T3 and BPA the result is even higher. Nevertheless, our results show that BPA upregulates TRα protein expression levels when E2 and/or THs are present in physiological levels in the samples.

TRβ

Compared to the TRα results, BPA had an increasing effect on the TRβ receptor protein expression in every treatment group, not just on the ntC and E2 cell cultures. The above result suggests that BPA has a strong upregulating effect on the TRβ receptor, possibly by inhibiting them directly (Delfosse *et al*., 2014) or by antagonizing T3 action at the transcriptional level (Moriyama *et al*., 2002). The mechanism of the ED is not dependent on the effects of the investigated hormones, although it is dependent on the presence of the glial tissue. In the Glia- cultures, the expression levels were ligand-dependent, and the overall results produced in the TRβ Glia- strongly correlated with the results from TRα Glia-. BPA alters the receptor protein expression of both receptors in the same manner, thus we can assume the effect is universal on the investigated TRs.

Compared to BPA alone, a significant change was found in two groups. E2+T3+BPA caused a stronger upregulation than BPA only. This can be explained by the synergistic estrogen effect of BPA and E2, which is strong enough to mask the downregulation caused by excess T3 on the TRs. However, E2+BPA does not caused an upregulation compared to BPA alone. The other outstanding result was produced by T4+BPA, possibly because the lack of T3 (the absence of deiodinase II) causes the cell to produce more TRs. However, this upregulative effect was missing from T4+E2+BPA. It seems that similarly to the effect found in Glia+, BPA determines an expression level of the TRs, whichever is further modified by T3 or T4, but their influence is

weak. E2 will weaken this effect, it will weaken the effect of T3 and lower the T4 caused upregulation. In summary, on the granule cells BPA acts like an E2 agonist, and the effect of the ED is stronger than the effect of estrogen. The publication of Ben-Jonathan *et al*. (1998) supports this alarming discovery.

ERβ

It is long known that BPA acts as an agonist on the ERβ receptors (Kurosawa *et al*., 2002). Our research confirms this attribute of the ED. BPA caused a strong upregulation of ERβ protein in all of the Glia+ treatment groups. We found no significant difference between the treatments, it seems that BPA influences the rate of the protein expression if glial cells are present, the effects of hormones are negligible. Interestingly almost all of the treatment groups are similar to this phenomenon in the Glia- cultures, except for T4+BPA and E2+T3+BPA. Those results are consistent with the measured expression levels from the TRα and TRβ experiments. BPA acts as an antagonist of T3 through suppression of TRα and TRβ in *Xenopus* tail in culture (Iwamuro *et al*., 2006). Our results can confirm this, on the cerebellar cells T3 had an antagonistic effect with BPA on the ERβ receptor expression, possibly by blocking or inhibiting some of the intracellular pathways activated by BPA exposure resulting in lower receptor protein levels after translation.

In summary according to our results it is safe to say that BPA elevates the TRβ and ERβ protein expression, but doesn't affect the TRα levels when glial cells are present *in vitro*, possible *in vivo* as well. Without the supporting effects from astroglia cells BPA elevated the protein expression in every experimental group. Due to the increased expression levels we can confirm our hypothesis: BPA acts as an ED on the physiology of the nerve cells.

5.3. Effects of ZEA

After ingestion ZEA affects the immune system, the physiology of the bones, liver, kidneys and the cells in the CNS. The most important and well researched targets of ZEA are the neuroendocrine and reproductive organs. In the latter severe anatomical and physiological disorders can be found after the exposure, leading to anoestrus, pseudopregnancy, elevated chance of stillbirth, and developmental disorders in the developing fetus of the pregnant animal. Additionally the mycotoxin has a carcinogenic effect. Several studies indicate that ZEA

stimulates the cell proliferation in tumors of the female reproductive tract and breast malignancies in humans (Withanage *et al*., 2001; Pillay *et al*., 2002b; Parveen *et al*., 2009).

In the CNS ZEA may alter the physiology of the BBB (Weidner *et al*., 2013), further strengthening the severity of the exposure to different EDs. The mycotoxin changes the expression of substances in the nerve fibers of the gastrointestinal such as vasoactive intestinal peptide, neuronal form of nitric oxide synthase, cocaine and amphetamine regulatory peptide, galanin, pituitary adenylate cyclase-activating peptide-27 and substance P (Gonkowski *et al*., 2015).

To the best of our knowledge, information about the effects of ZEA on the TRs cannot be found. It has a strong effect on the ERs however, and the THs might alter the estrogen signaling, thus ZEA may alter the TR expression indirectly.

Early studies suggested that the biological effects of ZEA may be due to their ability to bind to intracellular estrogen binding sites (Greenman *et al*., 1979; Takemura *et al*., 2007) or due to its ability to inhibit the intracellular MAPK enzyme (Pistol *et al*., 2015). Indeed, reporter gene assays demonstrated that ZEA and its metabolites α- and β-zearalenol exhibit just slightly less strong estrogenic potency than 17-β estradiol itself (Frizzell *et al*., 2011). Further study indicated that in the liver, ZEA could also bind to cellular proteins distinct from estrogen receptors (Mastri *et al*., 1985).

5.3.1. ZEA effects on mRNA transcription

Introduction; Glia+ vs. Glia-

ZEA inhibited the mRNA expression in every experimental groups compared to their ZEA untreated pairs. We found only minor differences between Glia+ vs. Glia-, however the differences between the strength of the downregulation were not significant. From the EDs used in the experiment of this thesis, ZEA was the only one with a strong global inhibitory effect on the mRNA expression levels.

TRα

Our present findings show that in the case of TRα, ZEA treatment alone decreased the receptor mRNA expression in Glia+ cell cultures. In the Glia- groups, treatments lack of E2 produced the same results. If E2 was added to the treatment, the changes between ZEA untreated and ZEA containing groups was not observable. Our results show a protective function of the astroglia, in the Glia+ cultures we observed only a slight inhibition (around 50%) induced by ZEA. Contrary to

the Glia+ results, in the glia reduced groups, where we observed a significant decrease, the magnitude of the reduction between untreated and ZEA treated pairs is more potent (nearly 75%). Half of the Glia- groups does not show a decrease in contrast to their Glia+ counterpart. This clearly shows a glia dependent modulation on the effect of ZEA.

TRβ

The **TRβ** receptor mRNA expression was suppressed in every ZEA containing experimental group compared to the ZEA untreated counterparts. The adverse effect of ZEA is more prominent on the Glia- cultures, glial modulation can be observed in the experimental results. The strongest ZEA effect on TRβ mRNA expression was found between the T3 vs. T3+ZEA groups. Hypothetically this may indicate a possible ZEA endocrine disruptor effect on the thyroid hormone system as well, it is not limited to estrogen signaling pathways.

ERβ

Mean values of **ERβ** mRNA expression levels are only higher in the comparison of Glia- vs. Glia+ cultures. According to Kuiper et al. (Kuiper *et al.*, 1998), ZEA can bind to ERβ and increase ERβ transcription. Our finding – administration of ZEA+E2+T3 in Glia+ leads to significantly reduced ERβ transcription compared to ZEA treatment alone – suggests that, at least in the cerebellum, it is the absence of the natural hormones (E2+T3), rather than the presence of ZEA that increases ERβ transcription. Adding to the aforementioned idea, the lack of significance in the difference between E2+T3 vs. E2+T3+ZEA in Glia- suggests that the determined hormone effects observed in Glia+ were exerted in glial cells.

In case of the EDs used in this study only ZEA had a strong inhibitory effect on all of the observed receptor mRNA expression levels. As it was mentioned in the introduction, this mycotoxin is a mixed agonist-antagonist of specific receptors (e.g.: ERα and ERβ). The process of activation and/or inhibition covers a broad range of intracellular processes (Kuiper *et al.*, 1998) thus the method of disruption is not clear yet. Our results are especially relevant to shed light onto the mechanisms in the above phenomenon due to the inhibition found in every case on the end-point (the transcription) of the affected intracellular pathways.

5.3.2. ZEA effects on protein translation

Introduction; Glia+ vs. Glia-

Regarding the effect of applied culture conditions on TRα translation, we observed an increased protein expression after ZEA treatment in every Glia- culture. In comparison in Glia+ this phenomenon was only detectable in the ntC and the E2 group. From the results we might assume a protective or modulatory TH effect, if the glial cells weren't suppressed and T3 or T4 was present in the experimental groups. In case of TRβ and ERβ we found no notable differences between Glia+ vs. Glia-.

mRNA vs. protein expression

The main difference found between the effect of ZEA on mRNA and protein expression was the direction of the effect. ZEA inhibited the mRNA expression in all examined receptors however the protein levels were elevated after ZEA treatment vs. the untreated pair in every experimental group.

TRα

In the Glia- cultures we found a strong upregulation of TRα after E2 treatment in T3+ZEA vs. E2+T3+ZEA. This is an indication of the E2 regulatory effect on TR expression. Disrupting the E2 pathways lead to serious consequences on the thyroid signaling pathways as well in pathological conditions where the supportive effect of the astroglia is not satisfactory. Interestingly in the Glia- groups after ZEA treatment TRα protein expression was higher after T4 than T3 treatment in both of the experimental pairs. According to the literature in the nervous tissue T4 can only be converted to T3 with help of the deiodinase II enzyme, which is localized intracellularly in the supporter astroglia cells. Only T3 should have a direct hormonal effect on the nerve cells. This means that T4 theoretically exhibit a modulatory effect on the expression of the TRα protein, and this route of regulation is different than the T3 pathway.

TRβ and ERβ

We found no significant difference between Glia+ and Glia- cultures regarding the ZEA induced effect on TRβ and ERβ receptor translation (except in TRβ glia+ E2+T4+ZEA vs. glia- E2+T4+ZEA but the trend is barely noticeable in the mentioned groups).

After ZEA treatment the receptor expression level increased in every experimental group, showing a strong modulatory effect on the receptor proteins thus indicating an ED characteristic.

5.4. Effects of As

Arsenic has a global potent cytotoxic effect. Intracellular arsenic effects include increased lipid peroxidation leading to damages in plasma membrane and the intracellular membrane system, furthermore As inhibits elimination of free radicals, interferes in methylation reactions and in coupling with thiol groups inducing DNA and protein damage (Yamanaka and Okada, 1994; Sundari *et al*., 1997; Zhong and Mass, 2001).

The exposure leads to a multitude of neurodevelopmental dysfunctions (Rodríguez-*Barranco et al*., 2013). In children, cognitive function could irreversibly decline (Rocha-Amador *et al*., 2007), deleterious effects cause deficit in verbal and performance domains (Calderón *et al*., 2001; Wasserman *et al*., 2011). There are a number of reports describing neurotoxic effects of As on rodent pups resulting in sever decrease of locomotor activity and behavioral disorders (Rodríguez *et al*., 2001). Most studies, however do not detail cellular and subcellular causes that are behind the above phenomena.

Arsenic affects the thyroid signaling system and vice versa, T4 treatment helps the animals to eliminate As through the kidneys (Rana and Allen, 2006). Hypothyroidism may protect the organism against As induced toxicity (Allen and Rana, 2007) but the exact mechanisms are still unknown. As exposure in low doses influences the transcriptional and translational levels of TRs furthermore serious As exposure causes apoptosis in the neural tissue (Fröhlich *et al*., 2008). Recent literature shows a suppressive effect on mRNA transcription both globally (Watson and Yager, 2007; Davey *et al*., 2008) and in case of TRs (Sun *et al*., 2016). It also reduces the expression of the gene coding type I and II deiodinases (Davey *et al*., 2008; Gibson *et al*., 2016). The damaging effect can even pass across the placental barrier to the fetal tissue (Chattopadhyay *et al*., 2002) and cause developmental problems during ontogenesis.

As may cause the inhibition of gene expression by influencing ER signaling pathways. Possibly it alters the PI3K, MAPK, and mitogen activated protein kinase pathways (Watson and Yager, 2007). An experiment by Bodwell et al. (2004) states that As acts as ED on receptors for progesterone, androgen and corticoids in a dose-dependent manner and enhance specific hormone-dependent gene transcription even at very low doses (Bodwell *et al*., 2004; 2006). ER were involved in these examinations later on, however demonstrating highly varying effects of As on ERβ expression. For example, Cimino-Reale *et al*. (2008) reported that As increased ERβ expression in bone marrow cells. In contrast, Chen *et al*. (2002) found that As only affected ERα, but not ERβ expression in breast carcinoma cells. Although information on As effects on ERβ is scarce, it appears that these As effects are tissue-dependent.

5.4.1. As effects on mRNA transcription

Introduction; Glia+ vs. Glia-

A strong glial modulatory effect is visible both on the change of the TRα TRβ and ERβ mRNA expression levels. The results from the cell culture containing granule cells co-cultured with glia shows no significant change between the experimental pairs. The difference in the pairs - between As threated and untreated cell cultures – were also negligible. In all of the glia- cultures however we found a strong inhibition vs. the As untreated pairs in every experimental group.

TRα and TRβ

Granule cells co-cultured with glia showed no significant change after As treatment in both TRα and TRβ. In all of the glia- cultures however we found two interesting phenomena. A noticeable difference was found between Glia- T3+As vs. T4+As, possibly due to the lack of type II deiodinase activity of the absent astroglia. Without the modulating effect of the glia both receptor subtypes demonstrated a strong downregulation in the respective mRNA expression in a hormone reduced environment (treatments without E2, T3 and T4). In tissues other than nervous tissue, As inhibits ongoing mRNA transcription in moderate doses, which supports our results of As mechanism in our cell culture (Davey *et al*., 2007, 2008; Sun *et al*., 2016), however during our experiment specific treatments produced different results. If we combined As with E2+T3 or E2+T4, we found a strong mRNA overexpression. This phenomenon cannot be explained according to the results of the recent literature. In the neural tissue in vivo As acts on the thyroid signaling pathways in a different way than in cell cultures produced only from specific cell types. My hypothesis is that As might cause oxidative stress in the neural tissue, and probably the adverse effect on the intracellular signaling can be modulated by THs. Conceivably this modulatory effect is more potent if E2 is also present in the tissues. This theory can be confirmed by a thesis written by Nagam (2013).

ERβ

Results in Glia+ ERβ mRNA expression levels after As and As+E2+T3 treatments showed a pattern comparable in trends with that found in BPA studies in Glia+ and to those seen in Zea studies in Glia-. It is noteworthy, however, that As+E2+T3 treatments did not lead to such robust activation of ERβ transcription as detected after BPA+E2+T3 treatments in Glia+. These observations raise the possibility that effects of As may lie on the grounds of a mechanism distinct from that used by BPA. The only outstanding difference was found in Glia- T3+As vs. T4+As similar to a phenomenon we found in our glia- TRβ mRNA expression experiment.

Our present results suggest that in the CNS, As alters ERβ transcription, regardless of the absence or presence of glia. Although at present we do not know the exact reason of the ERβ mRNA decreasing effect of As, it is possible that it caused a metabolic defect in the cell culture even during the relatively short, 6 hours duration of the treatment. The question of whether possible interaction exists between As and one or more other EDs warrants further experiments.

5.4.2. As effects on protein translation

Introduction; Glia+ vs. Glia-

On the protein expression level As induced a strong downregulation at mRNA level in most of our cell cultures. As is a strong cellular toxin which actively express new genes, proteins and activate caspase enzymes to induce apoptosis. As neurotoxicity is linked to cellular death caused by activation of different cellular pathways including MAP kinases. Inhibiting protein or RNA synthesis blocks the As-induced cerebellar neuron apoptosis (Namgung and Xia, 2001).
Hypothetically As with the absence of the glial modulation lowered the cell viability thus increased MAPK function to a level where all of the translational functions were stimulated, thus producing an increased receptor protein amount in each of our test samples.

mRNA vs. protein expression

All of the glia- mRNA expression results correlate with the data measured from the respective glia- receptor protein levels in case of ERβ. Compared to the changes in the mRNA expression, in Glia+ As lowered the expressed protein levels in ERβ vs. the ED untreated pairs. In case of TRα and TRβ the changes were inconsistent, only a minor alteration was found similar to the results from the TRα and TRβ mRNA values from PCR experiments. In Glia- the mRNA and protein changes were similar though the strength of the inhibition was reduced during the translation.

TRα

After As treatment both the TRα and TRβ receptor maintained an increased but almost equal level of protein expression in the Glia- experimental groups. A significant change was not found between any of the As treated probes in TRα however it was present between the untreated groups. In glia+ the only significant change induced by As was the increased TRα protein expression between ntC vs. As and E2 vs. E2+As. A 2-fold significant increase was found in both of the experimental pairs, but the results from untreated groups were lower than the TH

containing untreated groups, contrary to the results of Allen and Rana (2007). If the cell cultures contained T3 or T4, these differences were diminished, we found a constant protein level between all of the As containing and untreated groups.

As a consequence, As in Glia+ also equalized the TRα protein expression to a constant level in every experimental pair. It seems that TRα contributes to the apoptotic effect of As due to the disruption in its receptor protein expression which might result in a strong alteration in the physiology of the affected cells.

TRβ

The results from glia+ TRβ expression shows a decrease in protein expression after As treatment. The change is only significant in the T3 vs. T3+As pair, but all of the mean values show a decreasing trend. In case of glia- As increased the protein expression in all of the As treated samples compared to their respective ED untreated half. As a conclusion it is safe to assume that in the presence of glia the TRβ receptor and the signaling pathways starting with the TRβ receptor + ligand connection does not contribute in the As-induced apoptotic process. Although when the modulatory glial effect is unadequate in tissue As can act as an endocrine disruptor.

ERβ

Compared to the TR results we found an opposite As effect on the ERβ receptor expression. A downregulation was observed in every cell culture after As exposure, glia+ or glia- alike. The only exception was Glia- E2+T4 vs. E2+T4+As but the trend is similar. The rate of downregulation was significantly higher in the Glia+ group in contrast of the glia- cultures. Thus it is safe to say that the astroglia mediates the ERβ expression after As exposure.

Chen *et al*. (2002) found that lower doses of As significantly inhibited the ERα receptor protein expression but the expressed amount of ERβ remained unchanged in breast cancer cell lines. In mice hyperplastic uterine epithelium As caused an upregulation of ERs in the early lesions, although reversed effect was observed after progression (Waalkes *et al*., 2000). Most of the experimental results summarized in the review of Watson and Yager (Watson and Yager, 2007) are contradictory. It is safe to assume that the ERβ downregulatory effect of As is likely tissue specific, and according to our data, in the nervous tissue As inhibits ERβ protein expression.

As induced a strong downregulation in all of the cell cultures without astroglia. The glial cells likely reduce the apoptotic effects of As on the nerve cells. Under physiological conditions (In

Glia+) the effect of As was strong on the ERβ protein expression, but the translation in TRα and TRβ was negligible.

5.5. Effects of MBC

As an ED compound, MBC affects the estrogenic and thyroid pathways as well (Schlumpf *et al*., 2004; Schmutzler *et al*., 2004; Seidlová-Wuttke *et al*., 2006; Boas *et al*., 2012), but the exact mechanism-of-action is still unknown. It may alter the barrier function of the skin, causing an elevated risk of exposure simultaneously to any substance in contact of the organism. MBC exposure (mostly through ingestion) leads to convulsions, lethargy, ataxia, severe nausea and vomiting in small doses (Manoguerra *et al*., 2006). Additionally, seizures, apnea, asystole, circulatory collapse and death was observed in children (after small doses), or after an adequate dose in an adult (Bhaya and Beniwal, 2007; Khine et al., 2009).

In the CNS, after crossing the BBB MBC act as a stimulant with effects that range from mild CNS excitation to generalized seizures (Anthony S Manoguerra et al., 2006; MacKinney et al., 2015). Its effects on the developing CNS is only a little known, moreover those studies mostly focus on the hypothalamus–pituitary–gonad axis and the estrogen-related developmental changes in the early ages (Maerkel *et al*., 2007; Carou *et al*., 2009).

The estrogenic effect of the ED alters TSH, T3 and T4 levels in the serum in two different ways. One possible route may be elicited with the estrogenic effect of the ED by its own spectrum of alterations on multiple targets of the pathways in thyroid hormone action and metabolism (Schmutzler *et al*., 2004).

MBC increased serum TSH levels by inhibiting serum T4 trough the mechanisms of feedback regulation of the thyroid axis. An experiment by Seidlová-Wuttke *et al*. (2006) proved that MBC have an effect on several metabolic parameters (e.g. fat and lipid homeostasis) and on TH production, although these effects are not shared by E2 (Seidlová-Wuttke *et al*., 2006). The conclusions also support our hypothesis that other than estrogen-receptive mechanisms may be responsible for the modulation between transcription and translation of the observed receptors.

A study demonstrated that MBC showed no binding to ERβ in human endometrial cells, nevertheless it affected ER mediated events (Mueller *et al*., 2003). Interestingly, however, Schlumpf *et al*. (2004a) found that MBC displaced 16-alpha-125-I-estradiol from ERβ but not from ERα. Further studies also clarified that intrauterine exposure to MBC alters mRNA expression for both ERα and ERβ in uterine cells of the offspring (Durrer *et al*., 2005). A more detailed study by Schlumpf et al. (2004b) revealed that MBC exposure during the gestation

affects ERα, ERβ and progesterone receptor mRNA expression in various tissues, and also tissue-dependently, including the hypothalamus.

5.5.1. MBC effects on mRNA transcription

Introduction; Glia+ vs. Glia-

Adding to the afore-listed knowledge, probably the most prominent effect of MBC on our experimental model, among the other EDs used in this study, was its potency to increase ERβ mRNA expression in Glia+ cultures as compared to its respective ntC. We found notable differences between Glia+ vs. Glia- in the TRα and TRβ receptors as well, the direction of the disruption was different in Glia+ (mostly a receptor mRNA upregulation was found) than in Glia- (strong receptor mRNA downregulation).

TRα

According to our results, TRα mRNA was elevated by MBC in every glia+ and glia- cell cultures, except two glia suppressed experimental groups: ntC and the group treated with T3 only. Interestingly the measured transcription level in Glia- ntC was 6 times higher than Glia+ ntC, nevertheless MBC treatment equalized the measured mRNA levels.

TRβ

We observed only a moderate MBC effect on the TRβ receptor in the Glia+ cultures. In Glia- however a stronger suppression was observed than what we have found in the TRα cultures. The only significant change was produced by T3 in the Glia- cell cultures which was observed in case of the TRα receptor as well. Durrer *et al*. (2005) previously showed that MBC dose-dependently reduced the E2 induced up-regulation of progesterone receptor (PR) and insulin-like growth factor 1 (IGF-I) and down-regulation of ERα and androgen receptor. There is a strong possibility that the T3 induced TRα and TRβ upregulation was inhibited by MBC in our experiment as well.

ERβ

MBC increased ERβ mRNA expression in Glia+ cultures as compared to its respective ntC. We detected an almost linear further increase in ERβ transcription if E2+T3 were added to MBC that is consonant with our previous experiments stating that besides disrupting the estrogenic pathways, MBC also affects thyroid functions (Schlumpf *et al*., 2004b; Schmutzler *et al*., 2004). Our results suggest that the two endocrine effects might be synergistic that lies on the ground of a complex interplay between the estrogen and thyroid hormones and their receptors (Scalise *et*

al., 2012). In Glia- cultures, however, MBC decreased ERβ mRNA compared to its respective ntC, with more decreasing potency when combined with E2+T3.

5.5.2. MBC effects on protein translation

Introduction; Glia+ vs. Glia-

According to the recent literature MBC mostly affects the TH signaling pathways in the cells. In our cell cultures the substance strongly influenced the T3 or T4 treated experimental groups. In Glia+ a potent upregulatory effect was seen in TRα and ERβ. In TRβ the rate of upregulation was low or negligible. Without TH-s the overall effect in receptor protein upregulation was significantly less in TRα. In comparison with Glia+, in Glia- the results were similar in TRα, TRβ produced a visible pattern in the receptor expression after MBC treatment. In ERβ differences between the majority of MBC treated groups were not found (except in two specific pairs – discussed later).

mRNA vs. protein expression

The changes found in the Glia+ in the transcription and translation after MBC treatment were similar. However, in Glia- MBC had a modulatory effect on the receptor protein expression in TRβ compared to what we found on the level of transcription. The changes in TRα and ERβ were similar to the PCR experiments (except in one specific ERβ experimental group – discussed later).

TRα

Results in TRα protein expression levels show a reliable increase in the glia+ groups after MBC treatment. A noticeable trend is shown in every MBC treated sample. All of the glia+ MBC+hormone treated groups showed a lowered receptor expression compared to MBC only. Possibly all of our hormones used in the experiments lower the effect of the ED; however, the change in the expression remained high in every cell culture, in the overall expression level and the level compared to the respective untreated group (from a 4-fold increase to a 2-fold overexpression) as well. In glia- MBC showed a potency to elevate the TRα receptor expression to a 2.5 times higher level than the ntC in every cell culture, except the ones in MBC+T3. It seems that the direction of the modulation between the Glia- T3 and Glia T3+MBC treatment reversed itself between mRNA transcription and the protein translation, according to the western-blot results the MBC increased the protein expression rate.

TRβ

All of the treated cell cultures showed a slight elevation in TRβ receptor expression compared to the ntC in Glia+ cultures, and the rate of the increase was nearly the same. Based on our results we may assume that MBC does not alter TRβ receptor expression in the cell cultures if glial cells are present. In the Glia- group a difference can be seen between the groups without the disruptor and their MBC treated counterparts. MBC caused a minor non-significant increase in groups treated with only one hormone. However, in cell cultures with E2+T3 the resulting expression level was more intense after MBC exposure, maybe due to the lack of the deiodinase II this phenomenon was absent in the E2+T4 +MBC treated group.

Our results from the THRs suggest a potent connection between MBC and the thyroid regulatory system. Overall MBC showed a strong disruptor effect on both of TRα and TRβ receptor expression, indicating a potent ED nature. The effect of the disruption was only mediated by the Glia in case of the TRβ receptors. In the Glia+ experimental cultures the measured protein levels were identical to the „most physiological" Glia+ E2+T3 group. Interestingly this phenomenon was not manifested in the results from Glia- TRβ. It is a strong possibility that the supporting function of the astroglia mediates the deteriorative effect of MBC on the granule cells.

ERβ

ERβ protein expression values in MBC groups were nearly identical in Glia+ cultures. In the Glia- cultures however we found that compared to the MBC (only) treated group, the added hormones E2+MBC or T3+MBC lowered the ERβ protein expression, but if we applied them together in E2+T3+MBC, it caused a strong upregulation of ERβ receptors. It appears that MBC acts as an ED on both hormone systems, and if both hormones are present in a physiological level, it may worsen the effect of the endocrine disruption of MBC. All of the MBC treated groups show a 6x increase, except Glia- T3+MBC where the change in the expression level was only three times higher as the level measured in Glia- T3. The strong change in ERβ levels suggest that MBC has a really strong ED characteristic not just on the thyroid system but on the estrogen regulation as well.

Altogether, of the EDs tested in this study, MBC appears to influence TRα, TRβ and ERβ mRNA and protein expression via a mechanism distinct from that/those involved in BPA-Zea-As effects, nevertheless it is evident that MBC effects are also mediated by the glia. It has to be noted that while mean values in our results are considerably different, in some of the groups variances were also high and therefore some of the listed differences were not statistically significant.

Since, however, MBC effects on different cell types seems to highly vary (Schlumpf *et al*., 2004), our results still remain alarming with regard to MBC's influence on neural development.

5.6. All-ED Effects

Summarizing the results of the ED treatments, we detected changes in ERβ, and most of the TRα and TRβ mRNA expression levels after each of the EDs that we used, and these results suggest that there are distinct ways how the EDs used may alter receptor transcription. The observed versatility in ED effects, therefore, raised the question of how all the four EDs (All-EDs), co-added to the cultures, would affect receptor synthesis (on the mRNA and protein levels), since in their everyday lives, living organisms are exposed to a variety of EDs simultaneously.

If we compare the results from the "ED-cocktail" treated cell cultures with the different, individual ED treatments, we can separate or rule out specific routes of modulation or we can observe and identify the main – possibly the most dangerous – disruptor of the specific receptors.

For guidance to understand the comparison between individual ED and All-ED effects additional figures are presented. The figures are for representation, to ease the understanding of this chapter. The specific values of the bars, and levels of significance can be found in the Results chapter and in the Appendix. It can be generally stated that on the upper part of every picture results from Glia+, while in the bottom part results from Glia- are shown. On the Y axis the fold difference vs. ntC (Glia+) is plotted. All of the results are compared to the Glia+ ntC value, which we set arbitrary to „1". The bars on the graph are representing the fold changes compared to this "1" value measured after ED administration grouped by additional treatments. The specific treatments can be found below the Glia- graphs. Red bars are representing the All-ED treated data, bars with distinct colors are illustrating the individual ED effects.

5.6.1. All-ED effects on mRNA transcription

Glia+ vs. Glia-

Analyzing the compared results on Figure 18-20 we can observe differences among individual ED effects, and we can have a picture of the All-ED treatment effect in contrast to the individual EDs. The differences between the shown treatments were non-significant. Individual results does not show any uniformity, in many cases they have 1 or 2 magnitudes of differences

between them. This phenomenon was found only in of the results in the other experiments, but here, all of the groups produced inconclusive data, possibly because of the summative effect of different mechanisms of disruption (e.g.: agonist – antagonist interaction between EDs or blocking different parts of the receptor signalization pathways).

Due to the high differences in the variance of the values from different ED treatments, only a few conclusions can be drawn concerning the combined ED effects. One of the visible trends are the "loss of BPA effects" in the combined treatment. As already shown in Chapter 5.2.1. (BPA effects on mRNA transcription), BPA alone suppressed receptor mRNA expression compared to ntC, but concurrent hormone treatment resulted in increased mRNA expression in every BPA treated culture, both in Glia+ and in Glia-. However, from the All-ED effect this phenomenon is missing. It is safe to assume that the used EDs in combination somehow suppressed the individual BPA effect on the neurons both in presence and absence of glia.

The second most conspicuous trend concerning the effects of the applied „ED cocktail" was found in Glia+ TRα, TRβ and ERβ cultures. Samples without E2 (All-ED and T3+All-ED), and without T3 (All-ED and E2+All-ED) show a higher transcription compared to their corresponding ED untreated halfs than samples treated with the combined EDs, E2 and T3. To a lesser extent the same phenomenon was observable in the Glia- cultures as well. In all of the mentioned pairs we found a robust upregulation of receptor mRNA, but the SEM values were high as well.

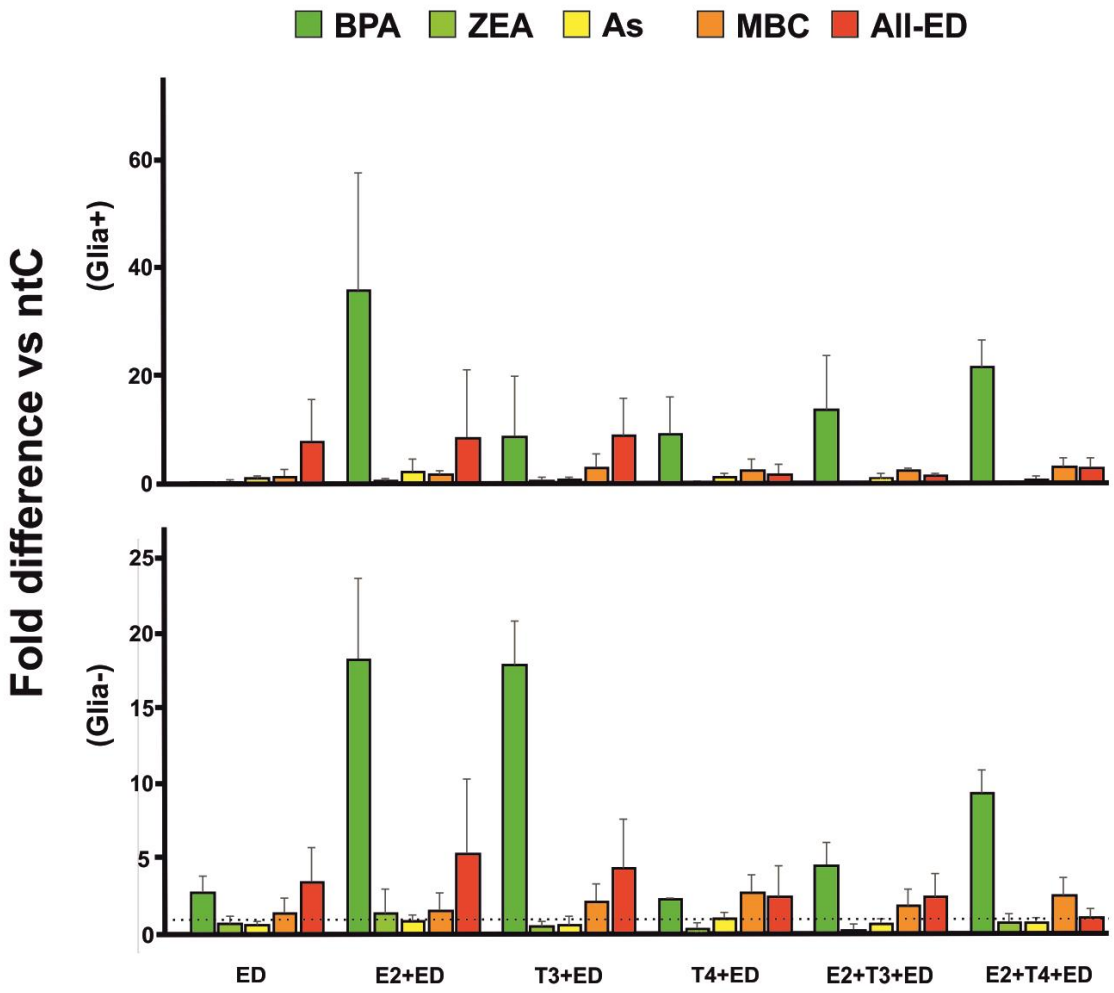

Figure 18: Comparing the effects of different ED treatments on the TRα receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

Combined ED effects were exerted when E2 and T3 levels were low, even in the presence of the glia. Since co-administration of E2 and T3 substantially reduced the robust effect of the mixture of EDs on receptor transcription, it is possible that under physiological circumstances, when young individuals are simultaneously exposed to multiple EDs, their neural development (in probably any CNS areas with the appropriate receptors), is more affected by environmental EDs when secretion of T3 and E2 is insufficient.

Supporting this idea, E2+T3, co-administered with All-EDs “only” doubled the receptor transcript values each of the three receptors in Glia+. This may result from the receptors higher affinity to the hormone ligands.

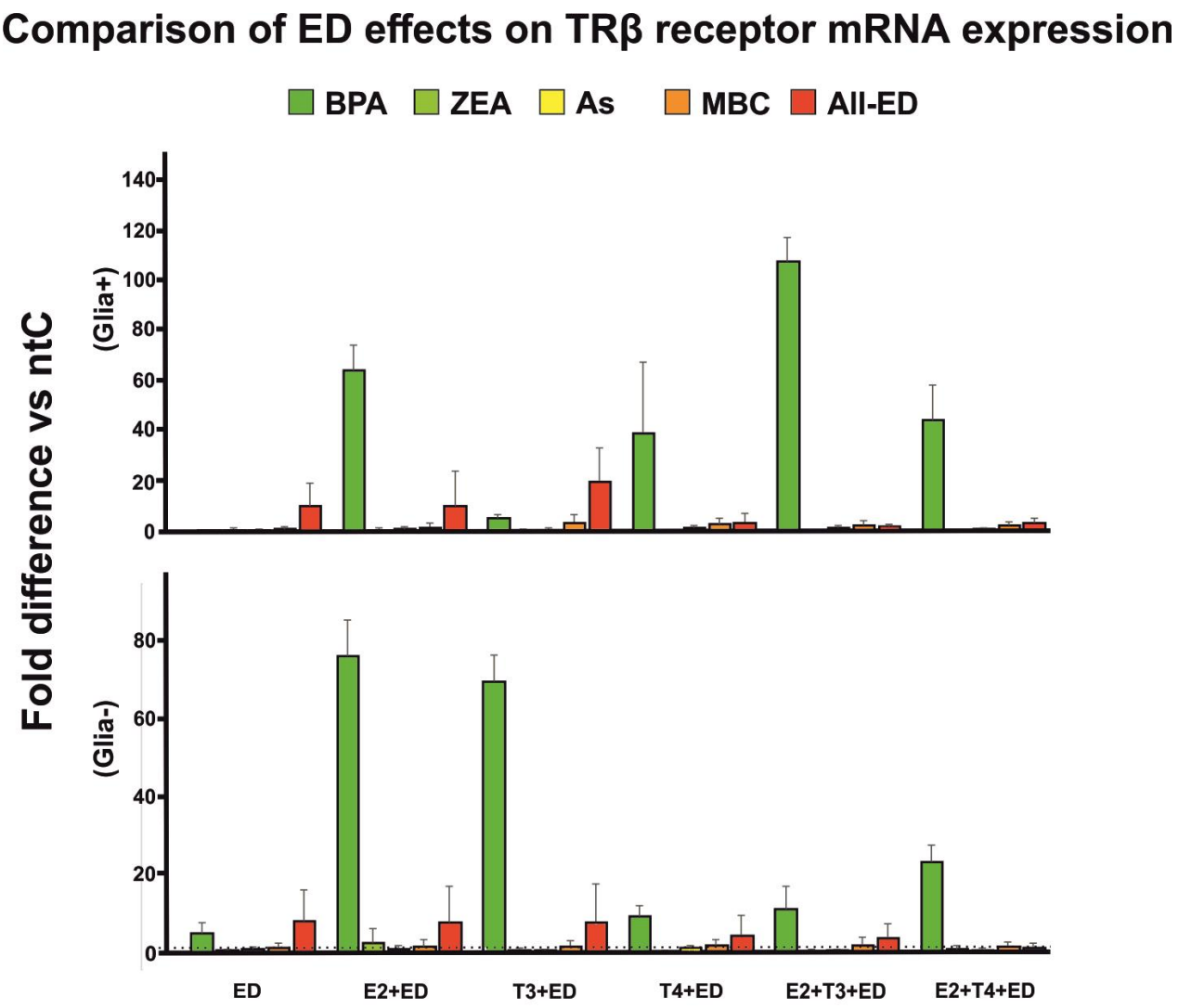

Figure 19: Comparing the effects of different ED treatments on the TRβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1” – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes compared to this “1” value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

Receptor mRNA expression values in All-ED+E2+T3 groups were nearly identical in Glia+ versus Glia- cultures, therefore it may appear that the glia did not play a role in the setting of the receptor transcript levels. It is also possible that under *in vitro* conditions and in the presence of the compounds used for these treatments, measured values represent an optimal receptor transcript level for survival that can be set in both major cell types, neurons and glia as well.

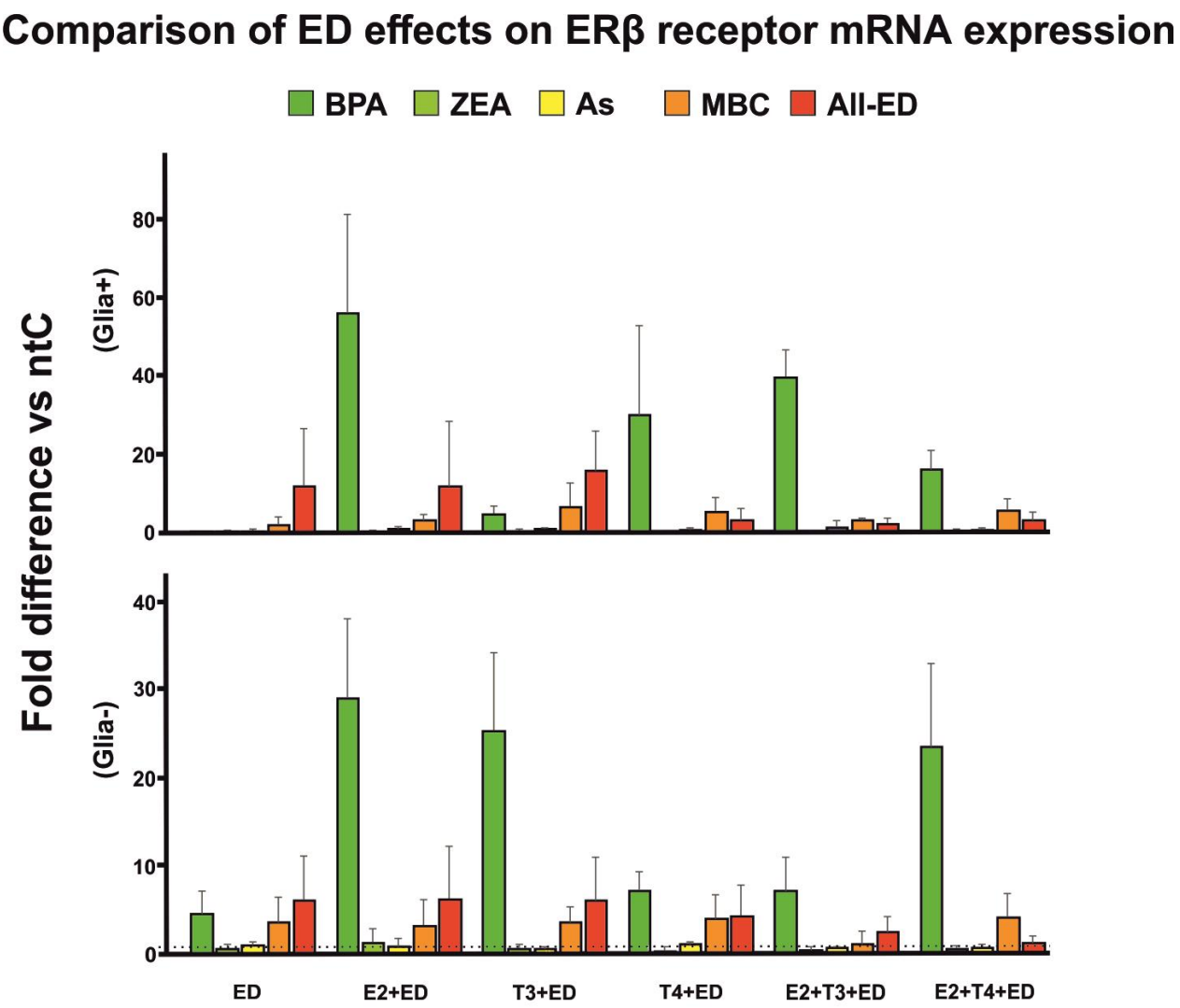

Figure 20: Comparing the effects of different ED treatments on the ERβ receptor mRNA expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

5.6.2. All-ED effects on protein translation

One of the most important aspects of the result analysis of the ED effects of protein expression is that we can observe a state, where the physiology of the cells are already disrupted, the interposing mechanisms between transcription and translation are already influenced by the substances of the experiment, “the damage has already been done”.

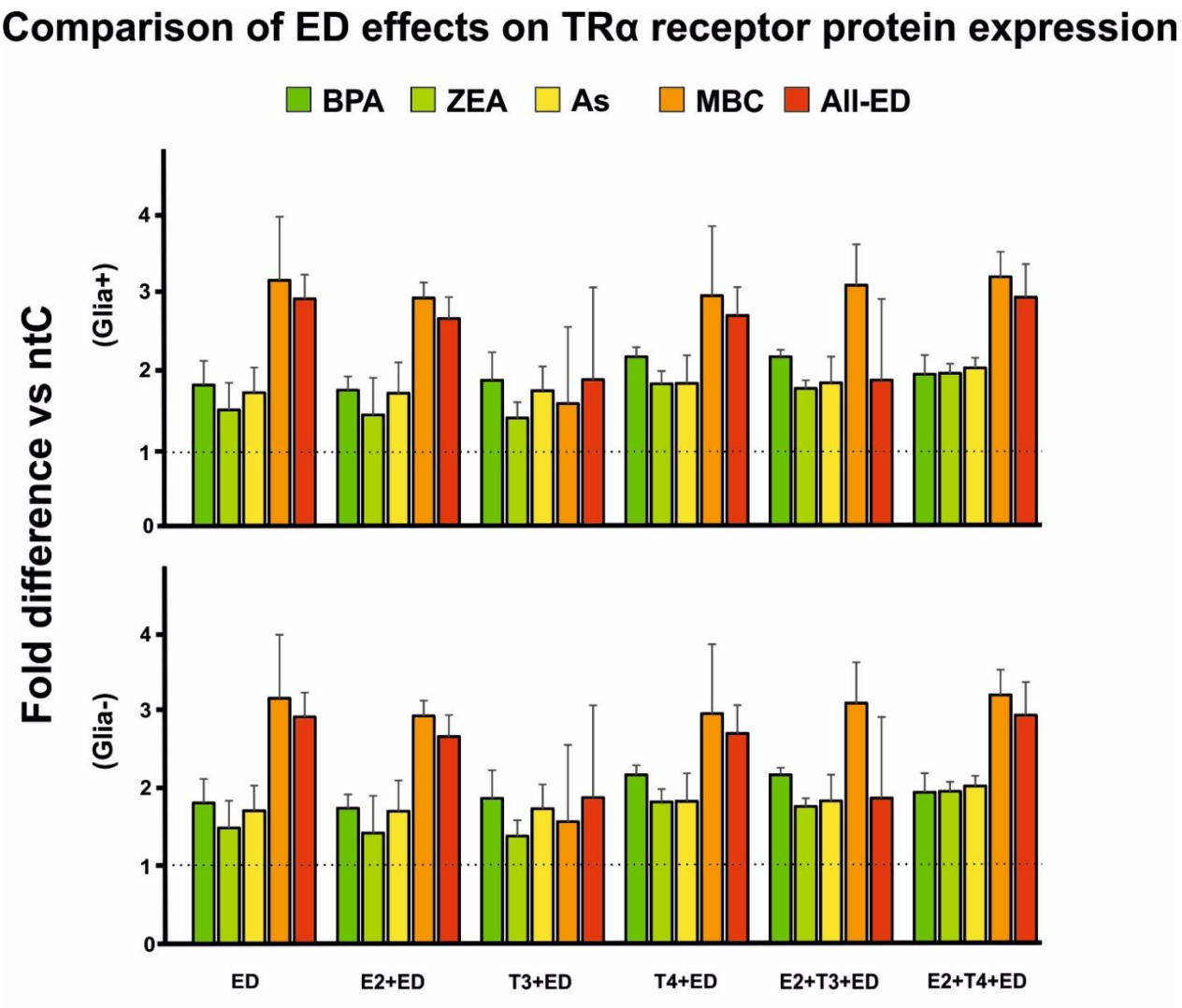

Figure 21: Comparing the effects of different ED treatments on the TRβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1” – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes compared to this “1” value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

TRα

Comparing the different ED treatments to those of All-ED treated samples, our findings clearly show a strong MBC influence on the TRα protein expression (Figure 21). When glial cells were co-cultured with neuron cells, All-ED treatments caused a strong upregulation of the tested receptors. The rate of upregulation was higher than the effect of BPA, ZEA or As. The fold difference compared to ntC cultures were similar to the MBC results in every treatment, indicating that MBC exert the strongest effects on the TRα protein expression modulating processes from the different EDs of the experiment. Another possibility is that MBC masks the other EDs effects.

MBC effects were also strong in the cell cultures without glial cells, when T3 levels were low. Only a minor receptor upregulation was found in case of the Glia- T3+MBC and Glia- E2+T3+MBC groups. The former phenomenon might be explained with the “MBC as primary modulator of TRα” hypothesis, due to the same observation found in the MBC experiment. However, the latter (The weak upregulation in Glia- E2+T3+All-ED compared to the T3 untreated All-ED groups) is a phenomenon only found in the All-ED experiment, conceivably the absence of astroglia lowered sensibility of the neurons.

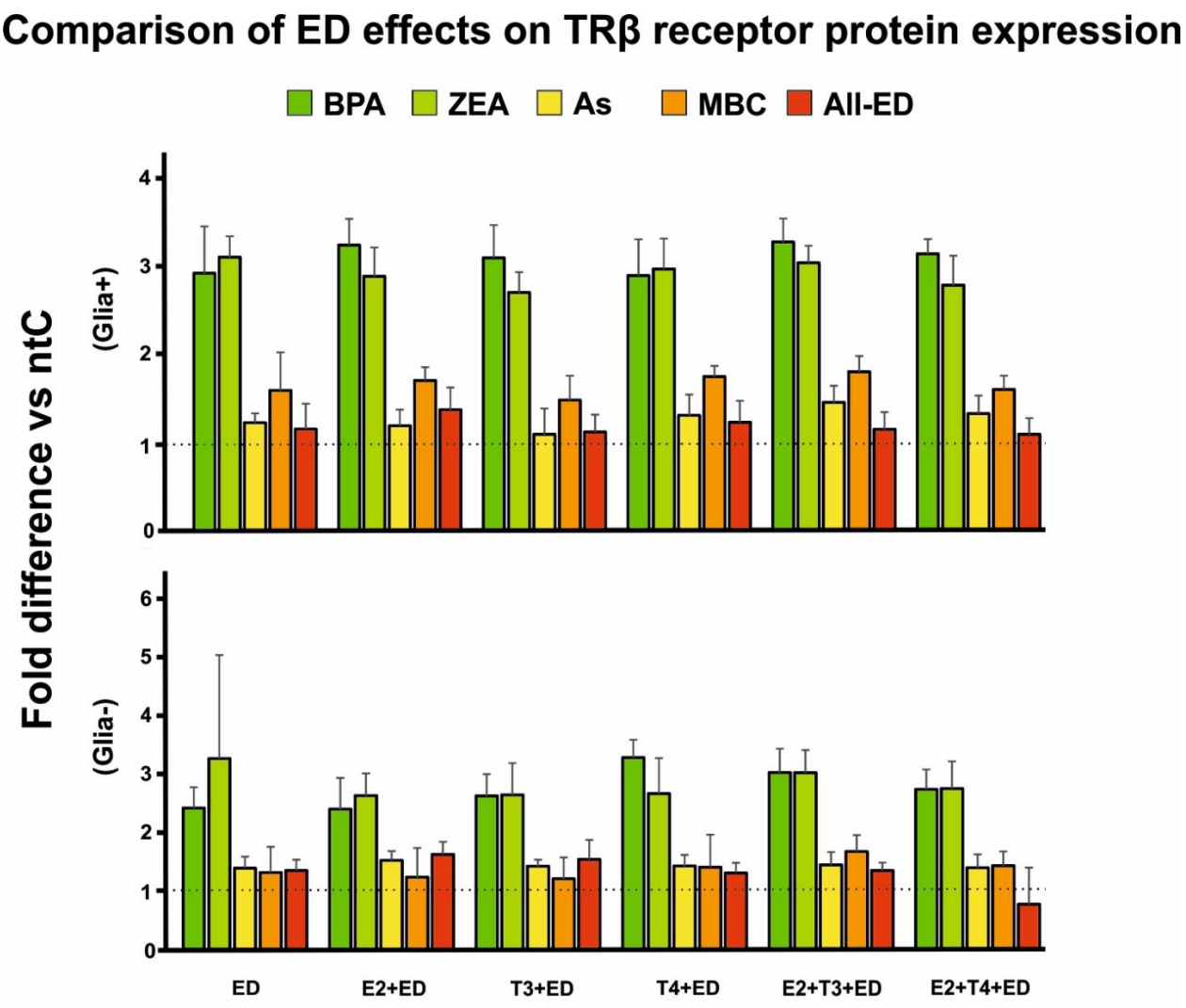

Figure 22: Comparing the effects of different ED treatments on the TRβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

TRβ

The effects on the TRβ receptor expression after All-ED treatment showed a strong similarity to the As or MBC treatment (Figure 22). The TRβ fold difference compared to ntC was negligible. The outcome of this experiment is exceptionally interesting because the All-ED treatment contained BPA and ZEA with MBC and As. BPA or ZEA alone caused a strong upregulation of the TRβ receptor protein. Hypothetically due to the lack of modulatory effects of MBC or As on receptor expression, BPA and ZEA should have an impact on different modulatory functions

leading to protein translation, furthermore the influenced functions shall compensate for one another. Another possibility is that MBC and/or As masks or inhibits the effects of BPA or ZEA, thus maintaining a normal protein expression.

Either way, the results of this experiment are particularly important, because it indicates that the ED "cocktail" has a negligible influence on the TRβ receptor expression of the nerve cells. Nevertheless, the disruption will alter other receptors, however this finding can help us understand the role of the TRβ receptor in the cell signalization pathways of the nerve cells.

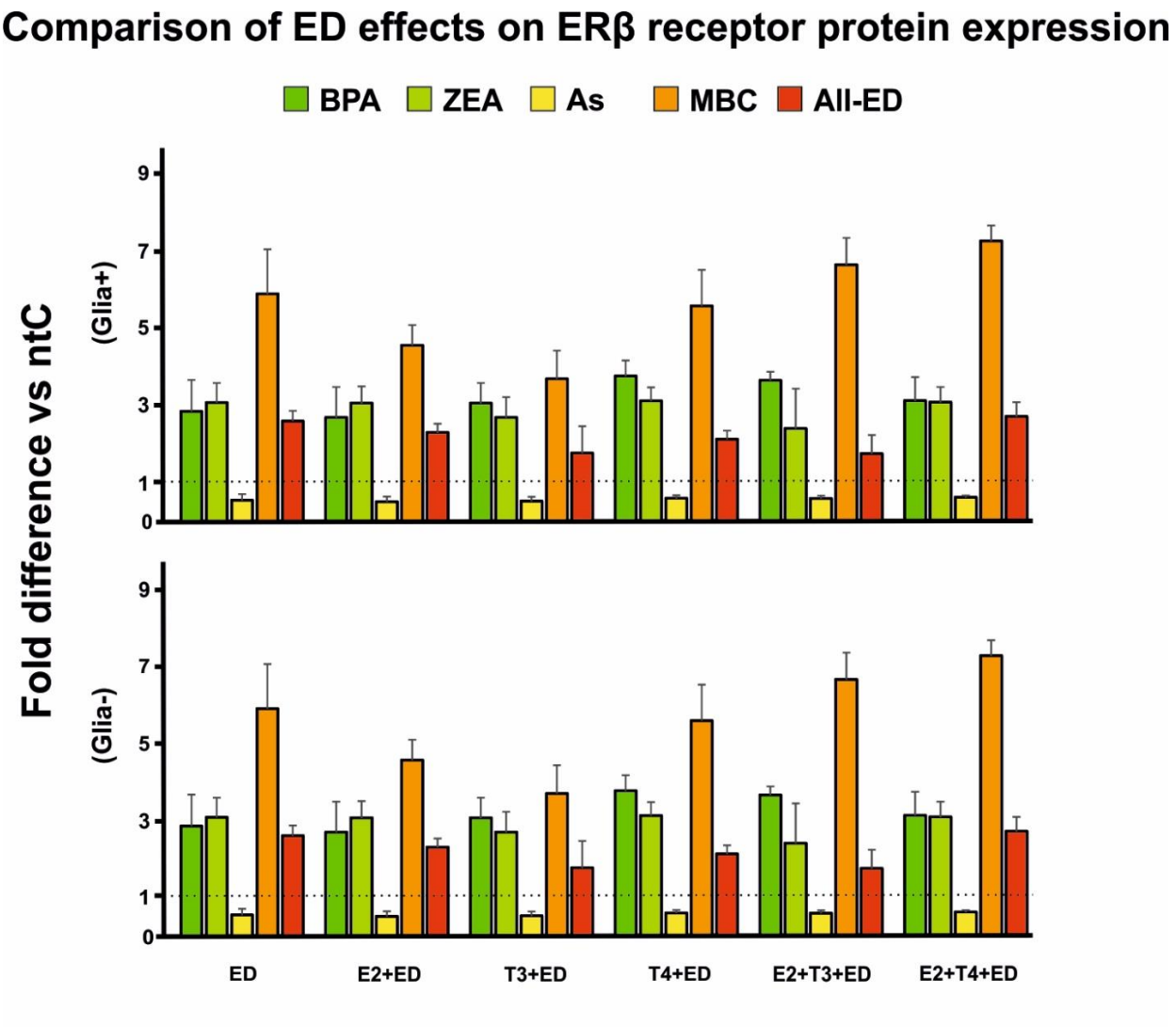

Figure 23: Comparing the effects of different ED treatments on the ERβ receptor protein expression in glia-containing or glia-suppressed cerebellar granule cell cultures. On the Y axis the fold difference vs. ntC (Glia+) – set arbitrary to „1" – is plotted. Glia+ ntC value is represented by a dotted line. The bars on the graph are representing the fold changes

compared to this “1” value measured after different treatments indicated by different letters on the X axis. Bars are color coded, representing the specified disruptors.

ERβ

Comparing the results of the ERβ protein expression modulating effects of the different EDs with the All-ED treatment a strong upregulation can be observed in all cell cultures (Figure 23). It is clear that both in the Glia+ and Glia- samples the All-ED effect on protein translation was distinct from the effects of MBC and As. All-ED caused a 2.5-fold upregulation in every ED treated cell culture compared to ntC and the corresponding untreated samples opposed to As (the fold difference was halved compared to the untreated samples) or MBC (6-7 fold upregulation compared to the untreated samples). The difference between All-ED and BPA or ZEA on the ERβ protein expression was negligible. Interpreting the results can be problematic because the final result of regulation during protein translation of the “ED cocktail” could have happened due to different processes:

1,) Simply the different effects of regulation may lead to a summarized fold difference value. As seen during the introduction of my thesis, EDs alter a myriad of intracellular components (signals, enzymes, receptors, etc.) of the estrogen signaling pathways. Possibly some of the aforementioned mechanisms are counterproductive, activating or inhibiting them by different specific ligands may lead to an equilibrium in the cell.

2,) Possibly the effects of MBC and As are weak compared to BPA and/or ZEA, or their influence are masked by the BPA and/or ZEA action in the cell culture.

3,) Hypothetically it is also possible that robust increases in translation may potentially exhaust cellular energy resources, thereby influencing other energy-dependent cellular processes, e.g.: the unknown “interposed mechanisms” or the translation as well. In my opinion the results produced during the MBC experiments refute this idea. If MBC can cause a 7 fold difference in upregulation, the maximum amount after All-ED exposure should be at least as robust to what we found during the previous (MBC) experiment.

Nevertheless, the „ED cocktail” did alter the receptor expression of TRα, TRβ and ERβ of the nerve cells, and there was a notable change in the receptor upregulation.

5.7. Conclusions

The results in this thesis clearly indicate that all of the EDs – alone or in combination – interfere with the physiological hormonal regulation of TRα, TRβ and ERβ mRNA and protein expression. Due to different results measured in Glia+ and Glia- cultures, and the disparity of ED effects between the receptor transcription and translation indicate that not just a specific, but a complex group of regulatory processes will be disrupted by the tested substances. The method of disruption depends on the type of ED, hypothetically all biochemical processes may be altered between the receptor activation and the gene transcription. EDs act as an agonist, or an antagonist of the target receptor, depending on the structure and biochemical traits of the substance.

This phenomenon clearly can be seen based on the results of this thesis. The impact of different EDs on the receptor expression were rather heterogeneous although we suspected a somewhat uniform effect due their properties to affect the thyroid and estrogen hormone system. From the experiments three important conclusions can be drawn: 1.) EDs' influential effects on TRα, TRβ and ERβ transcription and translation depends on the specific ED; 2.) THs and E2 affect the interference caused by endocrine disruption. Combined ED effects are exerted when E2 and T3 levels are low; 3.) The glia modulates ED effects on the mRNA and protein level of receptors in the cultured cell populations, and most probably the neuronal receptor transcription and translation as well.

Contrary to our hypothesis the effects of the combined ED exposure on the receptor protein expression of the target cells were not additive, even though the effect robustly altered the physiology of the neurons. It seems that specific EDs might mask each other's influence, inhibit the effect of each other or they might exhaust the energy storage of the cells, preventing further receptor expression.

The thesis clearly proves that all of the used EDs alone or in combination disrupt the neuroendocrine system. Disrupting the physiology of the neurons and the glia by the exposure of the test substances may lead to yet unknown – either beneficial or adverse – biological consequences in the CNS, and in the neuroendocrine organs of the organism.

6. NEW SCIENTIFIC RESULTS

A/1. TRα, TRβ and ERβ mRNA and protein expression were disrupted by different levels due to bisphenol-A (BPA), zearalenone (ZEA), arsenic (As) and 4-methylbenzylidene camphor (MBC) alone and in combination.

Compared to the ntC **mRNA levels** BPA greatly enhanced and ZEA halved the transcription; when the glia is limited in the cell cultures, mRNA levels are lowered by As; The effect of MBC on the transcription is strongly modulated by co-administered hormones.

The **receptor protein levels** are doubled by BPA and ZEA in case of TRβ and ERβ (TRα protein expression was only influenced by BPA when the glial effect was not potent). As reduced ERβ the expression in the presence of Glia. In glia reduced cultures expression level of TRα, TRβ and ERβ was decreased. MBC greatly elevated TRα and ERβ but TRβ was unaffected in the presence of glia.

A/2. Combined effects of disruption are receptor specific, but these effects were not additive. On the mRNA levels the combined treatment resulted in inconclusive data. MBC dominated in the disruption of TRα expression. Only As and MBC influenced the TRβ expression. ERβ was mainly modulated by BPA and ZEA.

B/1. 17-β-estradiol (E2) and thyroid hormones (T3, T4) altered the individual effect of investigated endocrine disruptors (BPA, ZEA, As, MBC) in a receptor specific, and an endocrine disruptor specific way. T3 lowered the TRα, TRβ and ERβ receptor mRNA expression in BPA treated cultures. The effects of As were not influenced by the hormones. T3 inhibited the effect of MBC on the protein expression.

B/2. Toxic interaction of BPA, ZEA, As and MBC was highly increased in the presence of low lewel of E2 and T3.

C. The presence of glial cells reduced the influence of endocrine disruptors on the receptor mRNA and protein expression levels. Modulatory effects of EDs were more potent in the absence of glial cells.

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8. THE AUTHOR'S PUBLICATIONS

8.1. Publications related to the topic of the present dissertation

8.1.1. Full text papers in peer-reviewed journal

Jocsak G., Kiss DS., Toth I., Goszleth G., Bartha T., Frenyo LV., Horvath TL., Zsarnovszky A.: **Comparison of Individual and Combined Effects of Four Endocrine Disruptors on Estrogen Receptor Beta Transcription in Cerebellar Cell Culture: The Modulatory Role of Estradiol and Triiodo-Thyronine**, *Int J Environ Res Public Health*, 13(6). pii: E619. doi: 10.3390/ijerph13060619., 2016. (IF: 2.035)

Somogyi V., Horvath TL., Toth I., Bartha T., Frenyo VL., Kiss DS., **Jocsak G**., Kerti A., Naftolin F., Zsarnovszky A.: **Influence of bisphenol A on thyroid hormone receptors in rat cerebellar cell culture**, *Acta Vet Hung,* 64(4):497-513, 2016. (IF: 0.871)

Jocsak G., Kiss DS., Toth I., Barany Z., Zsarnovszky A., Frenyo VL**.: A zearalenon, mint mikotoxin káros hatásai az emlős szervezetben: az utóbbi évtizedek eredményeinek rövid áttekintése. (Adverse biological effects of the mycotoxin zearalenone in mammals: a review.)**, *Magyar Állatorvosok Lapja,* 139(1):55-63, 2017*.* (IF: 0.185)

8.1.2. Poster and oral presentations on international conferences

Toth I., Kiss DS., **Jocsak G**., Bartha T., Frenyo VL., Zsarnovszky A.: **Effects of Bisphenol A on the regulation of estrogen- and thyroid hormone receptor expression in presence of estrogen and/or thyroid hormones on the developing cerebellum***,* P142, In: *International Brain Research Organization International Workshop,* Debrecen, Hungary, 2014.

Jocsak G., Toth I., Somogyi V., Bartha T., Frenyo VL., Zsarnovszky A. : **Effects of bisphenol a on the expression level of er and th receptors in the developing cerebellum**, FENS-0461, In: *9th Meeting of Federation of European Neuroscience Societies,* Milano, Italy, 2014.

Jocsak G., Somogyi V., Toth I., Goszleth G., Bartha T., Zsarnovszky A.: **Endocrine disruptor effect of Bisphenol A on the developing cerebellum, through estrogen and thyroid hormone receptor expression level changes,** p9.2, In: *Meeting of Hungarian Physiological Society - Federation of European Physiological Societies,* Budapest, Hungary, 2014; *Acta Phys. 211. 148,* 2014.

Jocsak G., Toth I., Somogyi V., Kiss DS., Bartha T., Frenyo VL., Horvath TL., Zsarnovszky A.: **Ligand-dependent estrogen and thyroid hormone receptor mRNA expression is modulated by the glia**, p1-71, In: *15th Meeting of the Hungarian Neuroscience Society,* Budapest, Hungary, 2015.

Jocsak G., Toth I., Somogyi V., Kiss DS., Bartha T., Frenyo VL., Zsarnovszky A.: **Effects of bisphenol-A on cell viability in developing cerebellar cell culture**, P6-6, In: *The Joint Meeting of the Federation of European Physiological Societies and the Baltic Physiological Societies,* Kaunas, Lithuania, 2015.

Jocsak G., Toth I., Kiss DS., Ashaber M., Barany Z., Bartha T. Frenyo VL., Zsarnovszky A.: **Effects of zearalenone on the expression level of estrogen- and thyroid hormone receptors in the developing cerebellum**, P2-125, In: *16th Meeting of the Hungarian Neuroscience Society; International Brain Research Organization International Workshop,* Budapest, Hungary, 2016.

Jocsak G., Toth I., Kiss DS., Bartha T., Frenyo VL., Zsarnovszky A.: **The effects of zearalenone on the expression level of thyroid- and estrogen hormone receptors in the developing cerebellum, a likely mechanism for endocrine disruption**, 1624-E009, In: *10th Meeting of Federation of European Neuroscience Societies,* Copenhagen, Denmark, 2016.

8.1.3. Oral presentations on Hungarian national conferences

Jócsák Gergely, Tóth István, Bartha Tibor, Frenyó V. László, Zsarnovszky Attila: **Ösztrogén, pajzsmirigyhormonok és biszfenol a hatása az ösztrogén- és pajzsmirigyhormon receptorok expressziójára fejlődő kisagyban**

Jócsák Gergely, Bartha Tibor, Goszleth Gréta, Zsarnovszky Attila: **Ösztrogén, pajzsmirigyhormonok és a zearalenon hatása az ösztrogén- és pajzsmirigyhormon receptorok expressziójára fejlődő kisagyban**

Jócsák Gergely, Tóth István, Bartha Tibor, Frenyó V. László, Zsarnovszky Attila: **A 4-metilbenzilidén kámfor hatása az ösztrogén- és pajzsmirigyhormon receptorok expressziójára fejlődő kisagyban, önmagában, ösztrogén és pajzsmirigyhormonok jelenlétében**

8.2. Publications not related to the topic of the present dissertation

8.2.1. Full text papers in peer-reviewed journal

Kiss DS., **Jocsak G**., Zsarnovszky A.: **Az inzulin szerepe a táplálékfölvétel központi idegrendszeri szabályozásában**, *Magyar Állatorvosok Lapja,* 134(10):635–640, 2012. (IF: 0.185)

Toth I., Kiss DS., **Jocsak G**., Somogyi V., Toronyi E., Bartha T., Frenyo VL., Horvath TL., Zsarnovszky A.: **Estrogen- and Satiety State-Dependent Metabolic Lateralization in the Hypothalamus of Female Rats**, *PLoSONE*, 10(9):e0137462, 2015. (IF: 3.234)

Borbély S., **Jocsak G**., Moldován K., Sedlak E., Preininger E., Boldizsar I., Toth A., Atlason PT., Molnar E., Vilagi I.: **Arctigenin reduces neuronal responses in the somatosensory cortex via the inhibition of non-NMDA glutamate receptors**, *Neurochem Int.* 97:83-90. 2016. (IF: 3.385)

Kiss DS., Toth I., **Jocsak G**., Sterczer A., Bartha T., Frenyo VL., Zsarnovszky A.: **Preparation of purified perikaryal and synaptosomal mitochondrial fractions from relatively small hypothalamic brain samples**, *MethodsX,* 3:417–429, 2016. (IF: -)

Barany Z., Sterczer A., **Jocsak G**., Frenyo VL., Kiss DS.: **A hepaticus encephalopathia kóroktana, patogenezisének újabb aspektusai (The etiology and some new**

pathophysiological aspects of hepatic encephalopathy) *Magyar Állatorvosok Lapja,*139(3):157-168, 2017. (IF: 0.185)

8.2.2. Poster and oral presentations on international conferences

Borbely S., **Jocsak G**., Sedlak E., Borsodi L., Boldizsar I., Atlason P., Molnar E., Vilagi I.: **Biologically active lignan arctigenin effectively reduces neuronal excitability,** *EPILEPSIA 50,* (S10) p. 79. 2009.

Borbely S., Toth A., **Jocsak G**., Molnar E., Detari L., and Vilagi I.: **Neuronal excitability is dose-dependently lessened by plant lignan arctigenin via inhibition of kainate receptors**. *Frontiers in Neuroscience,* vol: 4. 2010.

Vilagi I., Borbely S., **Jocsak G**., Sedlak E., Boldizsar I., Gyurjan I., Atlason P., Molnar E. Preininger E.: **Arctigenin, a plant lignan, dose-dependently reduce the neuronal excitability via inhibition of kainate receptors,** B34, *In: 7th Meeting of Federation of European Neuroscience Societies,* Amsterdam, The Netherlands, 2010.

Kiss DS., Toth I., **Jocsak G**., Bartha T., Frenyo VL., Zsarnovszky A.: **Mitochondrial metabolic lateralization in the rat hypothalamus***,* P141, In: *International Brain Research Organization International Workshop,* Debrecen, Hungary, 2014.

Toth I., Kiss DS., **Jocsak G**., Somogyi V., Bartha T., Frenyo VL., Zsarnovszky A.: **Metabolic lateralization in the hypothalamus: possible mechanism for cramming multiple functions into a small place**. FENS-0455, In: *9th Meeting of Federation of European Neuroscience Societies,* Milano, Italy, 2014.

Toth I., Kiss DS., **Jocsak G**., Frenyo VL., Zsarnovszky A.: **A possible way to decrease ”crowdedness” throughfunctional asymmetry in the hypothalamus***,* p10.19, In: *Meeting of Hungarian Physiological Society - Federation of European Physiological Societies,* Budapest, Hungary, 2014; *Acta Phys. 211. 148,* 2014.

Mandoki M., **Jocsak G**., Somogyi V., Kiss DS., Toth I., Bartha T.: **Use of virtual patients in teaching veterinary physiology at the Faculty of Veterinary Science**, conference speech, In: *Meeting of Hungarian Physiological Society - Federation of European Physiological Societies,* Budapest, 2014; *Acta Phys.* 211. 49, 2014.

Toth I., Szabo C., **Jocsak G**., Somogyi V., Kiss DS., Bartha T., Frenyo VL., Horvath TL., Zsarnovszky A.: **Impact of satiety state and testosterone on the metabolic lateralization in the male rat hypothalamus**, p1-72, In: *15th Meeting of the Hungarian Neuroscience Society,* Budapest, Hungary, 2015.

Toth I., Kiss DS., **Jocsak G**., Somogyi V., Bartha T., Frenyo VL., Zsarnovszky A.: **Satiety state dependent metabolic sidedness in the hypothalamus of male rats**, P6-5, In: *The Joint Meeting of the Federation of European Physiological Societies and the Baltic Physiological Societies,* Kaunas, Lithuania, 2015.

Toth I., Kiss DS., Ashaber M., **Jocsak G**., Barany Z., Bartha T., Frenyo VL., Zsarnovszky A.: **Metabolic asymmetry in the hypothalamic regulation of food-intake and reproductive processes in male and female rats,** P2-124, In: *16th Meeting of the Hungarian Neuroscience Society; International Brain Research Organization International Workshop,* Budapest, Hungary, 2016.

Barany Z., Kiss DS., Toth I., **Jocsak G**., Ashaber M., Sterczer A., Bartha T., Frenyo VL.,: **Effect of ammonia on alpha-ketoglutaramate production in primary astrocyte culture**, 1496-C108, In: *10th Meeting of Federation of European Neuroscience Societies,* Copenhagen, Denmark, 2016.

Toth I., Kiss DS., Ashaber M., **Jocsak G**., Barany Z., Barha T., Frenyo VL., Zsarnovszky A.: **Hypothalamic asymmetry in the regulation of food-intake and reproduction in male and female rats**, 2090-E007, In: *10th Meeting of Federation of European Neuroscience Societies,* Copenhagen, Denmark, 2016.

8.2.3. Oral presentations on Hungarian national conferences

Somogyi Virág, **Jócsák Gergely**, Tóth István, Kiss Dávid Sándor, Goszleth Gréta, Bartha Tibor, Frenyó V. László, Zsarnovszky Attila, Sterczer Ágnes: **A hepaticus encephalopathia hatása a fejlődő idegrendszerre: az ösztrogén- és pajzsmirigyhormon receptorok mRNS expressziójának vizsgálata kisagyi sejttenyészeten**

Kiss Dávid Sándor, Tóth István, **Jócsák Gergely**, Goszleth Gréta, Bartha Tibor, Frenyó V. László, Zsarnovszky Attila: **A jóllakottság és tesztoszteron hatása a hypothalamus metabolikus féloldaliságára hím patkányban**

Bárány Zoltán, **Jócsák Gergely**, Kiss Dávid Sándor, Sterczer Ágnes: **Az αlfa-ketoglutaramát, mint lehetséges biomarker hepaticus encephalopathia esetén**

8.3. Supervising of DVM theses

Haraszti Zsuzsanna: **A pajzsmirigy-és ösztrogén receptor proteinek expresszióját károsító anyag,a kámfor vizsgálata fejlődő primer kisagyi sejtkultúrán**, TDK dolgozat, 2016, Supervisors: **Jócsák G**., Toth I.

Bagó Bálint: **A zearaleon (ZEA) hatása az ER és TR kifejeződésére fejlődő primer kisagyi sejtkultúrában**, TDK dolgozat, scheduled time: 2017, Supervisors: Kiss DS., **Jócsák G**.

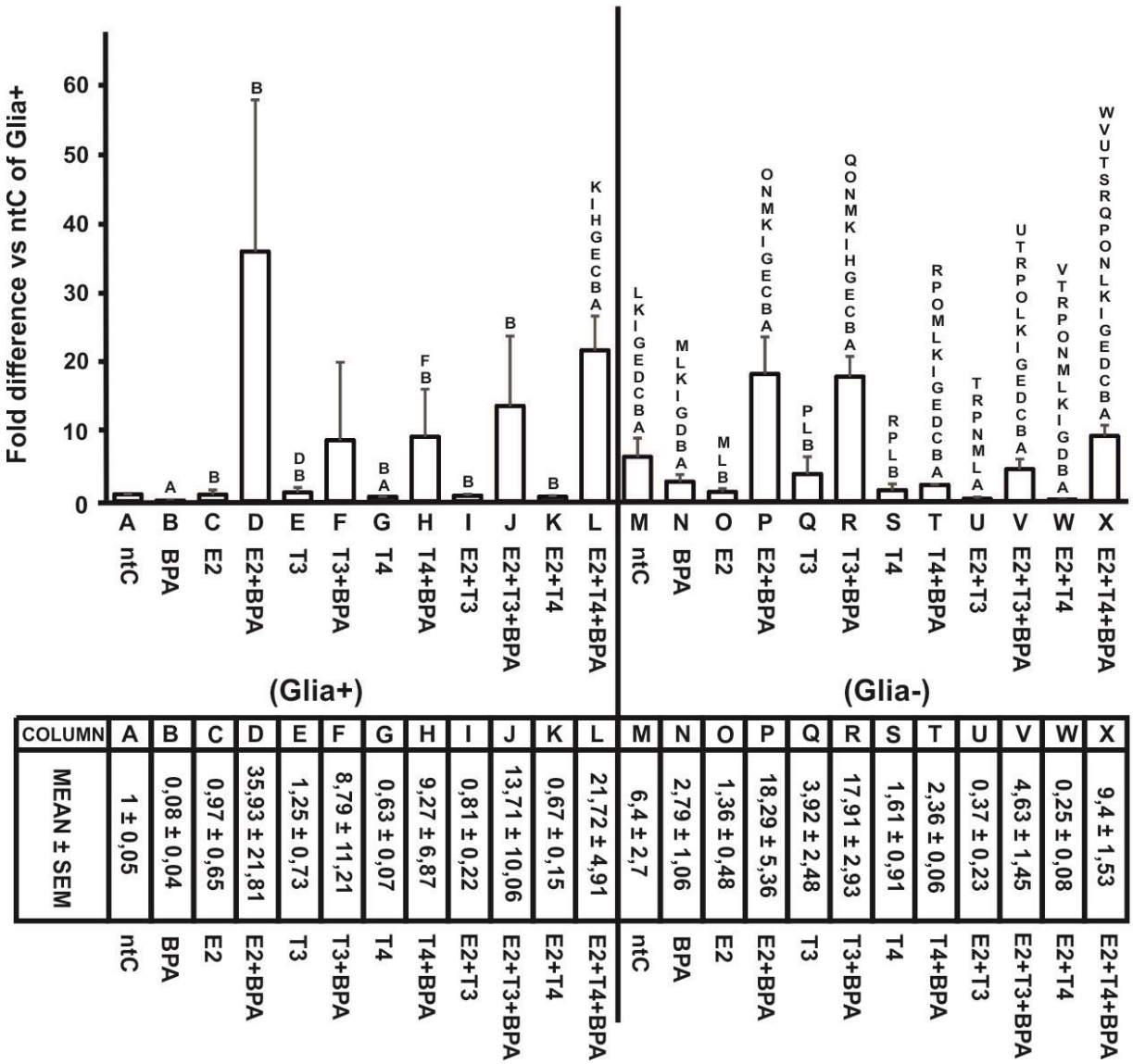
Fetter Viktória: **Asztrogliasejtek interleukin termelése oxidatív stress hatására**, TDK dolgozat, scheduled time: 2017, Supervisors: **Jócsák G**., Bárány Z.

9. APPENDIX

In the appendix figure captions are not included due to the size and complexity of the pictures. Generally it can be stated that the figures in the Appendix are consisted of data from every ED treatment combined with all of the hormone treatment, in the presence or absence of the glia. It can be generally stated that on the individual figures only the effect of **one specific disruptor** can be seen, affecting either the mRNA or the protein levels of one distinct receptor (TRα, TRβ or ERβ).

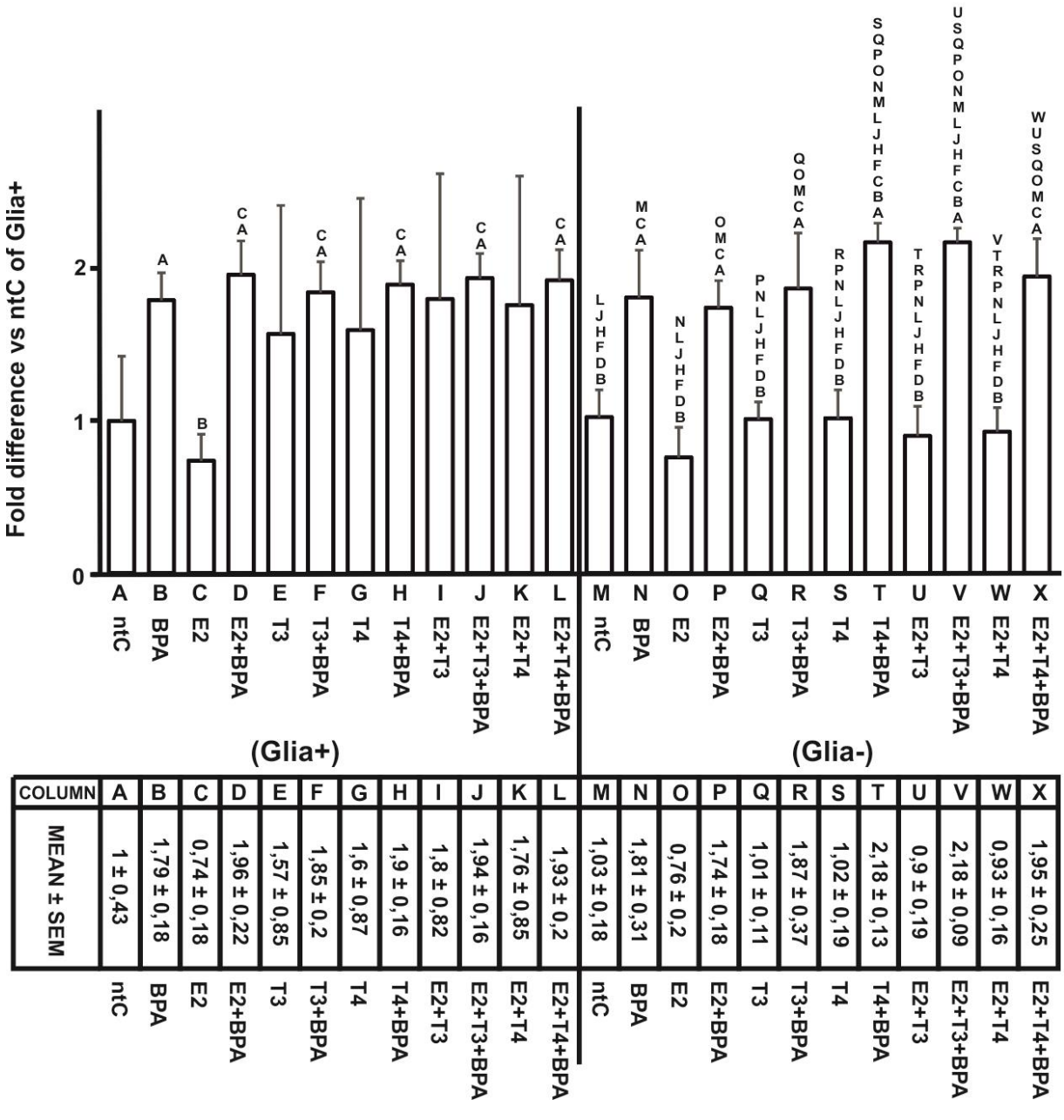
On the Y axis the fold difference vs. ntC (Glia+) is plotted. All of the results are compared to the Glia+ ntC value, which we set arbitrary to „1". The bars on the graph are representing the fold changes compared to this "1" value measured after different treatments indicated by different letters on the X axis. The specific treatments can be found below the graphs. ERβ). The title of the figure will indicate the type of examined receptor and disruptor. On the left side of the picture the results from glia containing (Glia+) groups, on the right side the results from glia-reduced (Glia-) groups are shown.

All data that have been presented are representative of at least three independent measurements. data under the bars show mean values +/- SEM. Statistical analyses were conducted using one-way Anova with Tukey's multiple comparison test (n=6). The level of statistical significance in differences between experimental groups is $p < 0.05$. Smaller size letters above the columns indicate significant differences from columns labeled with larger size letters.



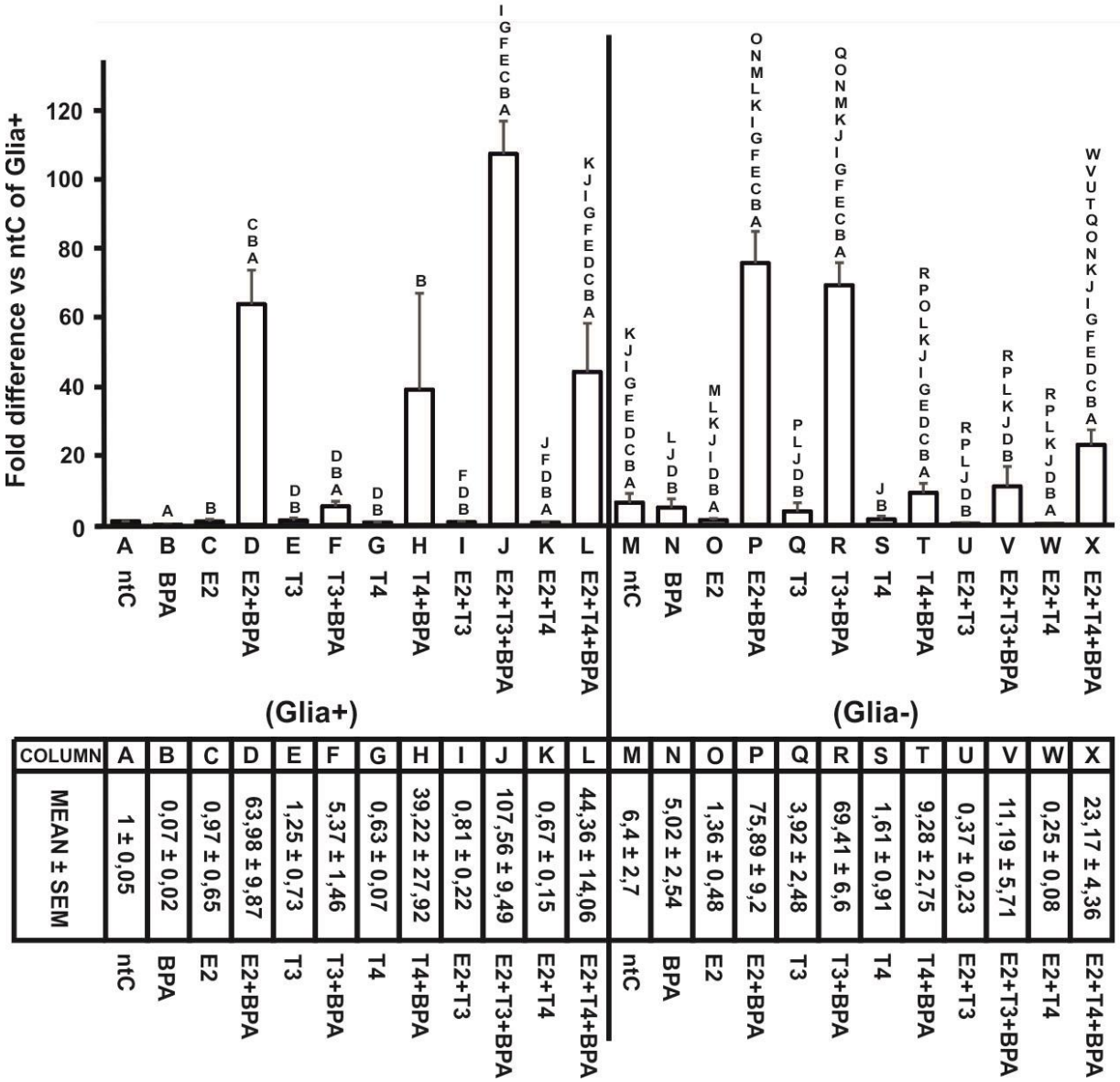
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	0,08 ± 0,04	0,97 ± 0,65	35,93 ± 21,81	1,25 ± 0,73	8,79 ± 11,21	0,63 ± 0,07	9,27 ± 6,87	0,81 ± 0,22	13,71 ± 10,06	0,67 ± 0,15	21,72 ± 4,91	6,4 ± 2,7	2,79 ± 1,06	1,36 ± 0,48	18,29 ± 5,36	3,92 ± 2,48	17,91 ± 2,93	1,61 ± 0,91	2,36 ± 0,06	0,37 ± 0,23	4,63 ± 1,45	0,25 ± 0,08	9,4 ± 1,53
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A1



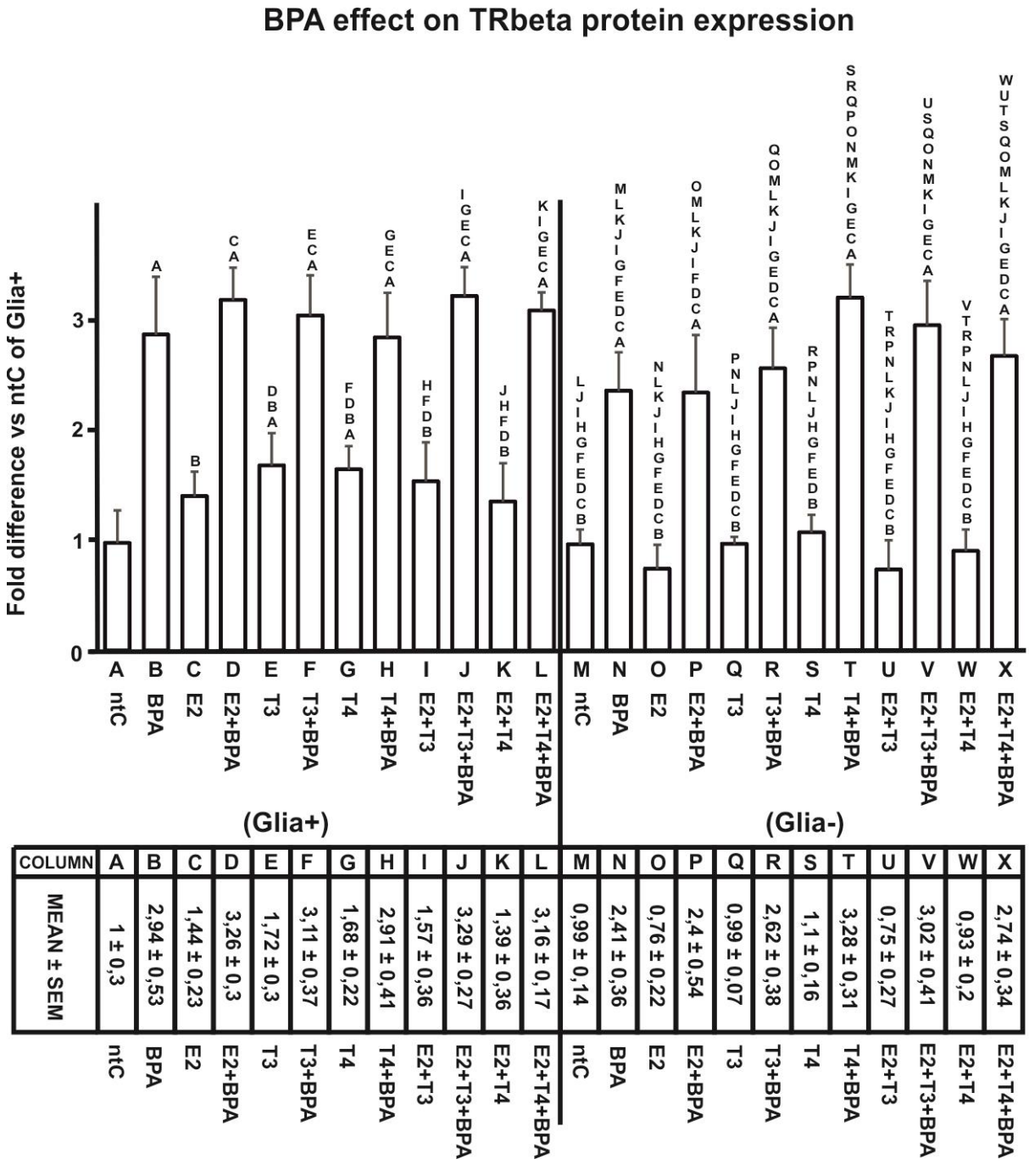
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,43	1,79 ± 0,18	0,74 ± 0,18	1,96 ± 0,22	1,57 ± 0,85	1,85 ± 0,2	1,6 ± 0,87	1,9 ± 0,16	1,8 ± 0,82	1,94 ± 0,16	1,76 ± 0,85	1,93 ± 0,2	1,03 ± 0,18	1,81 ± 0,31	0,76 ± 0,2	1,74 ± 0,18	1,01 ± 0,11	1,87 ± 0,37	1,02 ± 0,19	2,18 ± 0,13	0,9 ± 0,19	2,18 ± 0,09	0,93 ± 0,16	1,95 ± 0,25
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A2



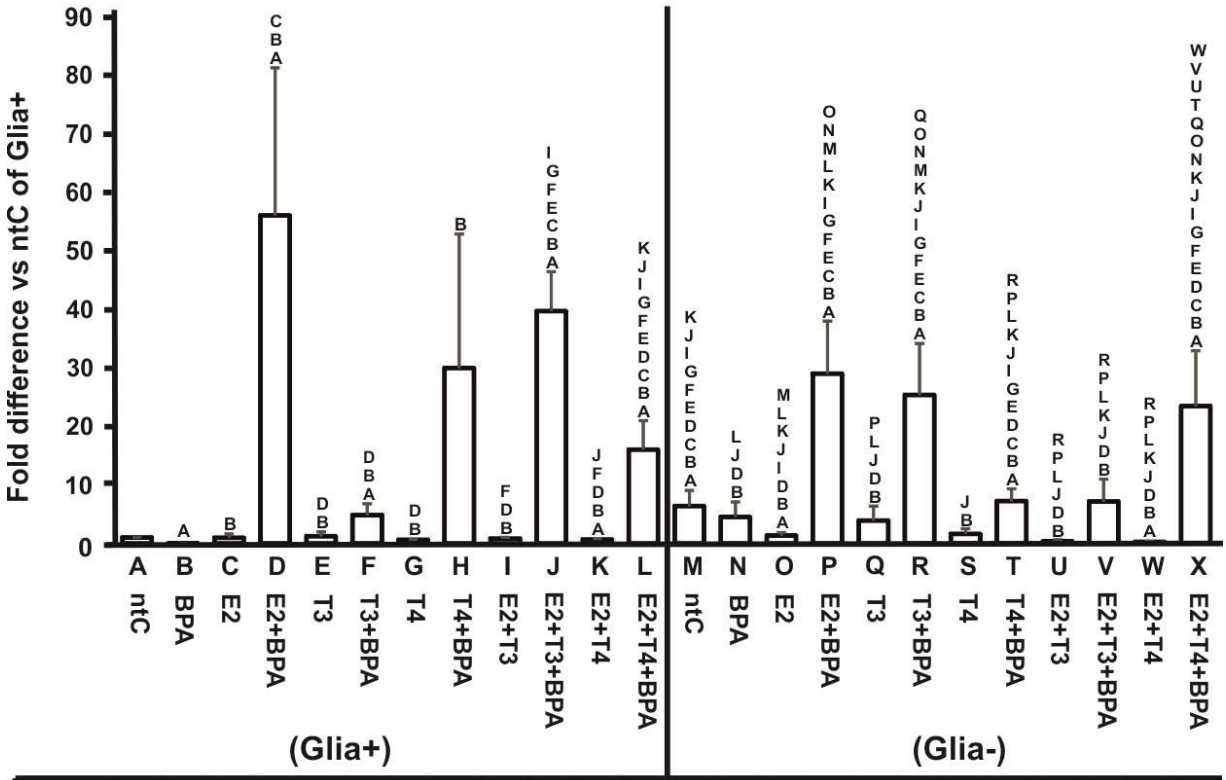
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	0,07 ± 0,02	0,97 ± 0,65	63,98 ± 9,87	1,25 ± 0,73	5,37 ± 1,46	0,63 ± 0,07	39,22 ± 27,92	0,81 ± 0,22	107,56 ± 9,49	0,67 ± 0,15	44,36 ± 14,06	6,4 ± 2,7	5,02 ± 2,54	1,36 ± 0,48	75,89 ± 9,2	3,92 ± 2,48	69,41 ± 6,6	1,61 ± 0,91	9,28 ± 2,75	0,37 ± 0,23	11,19 ± 5,71	0,25 ± 0,08	23,17 ± 4,36
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A3



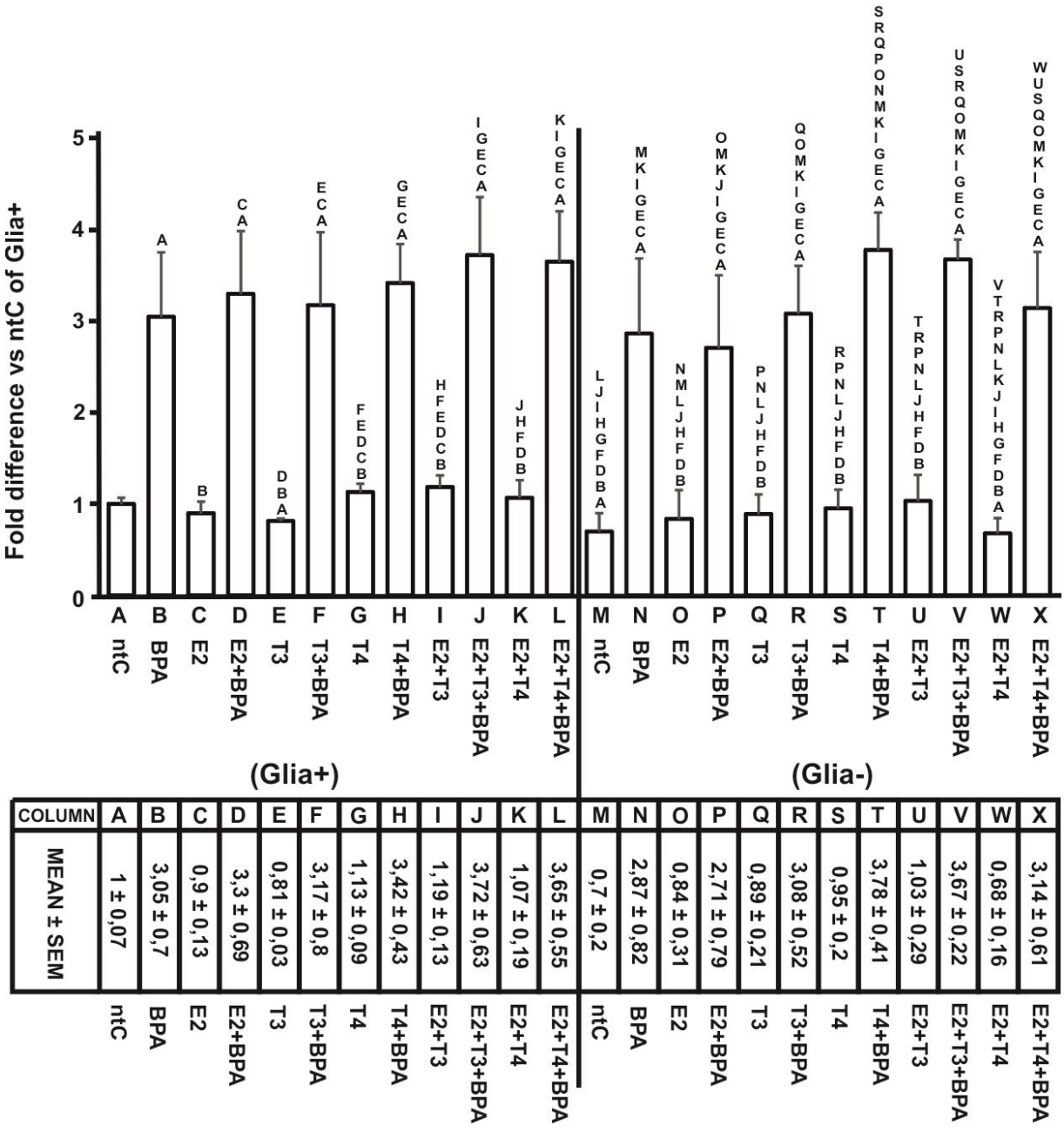
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,3	2,94 ± 0,53	1,44 ± 0,23	3,26 ± 0,3	1,72 ± 0,3	3,11 ± 0,37	1,68 ± 0,22	2,91 ± 0,41	1,57 ± 0,36	3,29 ± 0,27	1,39 ± 0,36	3,16 ± 0,17	0,99 ± 0,14	2,41 ± 0,36	0,76 ± 0,22	2,4 ± 0,54	0,99 ± 0,07	2,62 ± 0,38	1,1 ± 0,16	3,28 ± 0,31	0,75 ± 0,27	3,02 ± 0,41	0,93 ± 0,2	2,74 ± 0,34
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A4



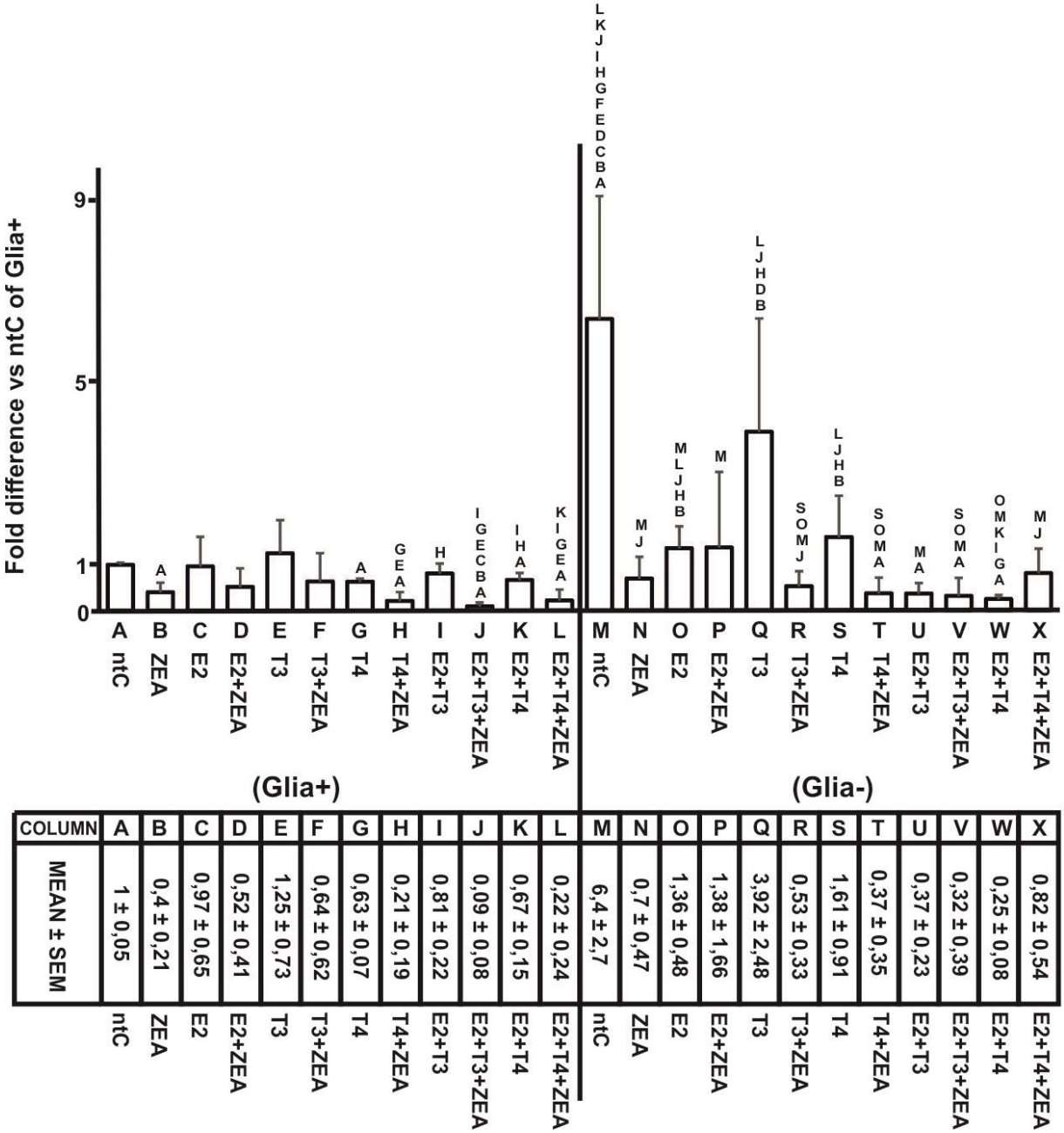
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	0,03 ± 0,01	0,97 ± 0,65	56,19 ± 25,31	1,25 ± 0,73	4,85 ± 1,95	0,63 ± 0,07	30,06 ± 22,95	0,81 ± 0,22	39,82 ± 6,75	0,67 ± 0,15	16,08 ± 4,96	6,4 ± 2,7	4,53 ± 2,56	1,36 ± 0,48	29,11 ± 8,98	3,92 ± 2,48	25,43 ± 8,85	1,61 ± 0,91	7,25 ± 2,1	0,37 ± 0,23	7,18 ± 3,87	0,25 ± 0,08	23,53 ± 9,48
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A5



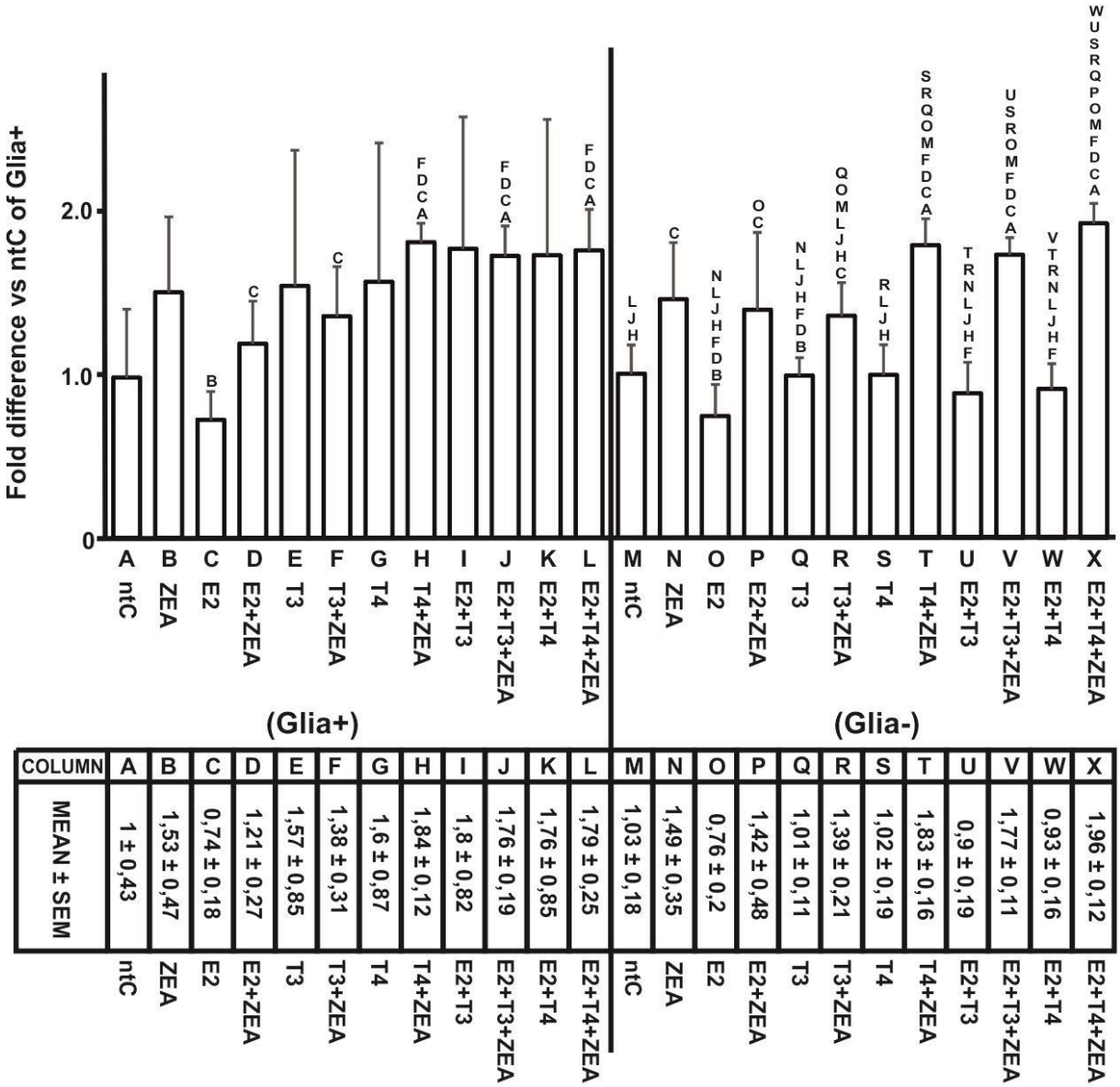
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,07	3,05 ± 0,7	0,9 ± 0,13	3,3 ± 0,69	0,81 ± 0,03	3,17 ± 0,8	1,13 ± 0,09	3,42 ± 0,43	1,19 ± 0,13	3,72 ± 0,63	1,07 ± 0,19	3,65 ± 0,55	0,7 ± 0,2	2,87 ± 0,82	0,84 ± 0,31	2,71 ± 0,79	0,89 ± 0,21	3,08 ± 0,52	0,95 ± 0,2	3,78 ± 0,41	1,03 ± 0,29	3,67 ± 0,22	0,68 ± 0,16	3,14 ± 0,61
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Figure A6



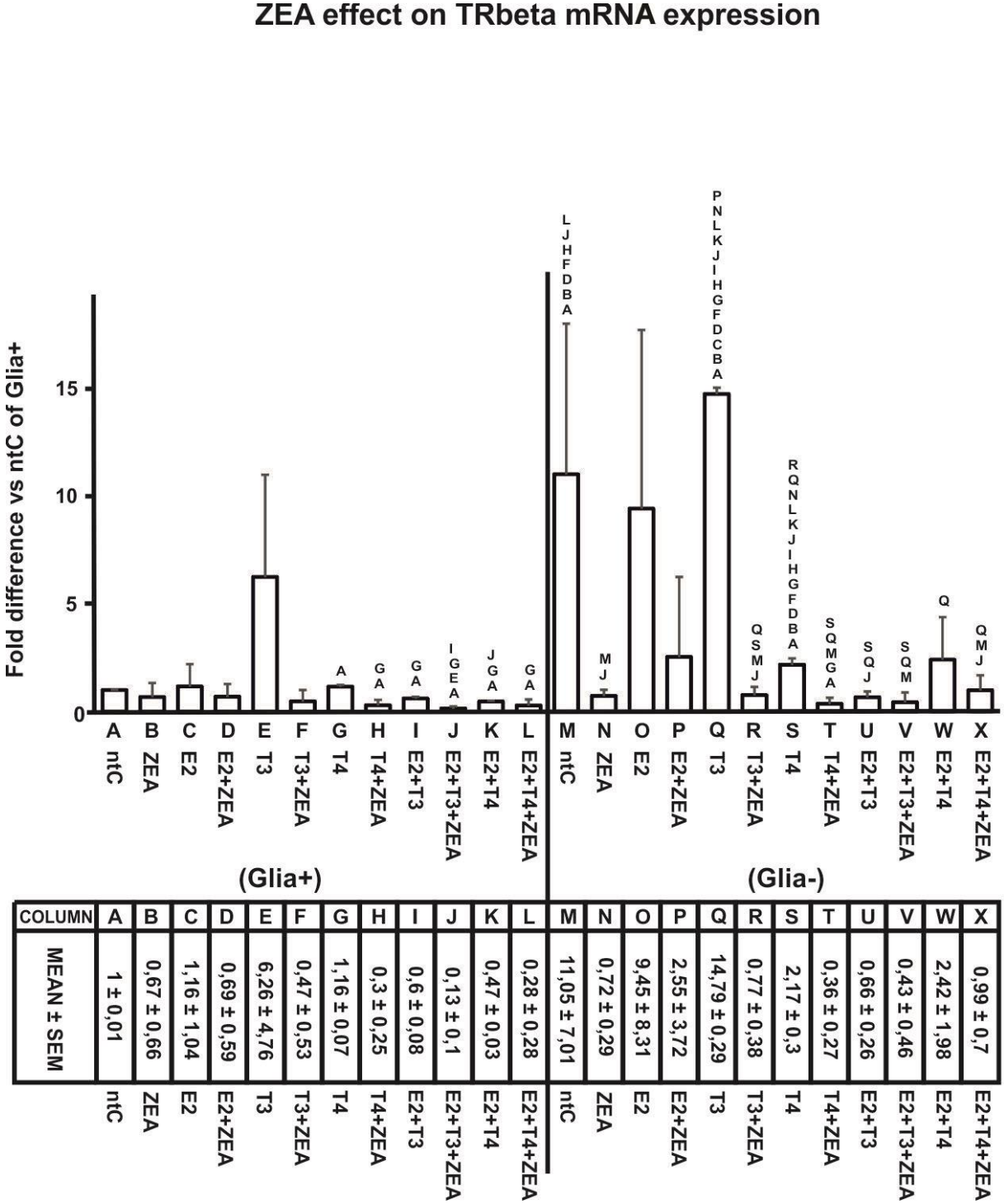
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	0,4 ± 0,21	0,97 ± 0,65	0,52 ± 0,41	1,25 ± 0,73	0,64 ± 0,62	0,63 ± 0,07	0,21 ± 0,19	0,81 ± 0,22	0,09 ± 0,08	0,67 ± 0,15	0,22 ± 0,24	6,4 ± 2,7	0,7 ± 0,47	1,36 ± 0,48	1,38 ± 1,66	3,92 ± 2,48	0,53 ± 0,33	1,61 ± 0,91	0,37 ± 0,35	0,37 ± 0,23	0,32 ± 0,39	0,25 ± 0,08	0,82 ± 0,54
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A7



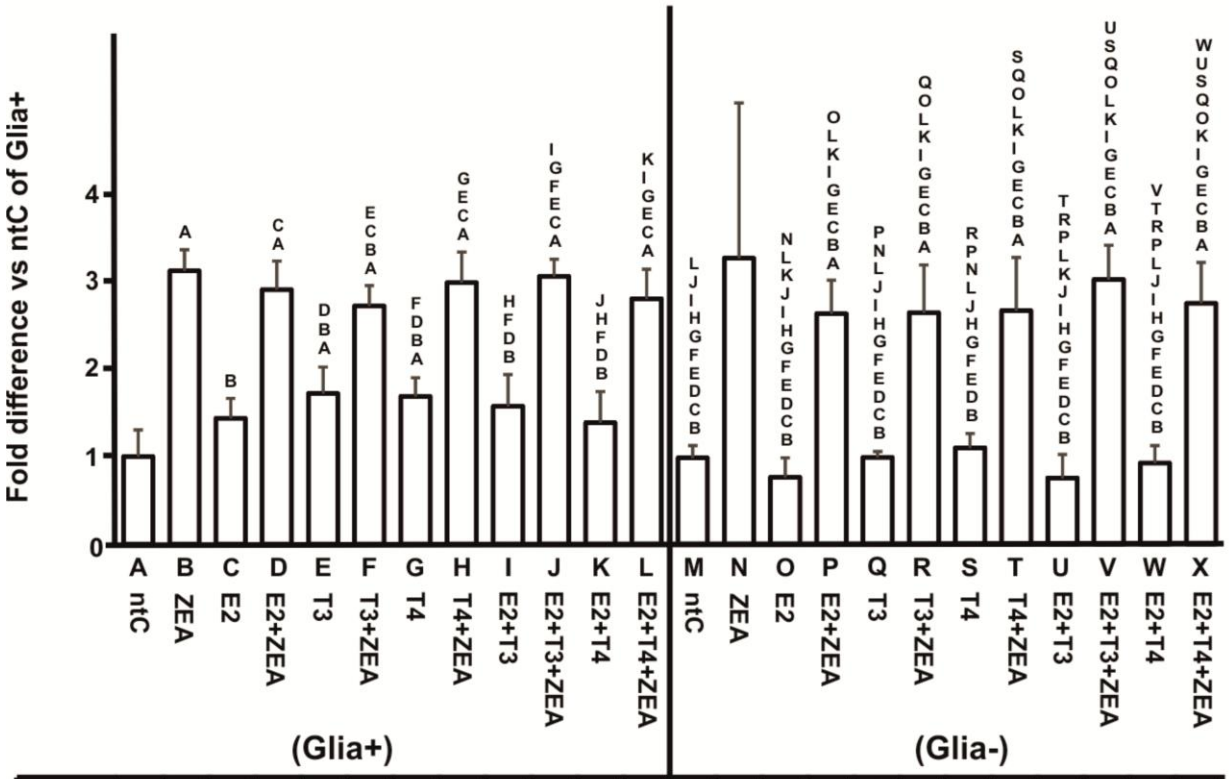
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,43	1,53 ± 0,47	0,74 ± 0,18	1,21 ± 0,27	1,57 ± 0,85	1,38 ± 0,31	1,6 ± 0,87	1,84 ± 0,12	1,8 ± 0,82	1,76 ± 0,19	1,76 ± 0,85	1,79 ± 0,25	1,03 ± 0,18	1,49 ± 0,35	0,76 ± 0,2	1,42 ± 0,48	1,01 ± 0,11	1,39 ± 0,21	1,02 ± 0,19	1,83 ± 0,16	0,9 ± 0,19	1,77 ± 0,11	0,93 ± 0,16	1,96 ± 0,12
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A8



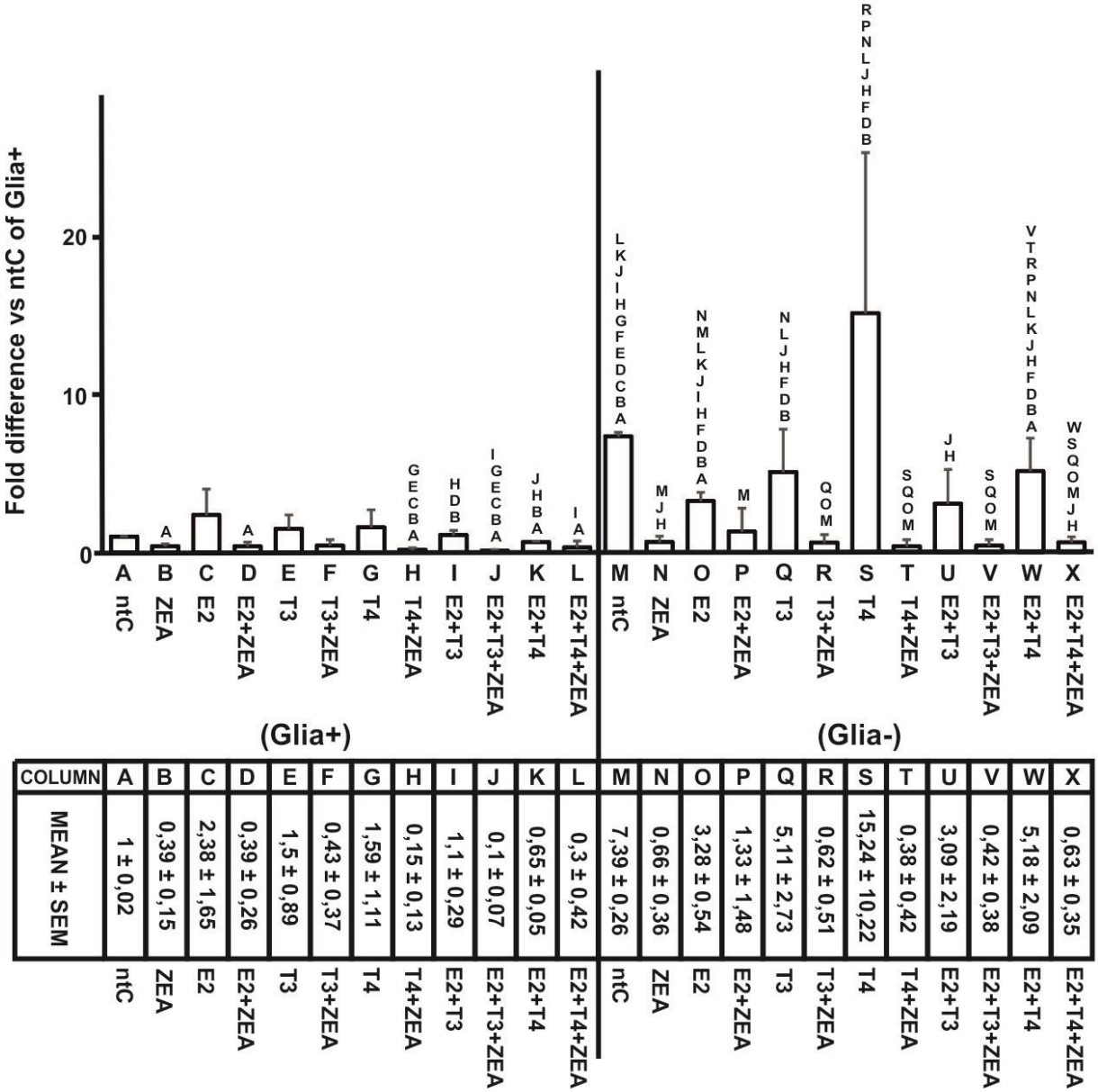
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,01	0,67 ± 0,66	1,16 ± 1,04	0,69 ± 0,59	6,26 ± 4,76	0,47 ± 0,53	1,16 ± 0,07	0,3 ± 0,25	0,6 ± 0,08	0,13 ± 0,1	0,47 ± 0,03	0,28 ± 0,28	11,05 ± 7,01	0,72 ± 0,29	9,45 ± 8,31	2,55 ± 3,72	14,79 ± 0,29	0,77 ± 0,38	2,17 ± 0,3	0,36 ± 0,27	0,66 ± 0,26	0,43 ± 0,46	2,42 ± 1,98	0,99 ± 0,7
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A9



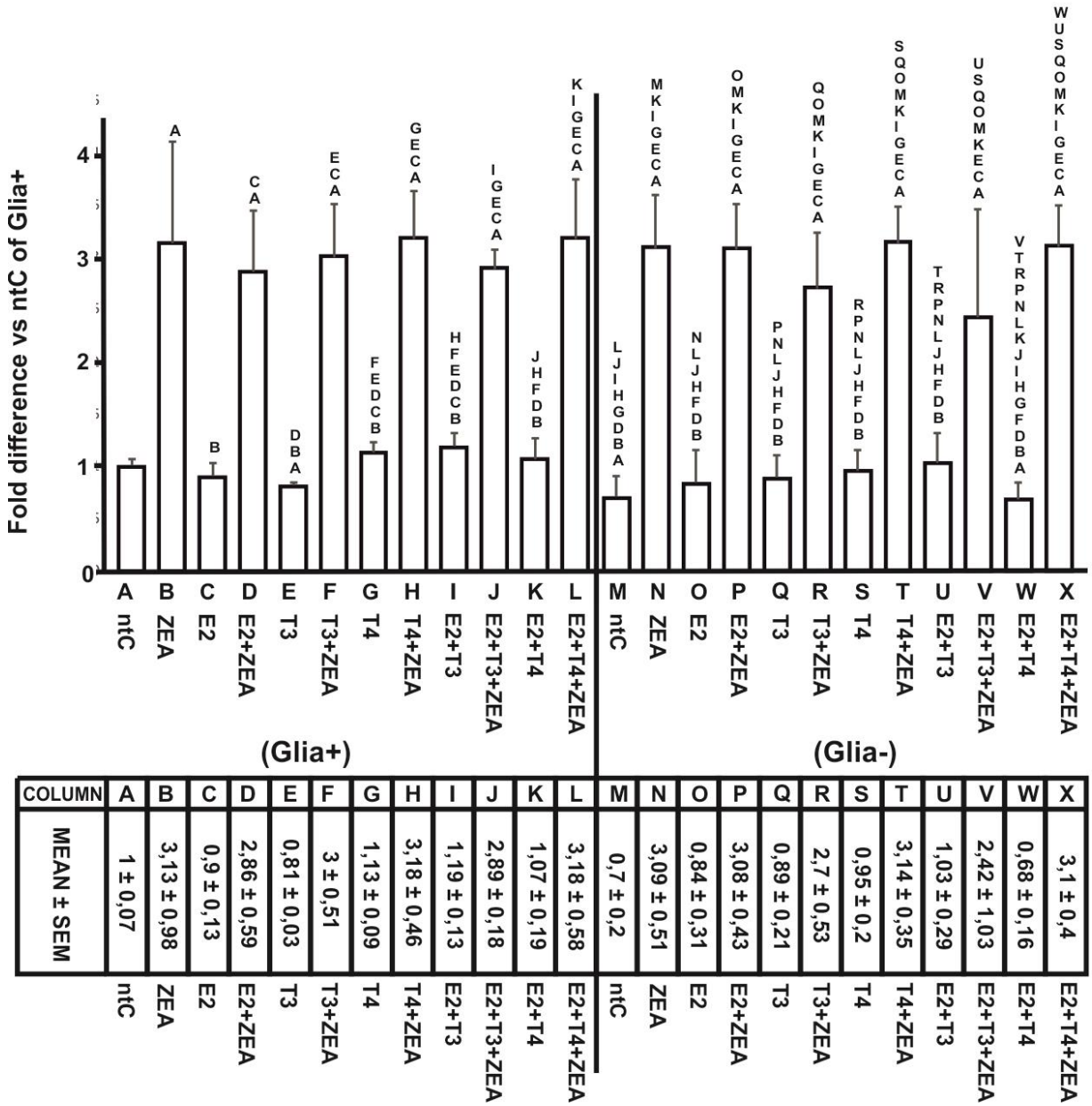
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,3	3,12 ± 0,24	1,44 ± 0,23	2,9 ± 0,33	1,72 ± 0,3	2,72 ± 0,23	1,68 ± 0,22	2,98 ± 0,35	1,57 ± 0,36	3,05 ± 0,2	1,39 ± 0,36	2,8 ± 0,34	0,99 ± 0,14	3,26 ± 1,77	0,76 ± 0,22	2,63 ± 0,38	0,99 ± 0,07	2,64 ± 0,55	1,1 ± 0,16	2,66 ± 0,61	0,75 ± 0,27	3,02 ± 0,39	0,93 ± 0,2	2,75 ± 0,46
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A10



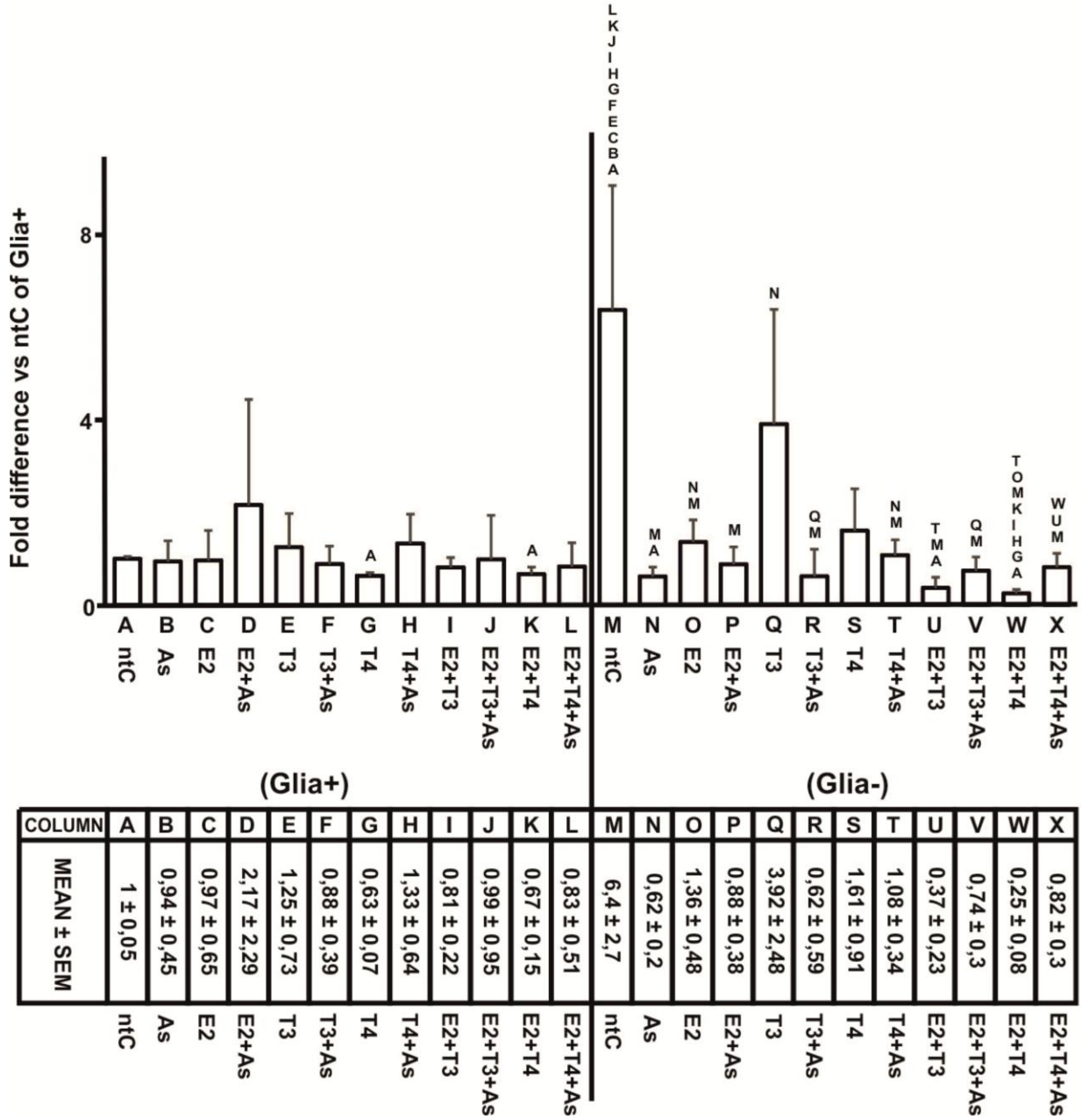
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,02	0,39 ± 0,15	2,38 ± 1,65	0,39 ± 0,26	1,5 ± 0,89	0,43 ± 0,37	1,59 ± 1,11	0,15 ± 0,13	1,1 ± 0,29	0,1 ± 0,07	0,65 ± 0,05	0,3 ± 0,42	7,39 ± 0,26	0,66 ± 0,36	3,28 ± 0,54	1,33 ± 1,48	5,11 ± 2,73	0,62 ± 0,51	15,24 ± 10,22	0,38 ± 0,42	3,09 ± 2,19	0,42 ± 0,38	5,18 ± 2,09	0,63 ± 0,35
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A11



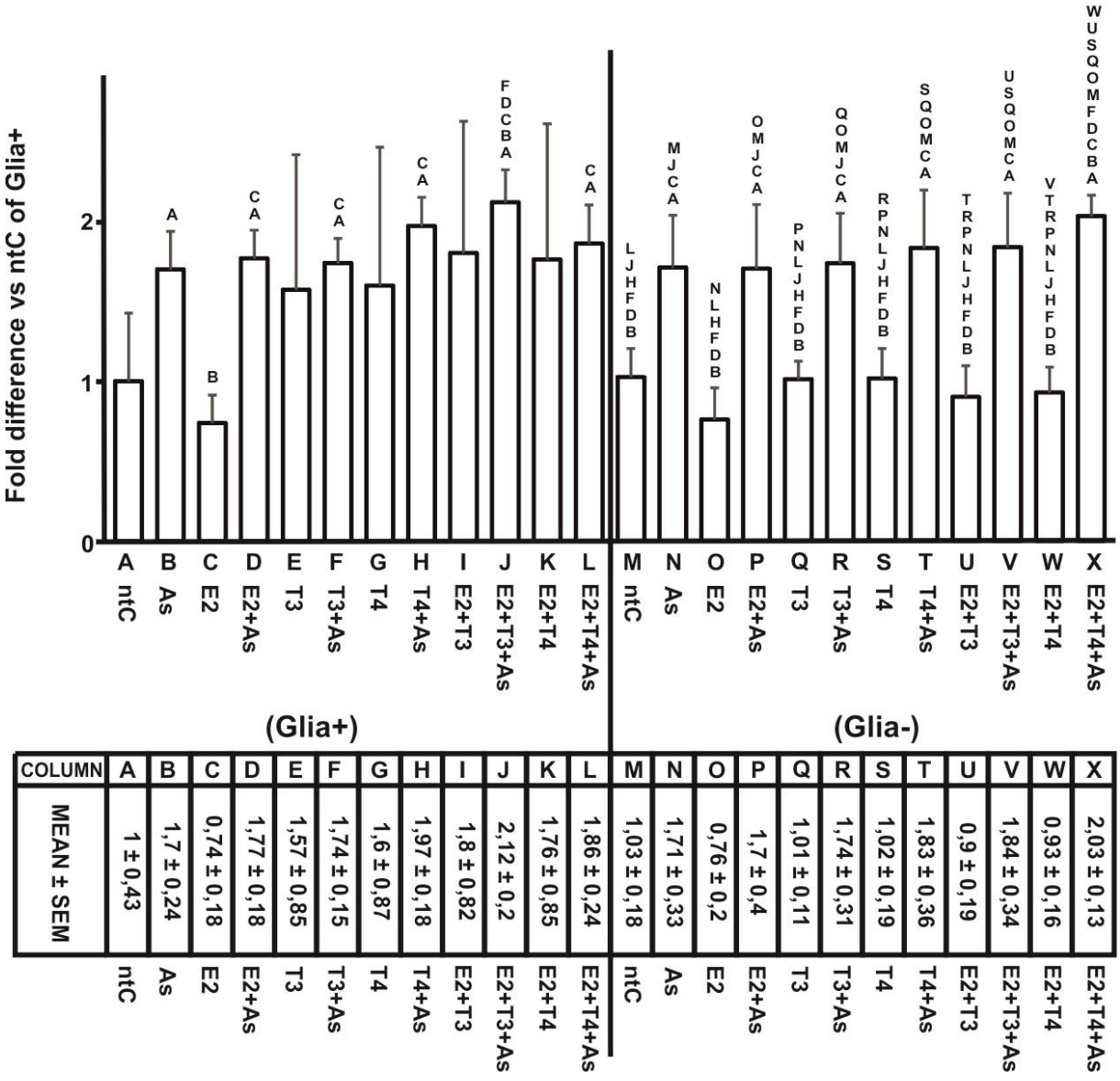
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,07	3,13 ± 0,98	0,9 ± 0,13	2,86 ± 0,59	0,81 ± 0,03	3 ± 0,51	1,13 ± 0,09	3,18 ± 0,46	1,19 ± 0,13	2,89 ± 0,18	1,07 ± 0,19	3,18 ± 0,58	0,7 ± 0,2	3,09 ± 0,51	0,84 ± 0,31	3,08 ± 0,43	0,89 ± 0,21	2,7 ± 0,53	0,95 ± 0,2	3,14 ± 0,35	1,03 ± 0,29	2,42 ± 1,03	0,68 ± 0,16	3,1 ± 0,4
	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA	ntC	ZEA	E2	E2+ZEA	T3	T3+ZEA	T4	T4+ZEA	E2+T3	E2+T3+ZEA	E2+T4	E2+T4+ZEA

Figure A12



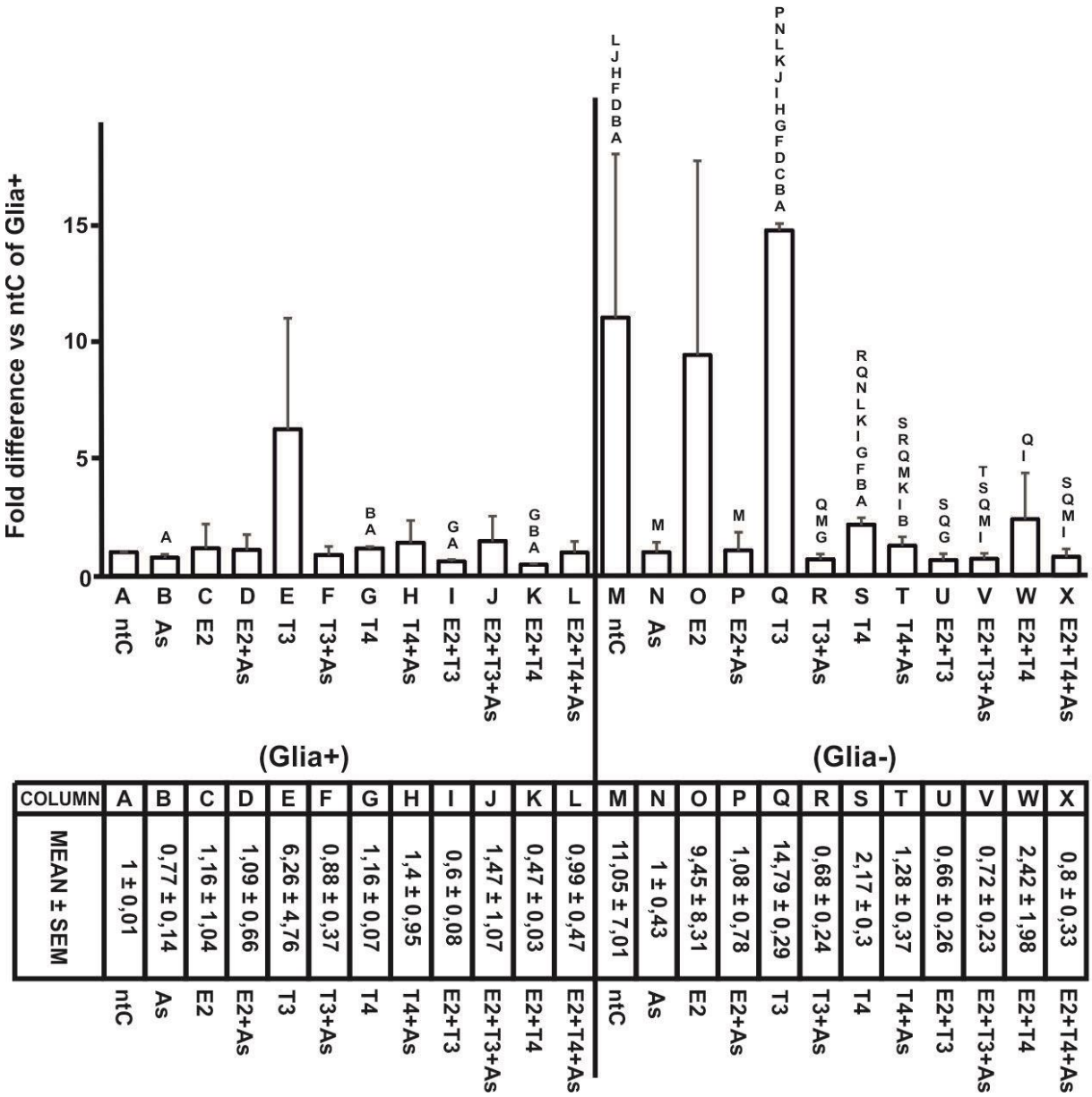
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	0,94 ± 0,45	0,97 ± 0,65	2,17 ± 2,29	1,25 ± 0,73	0,88 ± 0,39	0,63 ± 0,07	1,33 ± 0,64	0,81 ± 0,22	0,99 ± 0,95	0,67 ± 0,15	0,83 ± 0,51	6,4 ± 2,7	0,62 ± 0,2	1,36 ± 0,48	0,88 ± 0,38	3,92 ± 2,48	0,62 ± 0,59	1,61 ± 0,91	1,08 ± 0,34	0,37 ± 0,23	0,74 ± 0,3	0,25 ± 0,08	0,82 ± 0,3
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A13



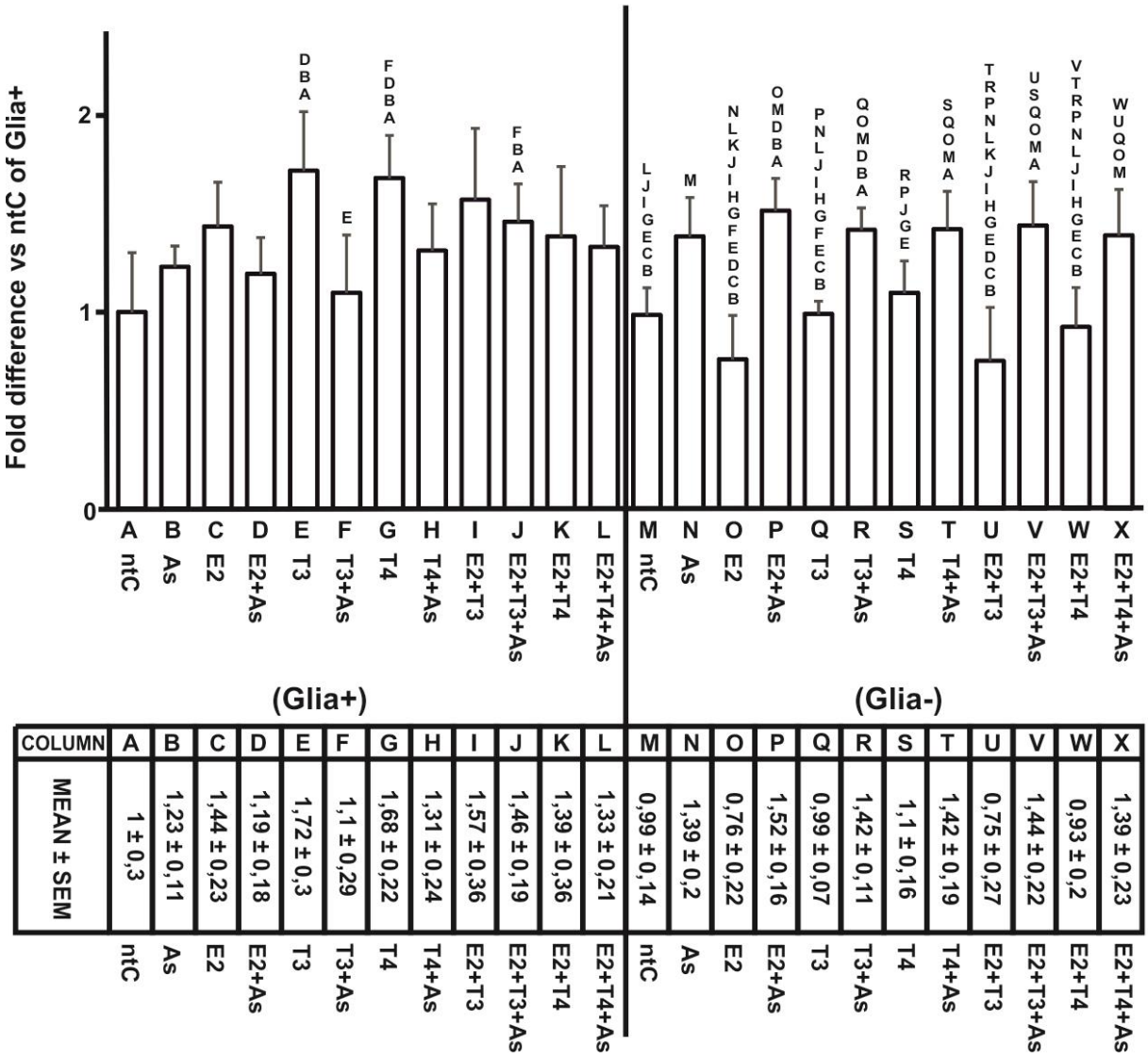
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,43	1,7 ± 0,24	0,74 ± 0,18	1,77 ± 0,18	1,57 ± 0,85	1,74 ± 0,15	1,6 ± 0,87	1,97 ± 0,18	1,8 ± 0,82	2,12 ± 0,2	1,76 ± 0,85	1,86 ± 0,24	1,03 ± 0,18	1,71 ± 0,33	0,76 ± 0,2	1,7 ± 0,4	1,01 ± 0,11	1,74 ± 0,31	1,02 ± 0,19	1,83 ± 0,36	0,9 ± 0,19	1,84 ± 0,34	0,93 ± 0,16	2,03 ± 0,13
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A14



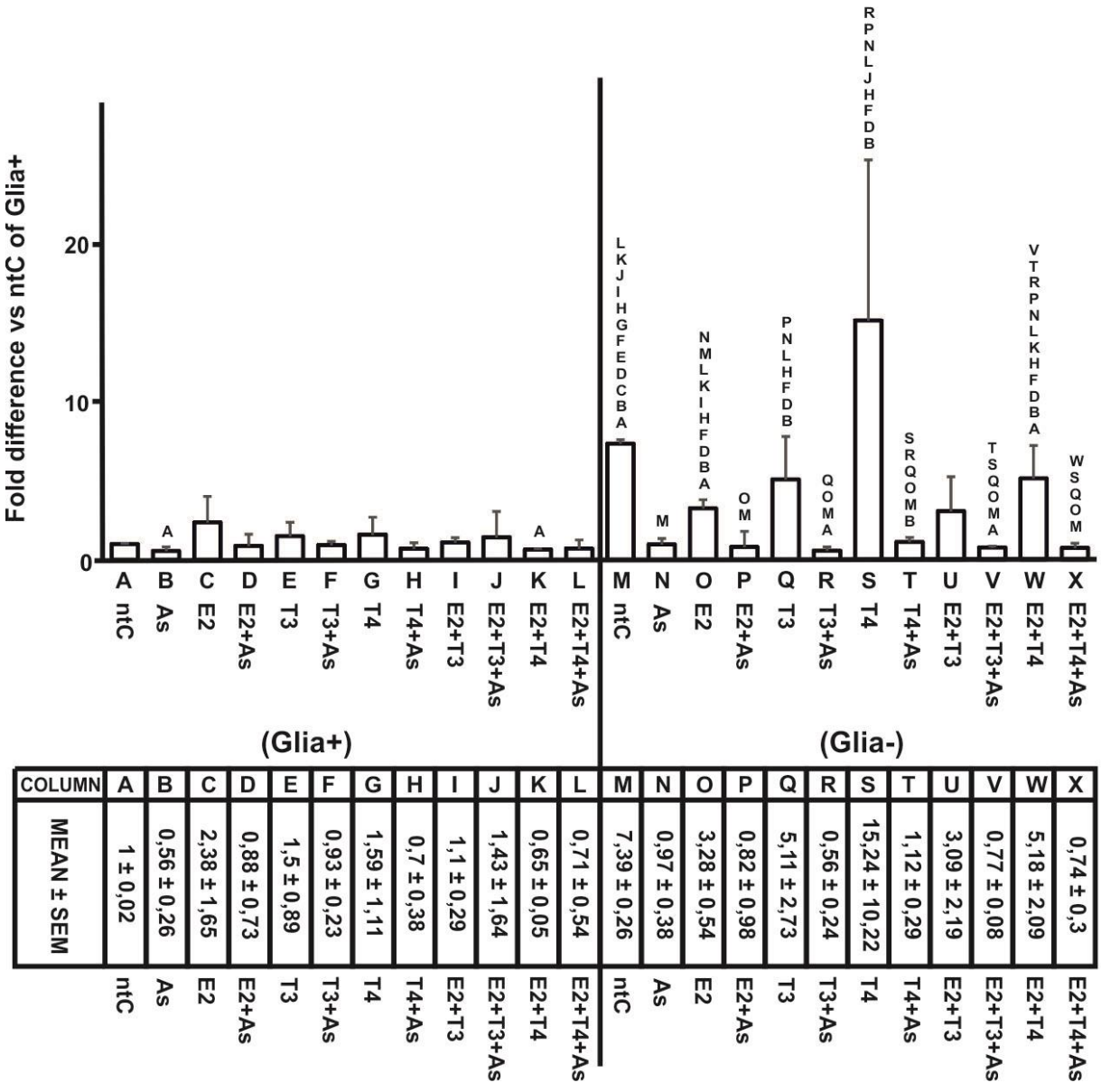
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,01	0,77 ± 0,14	1,16 ± 1,04	1,09 ± 0,66	6,26 ± 4,76	0,88 ± 0,37	1,16 ± 0,07	1,4 ± 0,95	0,6 ± 0,08	1,47 ± 1,07	0,47 ± 0,03	0,99 ± 0,47	11,05 ± 7,01	1 ± 0,43	9,45 ± 8,31	1,08 ± 0,78	14,79 ± 0,29	0,68 ± 0,24	2,17 ± 0,3	1,28 ± 0,37	0,66 ± 0,26	0,72 ± 0,23	2,42 ± 1,98	0,8 ± 0,33
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A15



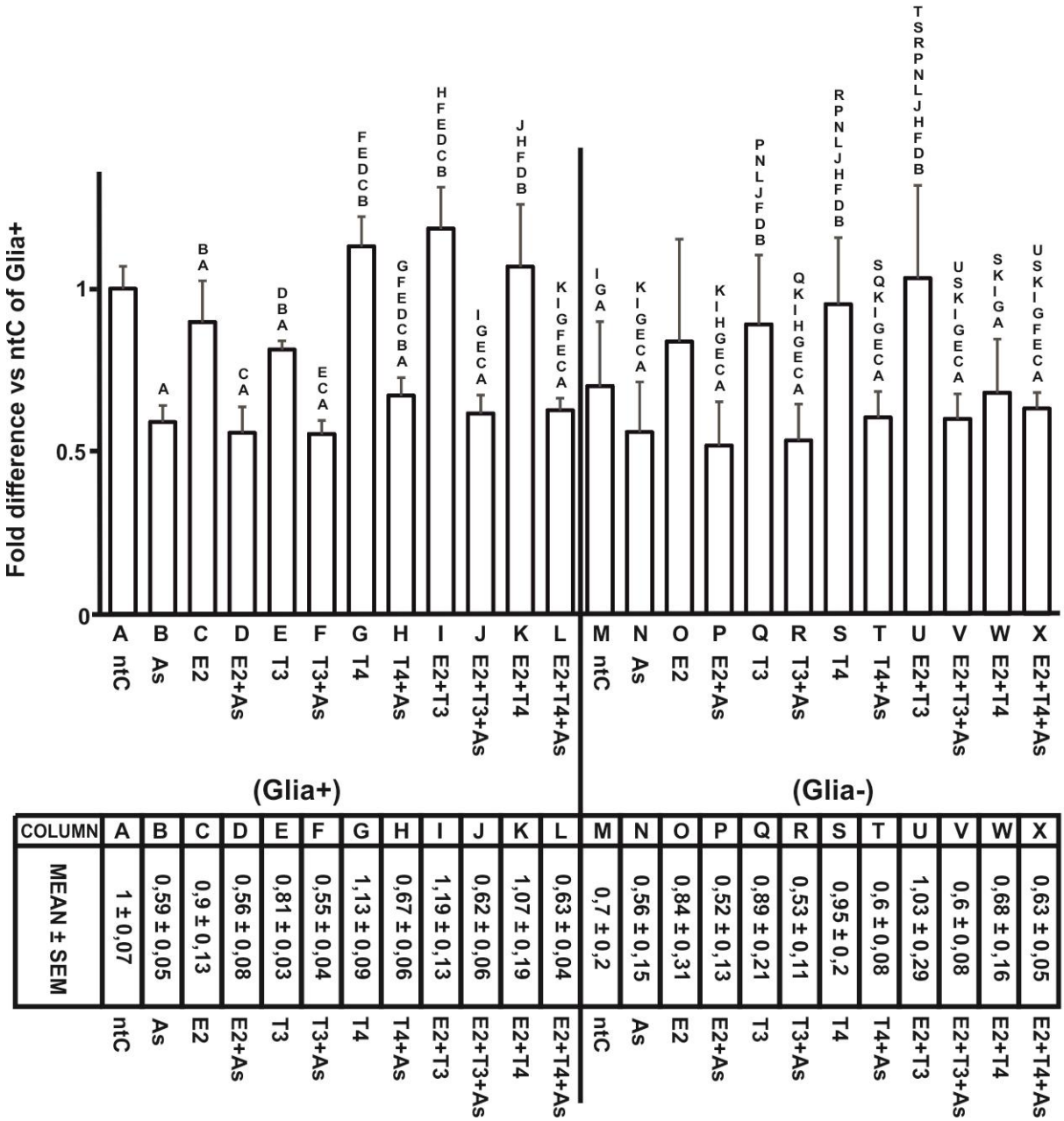
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,3	1,23 ± 0,11	1,44 ± 0,23	1,19 ± 0,18	1,72 ± 0,3	1,1 ± 0,29	1,68 ± 0,22	1,31 ± 0,24	1,57 ± 0,36	1,46 ± 0,19	1,39 ± 0,36	1,33 ± 0,21	0,99 ± 0,14	1,39 ± 0,2	0,76 ± 0,22	1,52 ± 0,16	0,99 ± 0,07	1,42 ± 0,11	1,1 ± 0,16	1,42 ± 0,19	0,75 ± 0,27	1,44 ± 0,22	0,93 ± 0,2	1,39 ± 0,23
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A16



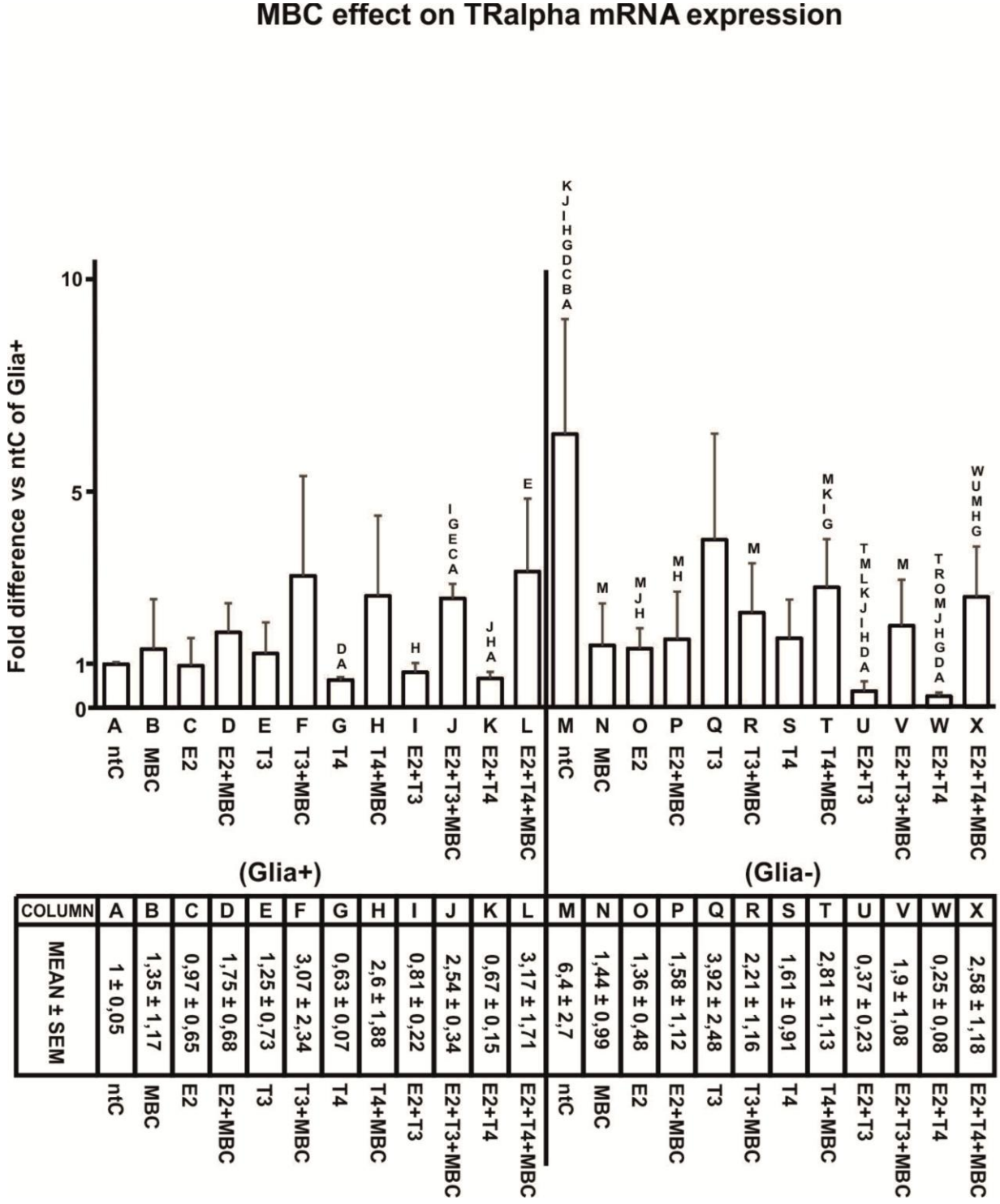
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,02	0,56 ± 0,26	2,38 ± 1,65	0,88 ± 0,73	1,5 ± 0,89	0,93 ± 0,23	1,59 ± 1,11	0,7 ± 0,38	1,1 ± 0,29	1,43 ± 1,64	0,65 ± 0,05	0,71 ± 0,54	7,39 ± 0,26	0,97 ± 0,38	3,28 ± 0,54	0,82 ± 0,98	5,11 ± 2,73	0,56 ± 0,24	15,24 ± 10,22	1,12 ± 0,29	3,09 ± 2,19	0,77 ± 0,08	5,18 ± 2,09	0,74 ± 0,3
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A17



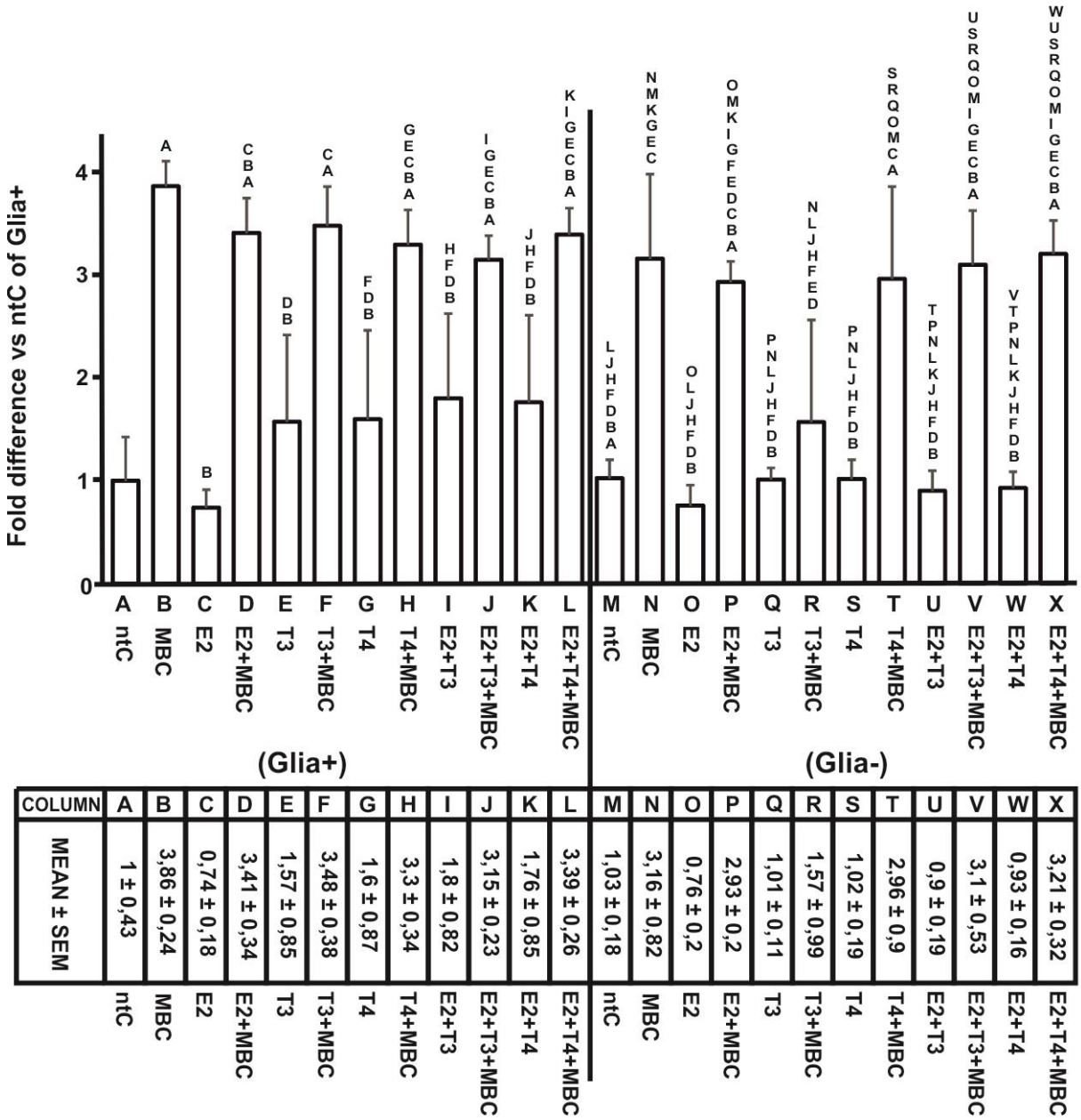
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,07	0,59 ± 0,05	0,9 ± 0,13	0,56 ± 0,08	0,81 ± 0,03	0,55 ± 0,04	1,13 ± 0,09	0,67 ± 0,06	1,19 ± 0,13	0,62 ± 0,06	1,07 ± 0,19	0,63 ± 0,04	0,7 ± 0,2	0,56 ± 0,15	0,84 ± 0,31	0,52 ± 0,13	0,89 ± 0,21	0,53 ± 0,11	0,95 ± 0,2	0,6 ± 0,08	1,03 ± 0,29	0,6 ± 0,08	0,68 ± 0,16	0,63 ± 0,05
	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As	ntC	As	E2	E2+As	T3	T3+As	T4	T4+As	E2+T3	E2+T3+As	E2+T4	E2+T4+As

Figure A18



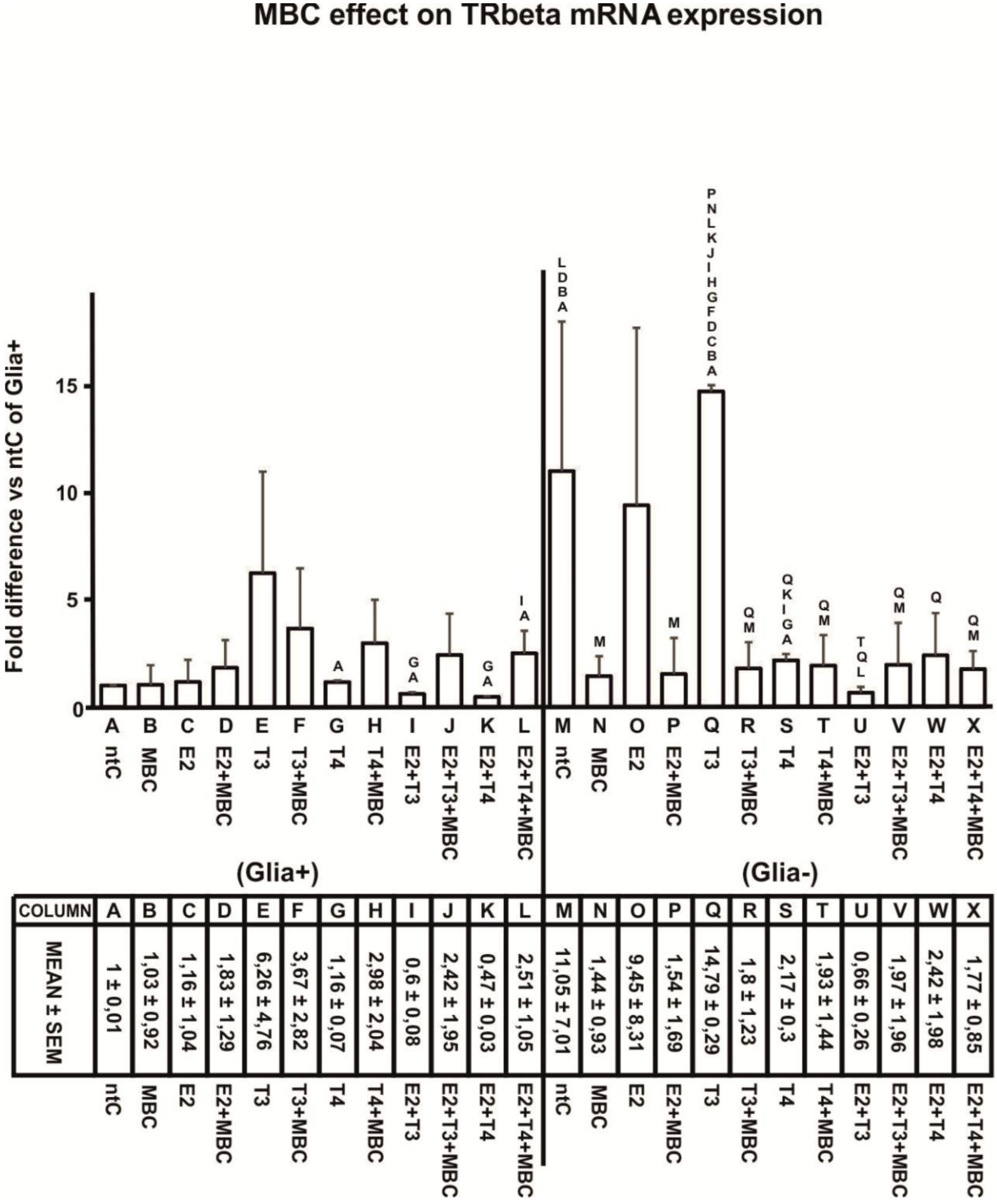
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	1,35 ± 1,17	0,97 ± 0,65	1,75 ± 0,68	1,25 ± 0,73	3,07 ± 2,34	0,63 ± 0,07	2,6 ± 1,88	0,81 ± 0,22	2,54 ± 0,34	0,67 ± 0,15	3,17 ± 1,71	6,4 ± 2,7	1,44 ± 0,99	1,36 ± 0,48	1,58 ± 1,12	3,92 ± 2,48	2,21 ± 1,16	1,61 ± 0,91	2,81 ± 1,13	0,37 ± 0,23	1,9 ± 1,08	0,25 ± 0,08	2,58 ± 1,18
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A19



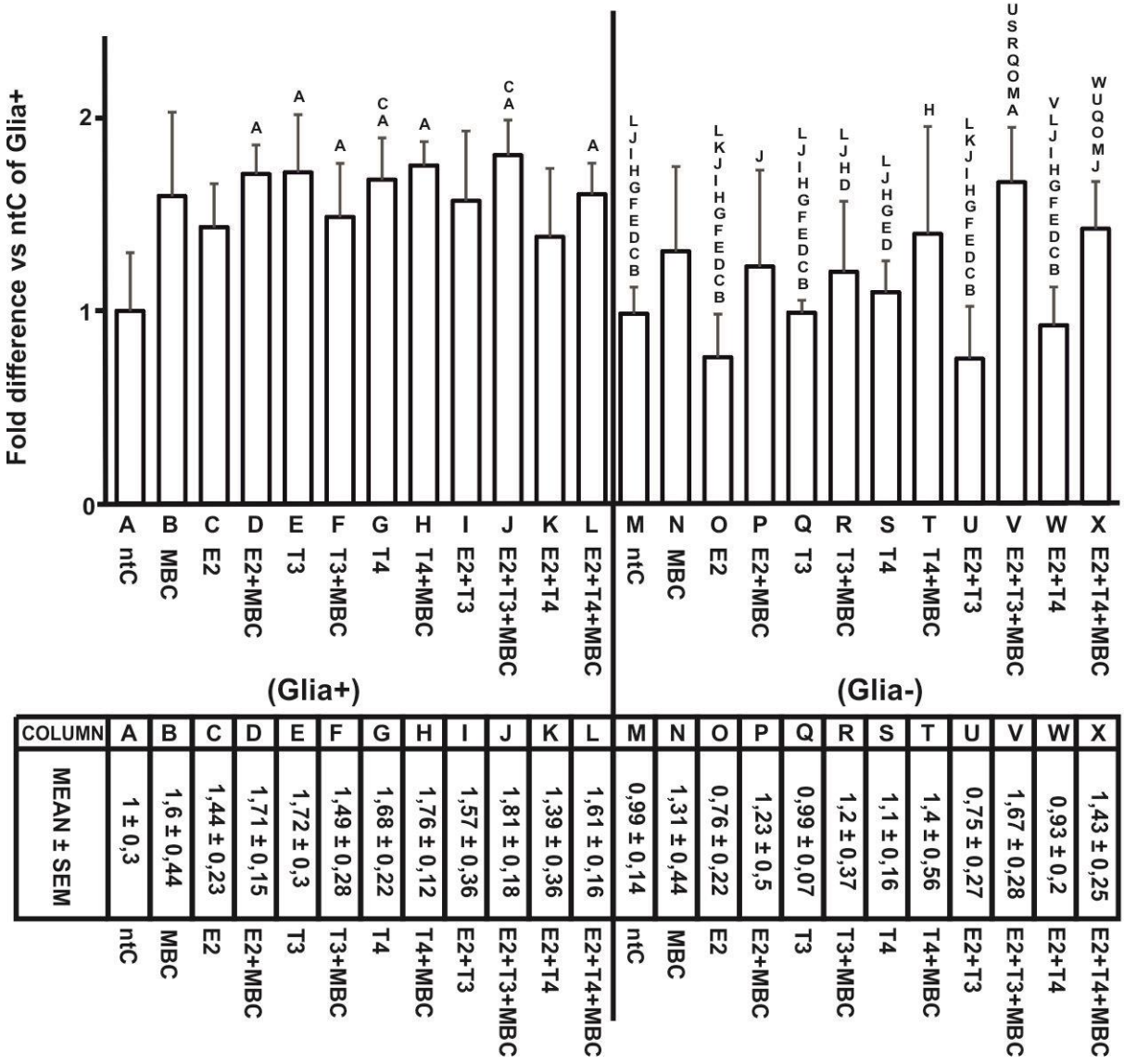
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,43	3,86 ± 0,24	0,74 ± 0,18	3,41 ± 0,34	1,57 ± 0,85	3,48 ± 0,38	1,6 ± 0,87	3,3 ± 0,34	1,8 ± 0,82	3,15 ± 0,23	1,76 ± 0,85	3,39 ± 0,26	1,03 ± 0,18	3,16 ± 0,82	0,76 ± 0,2	2,93 ± 0,2	1,01 ± 0,11	1,57 ± 0,99	1,02 ± 0,19	2,96 ± 0,9	0,9 ± 0,19	3,1 ± 0,53	0,93 ± 0,16	3,21 ± 0,32
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A20



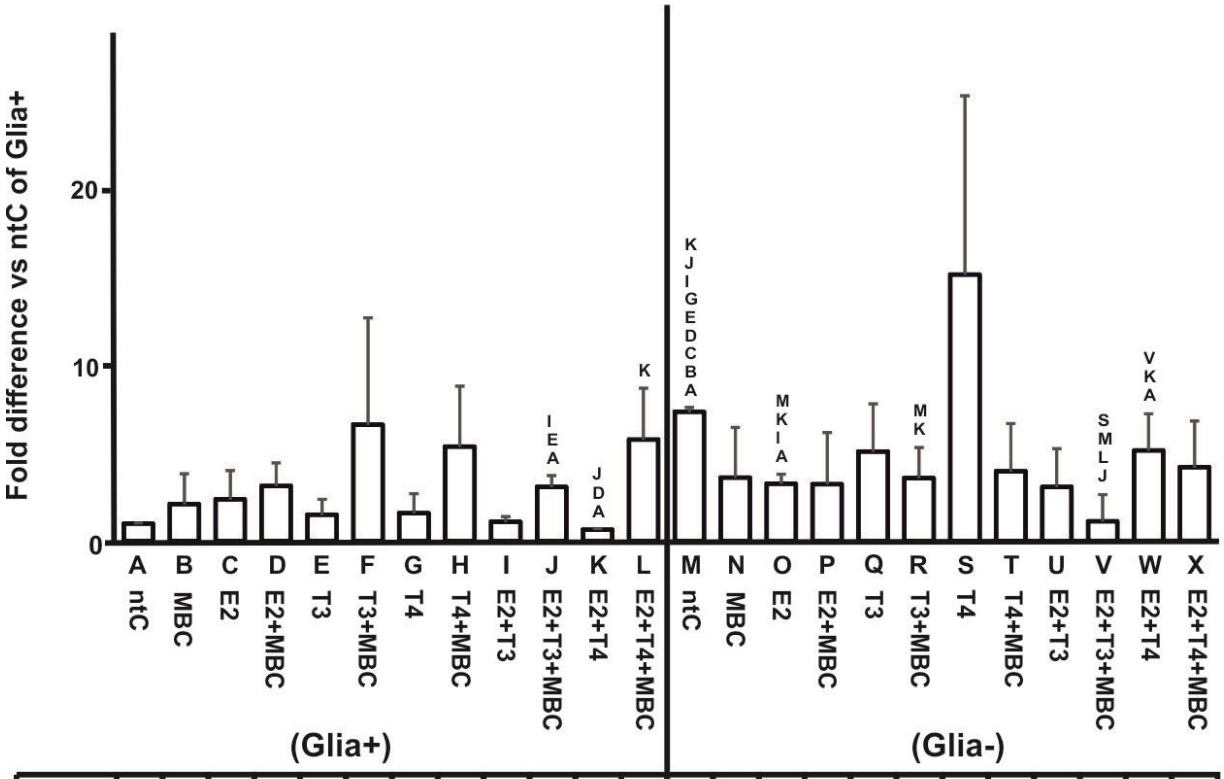
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,01	1,03 ± 0,92	1,16 ± 1,04	1,83 ± 1,29	6,26 ± 4,76	3,67 ± 2,82	1,16 ± 0,07	2,98 ± 2,04	0,6 ± 0,08	2,42 ± 1,95	0,47 ± 0,03	2,51 ± 1,05	11,05 ± 7,01	1,44 ± 0,93	9,45 ± 8,31	1,54 ± 1,69	14,79 ± 0,29	1,8 ± 1,23	2,17 ± 0,3	1,93 ± 1,44	0,66 ± 0,26	1,97 ± 1,96	2,42 ± 1,98	1,77 ± 0,85
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A21



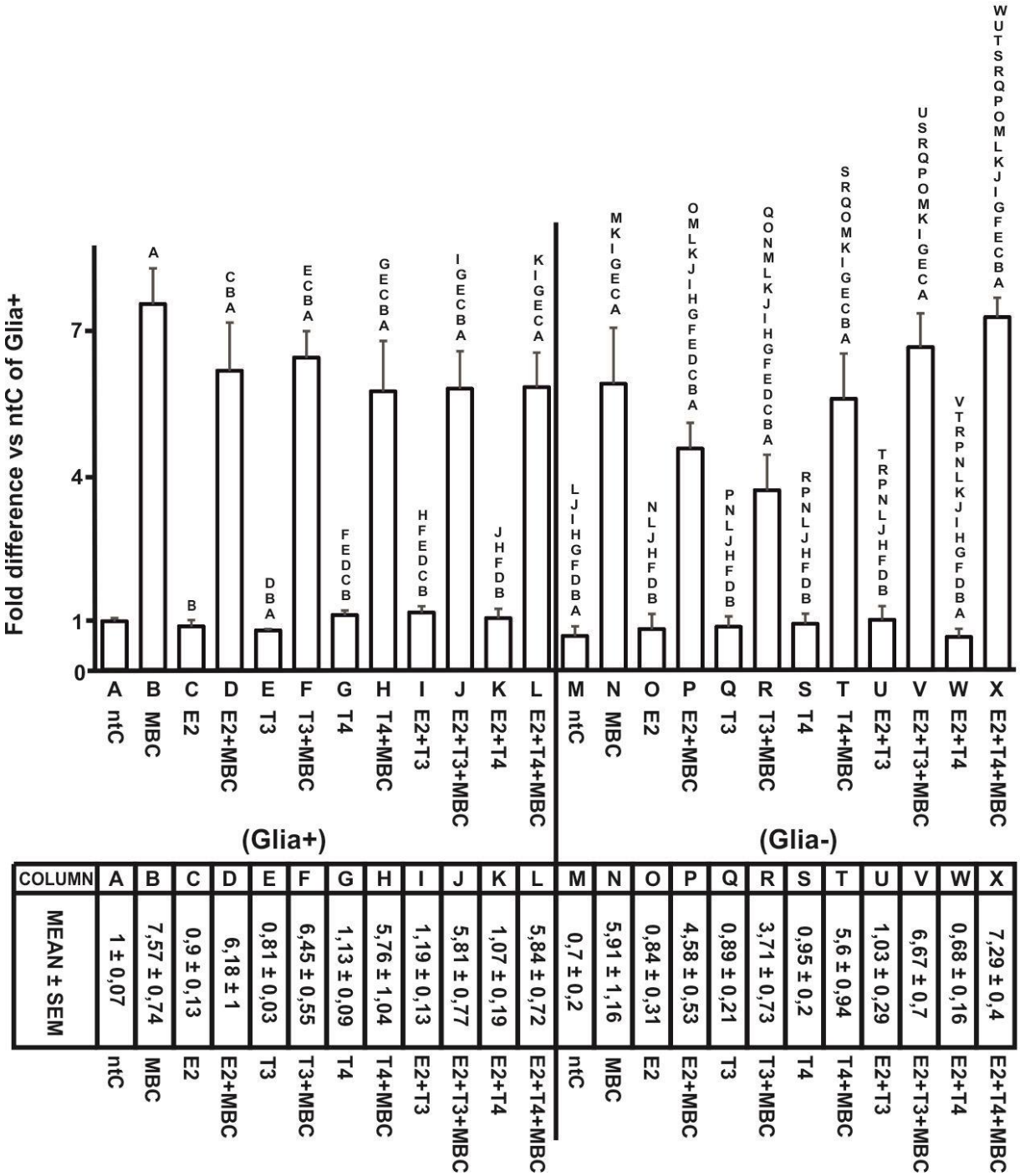
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,3	1,6 ± 0,44	1,44 ± 0,23	1,71 ± 0,15	1,72 ± 0,3	1,49 ± 0,28	1,68 ± 0,22	1,76 ± 0,12	1,57 ± 0,36	1,81 ± 0,18	1,39 ± 0,36	1,61 ± 0,16	0,99 ± 0,14	1,31 ± 0,44	0,76 ± 0,22	1,23 ± 0,5	0,99 ± 0,07	1,2 ± 0,37	1,1 ± 0,16	1,4 ± 0,56	0,75 ± 0,27	1,67 ± 0,28	0,93 ± 0,2	1,43 ± 0,25
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A22



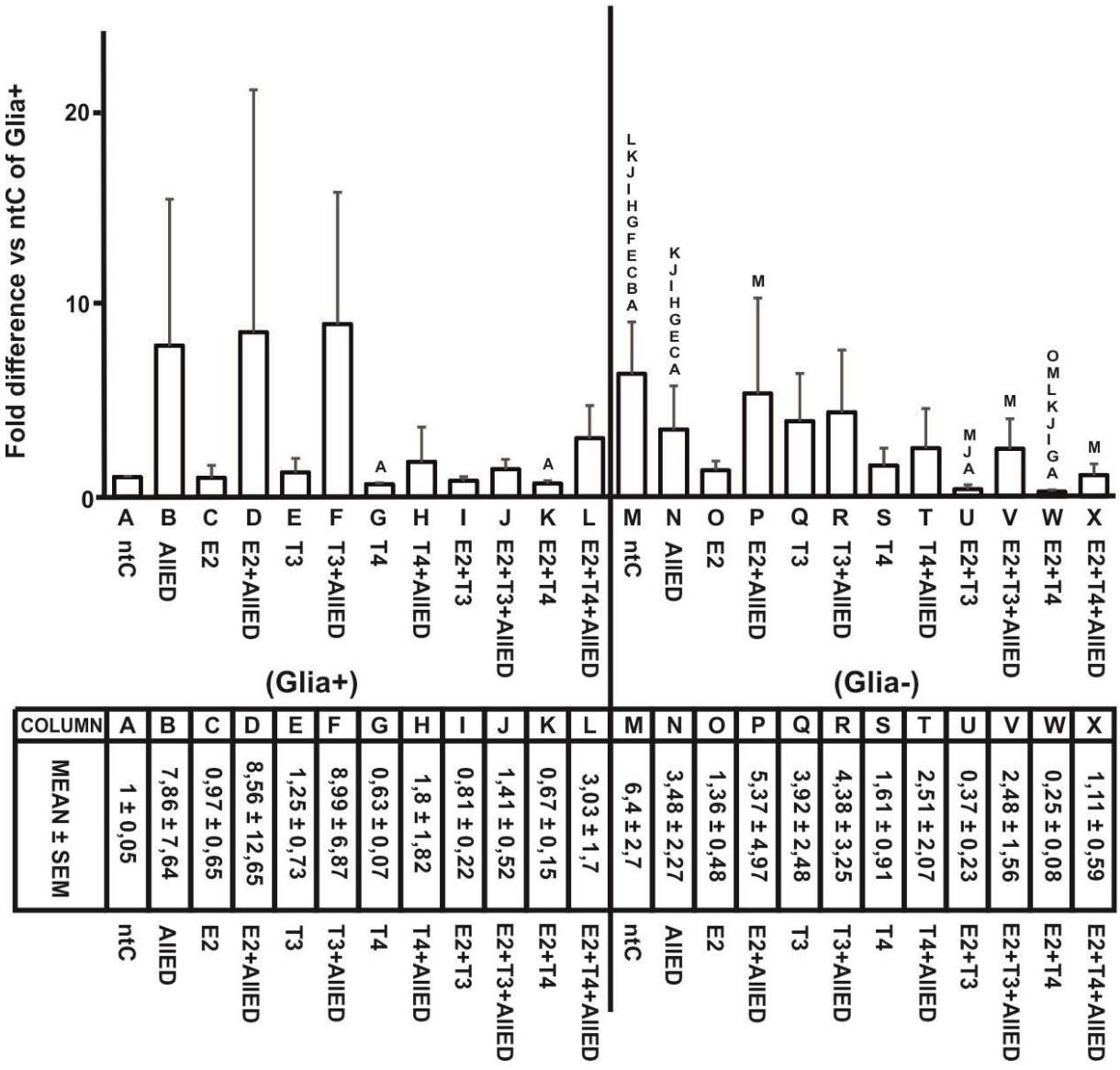
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,02	2,11 ± 1,74	2,38 ± 1,65	3,15 ± 1,32	1,5 ± 0,89	6,66 ± 6,11	1,59 ± 1,11	5,39 ± 3,46	1,1 ± 0,29	3,1 ± 0,65	0,65 ± 0,05	5,8 ± 2,94	7,39 ± 0,26	3,62 ± 2,87	3,28 ± 0,54	3,25 ± 2,95	5,11 ± 2,73	3,6 ± 1,75	15,24 ± 10,22	3,99 ± 2,72	3,09 ± 2,19	1,13 ± 1,52	5,18 ± 2,09	4,23 ± 2,63
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A23



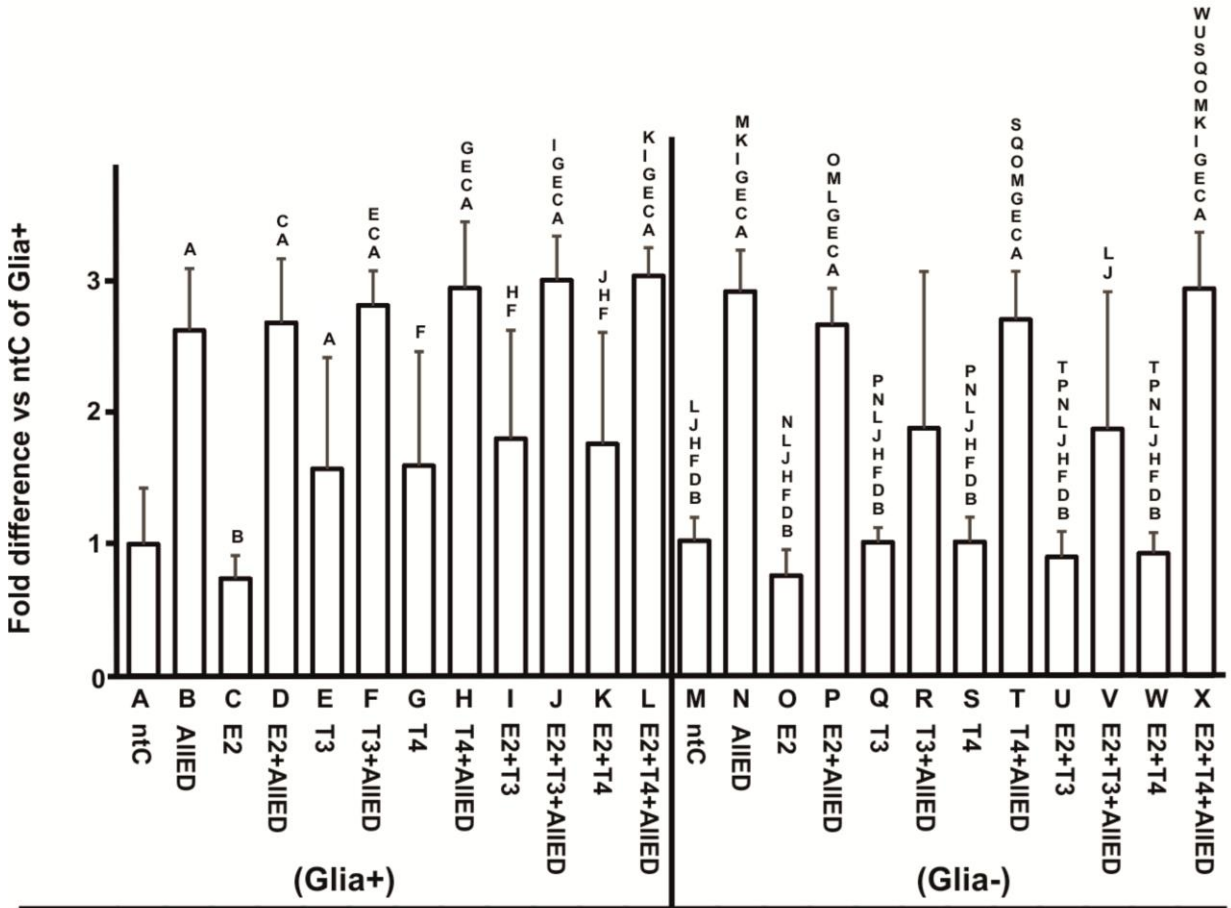
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,07	7,57 ± 0,74	0,9 ± 0,13	6,18 ± 1	0,81 ± 0,03	6,45 ± 0,55	1,13 ± 0,09	5,76 ± 1,04	1,19 ± 0,13	5,81 ± 0,77	1,07 ± 0,19	5,84 ± 0,72	0,7 ± 0,2	5,91 ± 1,16	0,84 ± 0,31	4,58 ± 0,53	0,89 ± 0,21	3,71 ± 0,73	0,95 ± 0,2	5,6 ± 0,94	1,03 ± 0,29	6,67 ± 0,7	0,68 ± 0,16	7,29 ± 0,4
	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC	ntC	MBC	E2	E2+MBC	T3	T3+MBC	T4	T4+MBC	E2+T3	E2+T3+MBC	E2+T4	E2+T4+MBC

Figure A24



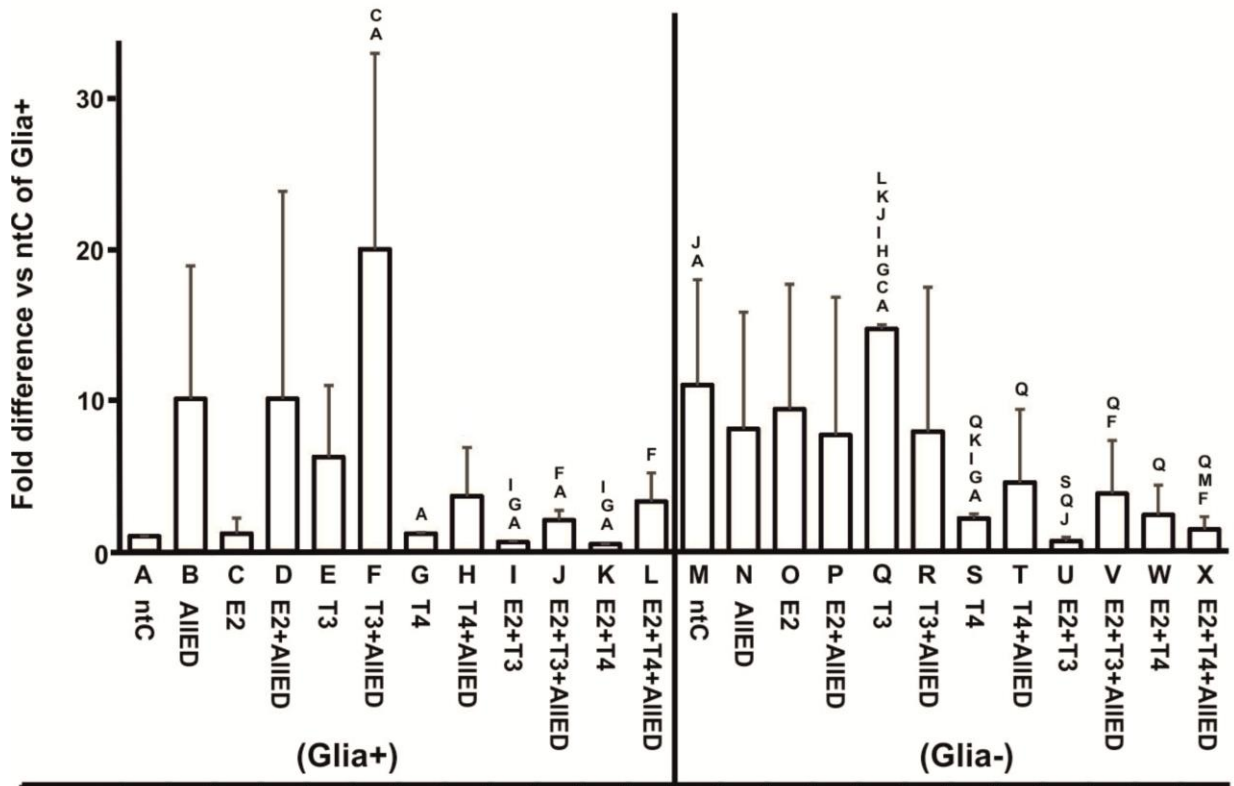
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,05	7,86 ± 7,64	0,97 ± 0,65	8,56 ± 12,65	1,25 ± 0,73	8,99 ± 6,87	0,63 ± 0,07	1,8 ± 1,82	0,81 ± 0,22	1,41 ± 0,52	0,67 ± 0,15	3,03 ± 1,7	6,4 ± 2,7	3,48 ± 2,27	1,36 ± 0,48	5,37 ± 4,97	3,92 ± 2,48	4,38 ± 3,25	1,61 ± 0,91	2,51 ± 2,07	0,37 ± 0,23	2,48 ± 1,56	0,25 ± 0,08	1,11 ± 0,59
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A25



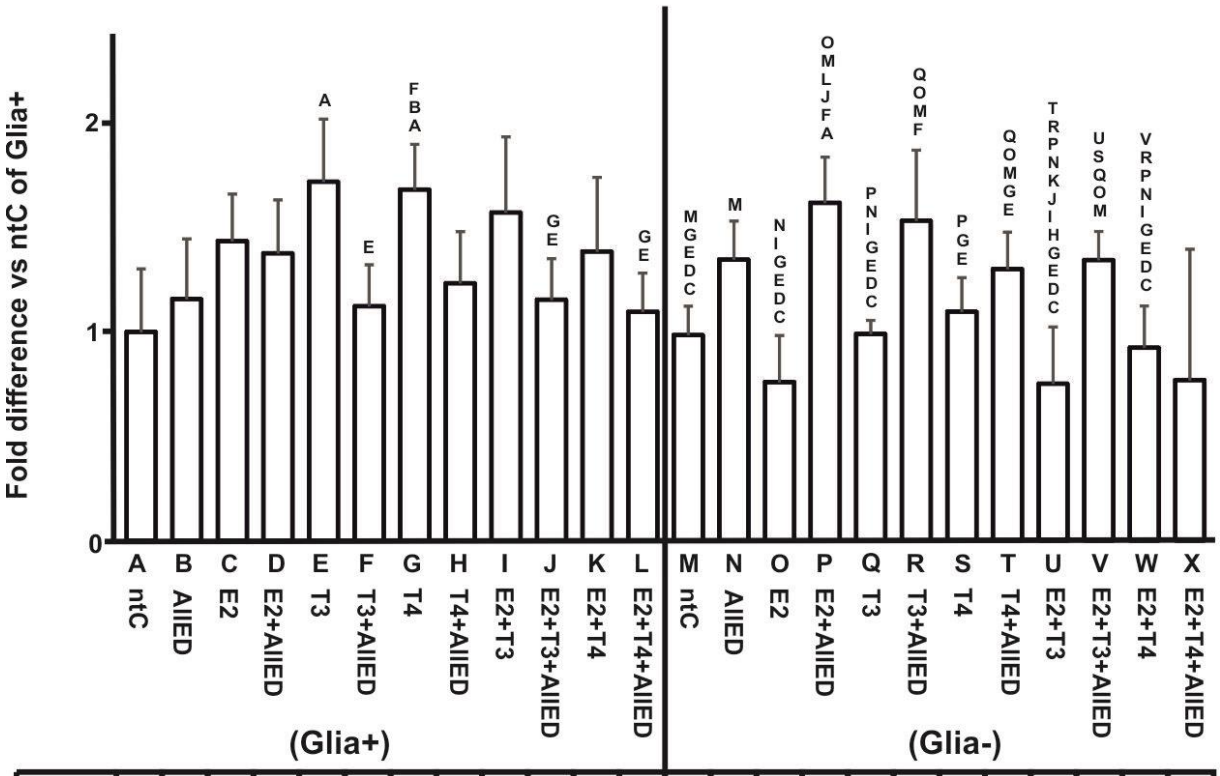
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,43	2,63 ± 0,47	0,74 ± 0,18	2,68 ± 0,49	1,57 ± 0,85	2,82 ± 0,26	1,6 ± 0,87	2,95 ± 0,5	1,8 ± 0,82	3,01 ± 0,34	1,76 ± 0,85	3,04 ± 0,21	1,03 ± 0,18	2,92 ± 0,31	0,76 ± 0,2	2,67 ± 0,28	1,01 ± 0,11	1,88 ± 1,19	1,02 ± 0,19	2,71 ± 0,37	0,9 ± 0,19	1,88 ± 1,04	0,93 ± 0,16	2,94 ± 0,43
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A26



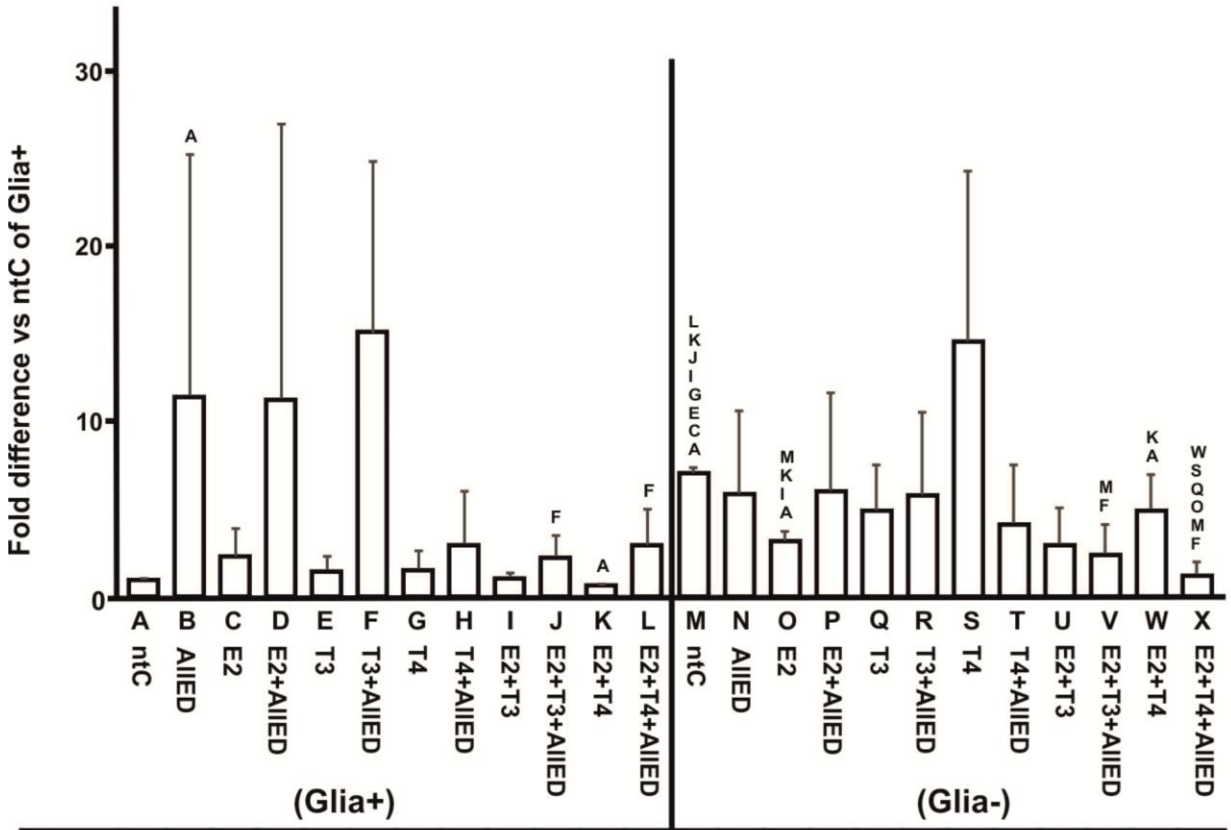
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,01	10,13 ± 8,85	1,16 ± 1,04	10,15 ± 13,8	6,26 ± 4,76	20,09 ± 13,05	1,16 ± 0,07	3,66 ± 3,25	0,6 ± 0,08	2,06 ± 0,65	0,47 ± 0,03	3,31 ± 1,9	11,05 ± 7,01	8,15 ± 7,75	9,45 ± 8,31	7,74 ± 9,17	14,79 ± 0,29	7,96 ± 9,6	2,17 ± 0,3	4,56 ± 4,88	0,66 ± 0,26	3,84 ± 3,53	2,42 ± 1,98	1,44 ± 0,84
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A27



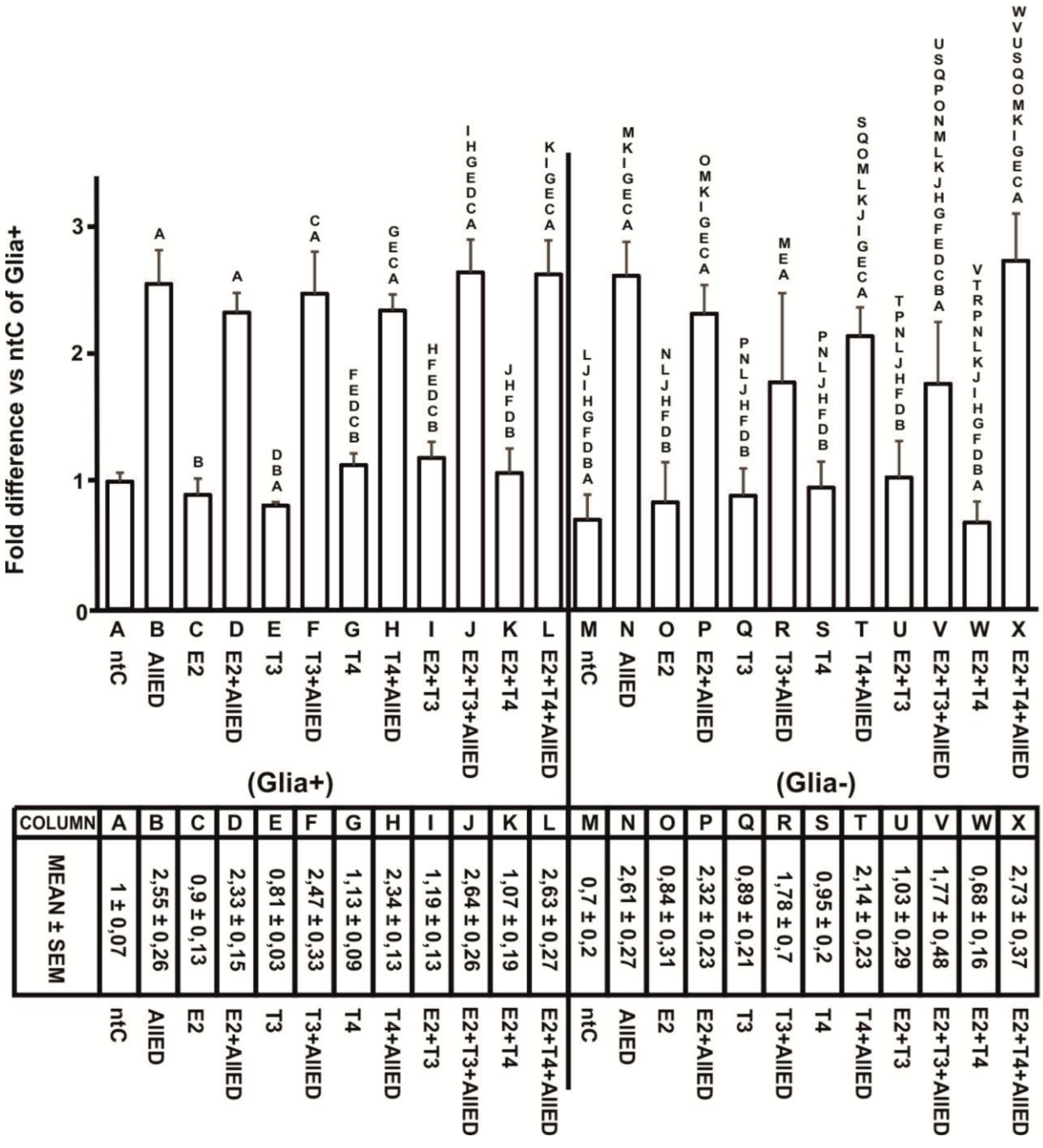
COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,3	1,16 ± 0,29	1,44 ± 0,23	1,38 ± 0,26	1,72 ± 0,3	1,12 ± 0,2	1,68 ± 0,22	1,23 ± 0,25	1,57 ± 0,36	1,15 ± 0,2	1,39 ± 0,36	1,1 ± 0,19	0,99 ± 0,14	1,35 ± 0,18	0,76 ± 0,22	1,62 ± 0,22	0,99 ± 0,07	1,53 ± 0,34	1,1 ± 0,16	1,3 ± 0,18	0,75 ± 0,27	1,34 ± 0,14	0,93 ± 0,2	0,77 ± 0,63
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A28



COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,02	12 ± 14,47	2,38 ± 1,65	11,82 ± 16,51	1,5 ± 0,89	15,79 ± 10,29	1,59 ± 1,11	3,07 ± 3,17	1,1 ± 0,29	2,35 ± 1,22	0,65 ± 0,05	3,05 ± 2,16	7,39 ± 0,26	6,13 ± 4,94	3,28 ± 0,54	6,31 ± 5,9	5,11 ± 2,73	6,05 ± 4,93	15,24 ± 10,22	4,33 ± 3,53	3,09 ± 2,19	2,52 ± 1,79	5,18 ± 2,09	1,26 ± 0,76
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A29



COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN ± SEM	1 ± 0,07	2,55 ± 0,26	0,9 ± 0,13	2,33 ± 0,15	0,81 ± 0,03	2,47 ± 0,33	1,13 ± 0,09	2,34 ± 0,13	1,19 ± 0,13	2,64 ± 0,26	1,07 ± 0,19	2,63 ± 0,27	0,7 ± 0,2	2,61 ± 0,27	0,84 ± 0,31	2,32 ± 0,23	0,89 ± 0,21	1,78 ± 0,7	0,95 ± 0,2	2,14 ± 0,23	1,03 ± 0,29	1,77 ± 0,48	0,68 ± 0,16	2,73 ± 0,37
	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED	ntC	AllED	E2	E2+AllED	T3	T3+AllED	T4	T4+AllED	E2+T3	E2+T3+AllED	E2+T4	E2+T4+AllED

Figure A30

10. ACKNOWLEDGEMENT

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