Journal of Neuroimmunology 328 (2019) 105–107

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short communication

Muscarinic acetylcholine receptor expression in brain and immune cells of *Oreochromis niloticus*

Covantes-Rosales C.E.[a], Toledo-Ibarra G.A.[a], Díaz-Resendíz K.J.G.[a], Ventura-Ramón G.H.[b], Girón-Pérez M.I.[a,b,*]

[a] Universidad Autónoma de Nayarit, Secretaría de Investigación y Posgrado, Laboratorio de Inmunotoxicología, Boulevard Tepic-Xalisco s/n. Cd, de la Cultura Amado Nervo, C.P. 63000 Tepic, Nayarit, Mexico

[b] Centro Nayarita de Innovación y Transferencia de Tecnología A.C., Laboratorio Nacional para la Investigación en Inocuidad Alimentaria-Unidad Nayarit, Calle Tres s/n. Cd Industrial, Tepic, Nayarit, Mexico

ARTICLE INFO

Keywords:
Acetylcholine
Acetylcholine receptors
Brain
Leukocytes
Neuroimmunomodulation
Non-neuronal cholinergic system

ABSTRACT

Nervous and immune systems maintain a bidirectional communication, expressing receptors for neurotransmitters and cytokines. Despite being well established in mammals, this has been poorly described in lower vertebrates as fishes. Experimental evidence shows that the neurotransmitter acetylcholine (ACh) regulates the immune response. In this research, we evaluated mRNA levels of muscarinic acetylcholine receptor (mAChR) in spleen mononuclear cells of Nile tilapia (*Oreochromis niloticus*) and compared the expression levels of immune cells with the brain. The mAChR subtypes (M2-M5A) were detected in both tissues, but mAChRs mRNA levels were higher in immune cells. This data have a potential use in biomedical and comparative immunology fields.

1. Introduction

The existence of "neuro-immunological interactions" in mammals has been demonstrated to consist of a constant and bidirectional communication between the immune system and the nervous system: an essential relationship for the maintenance of homeostasis (Romero et al., 2004). In this sense, the activation of the neuroendocrine-immune interactions serve to maintain an adequate function of the organism in the presence of the stressful stimulus, allowing response to minimize infections or injuries to the body, to recover health (Kepka et al., 2013; Verburg-van Kemenade et al., 2013).

Several studies show how elements of the nervous system regulate the immune system and vice versa, depending on a plurality of molecules such as cytokines and neurotransmitters as acetylcholine (ACh), produced by neurons and leukocytes. This molecule influences the immune system through specific receptors expressed in leukocytes, as muscarinic acetylcholine receptors (mAChRs) (Verburg-van Kemenade et al., 2013). Previously reported results by our research group show that Nile tilapia fish leukocytes, possess essential components of a non-neuronal cholinergic system, like ACh and acetylcholinesterase (AChE) (Toledo-Ibarra et al., 2014), which could be related to the functionality of leukocytes and therefore regulate the immunocompetence of the organism (Kawashima et al., 2012).

This study has a phylogenetic and biomedical importance. Teleost fishes are the first lower vertebrates to develop mechanisms of innate and adaptive immunity (Rauta et al., 2012; Toledo-Ibarra et al., 2013). In consequence, studying the elements of neuroendocrine communication in fish is important to know the evolution of this axis. On the other hand, the study of the leukocyte cholinergic system could be linked to inflammatory and immunocompetence diseases (autoimmunity, hypersensibility, and major susceptibility to infection) (Kawashima et al., 2012; Díaz-Resendiz et al., 2015). For that reason, the aim of this paper is to compare mRNA levels of mAChR subtypes in spleen mononuclear cells (SMNC) with expression levels in the brain of Nile tilapia (*O. niloticus*).

2. Materials and Methods

Male Nile tilapia fish ($n = 7$) (273 ± 43 g and 20 ± 3 cm) were euthanized by submersion in ice bath and the brains were immediately aseptically dissected and placed in 1 mL TRIzol Reagent (Invitrogen™). The SMNC were isolated following the method by Toledo-Ibarra et al., 2016 while total RNA was obtained according to the methodology by Rio et al., 2010. Total RNA was reverse-transcribed at a final concentration of 25 ng/μL using TaqMan™ Reverse Transcription Reagents (Applied Biosystems) with oligo $d(T)_{16}$. Gene expression analysis was

* Corresponding author at: Universidad Autónoma de Nayarit, Secretaría de Investigación y Posgrado, Laboratorio de Inmunotoxicología, Boulevard Tepic-Xalisco s/n. Cd, de la Cultura Amado Nervo, C.P. 63000 Tepic, Nayarit, Mexico
E-mail address: ivangiron@uan.edu.mx (M.I. Girón-Pérez).

https://doi.org/10.1016/j.jneuroim.2019.01.012
Received 11 January 2019; Received in revised form 21 January 2019; Accepted 22 January 2019

Table 1
Oligonucleotides used for amplification of specific gene products.

Target	Sense (5′-3′)	Antisense (5′-3′)	Product (bp)	Reference
mAChR-M2	GGCAGATCTCCAGAGCAAGCAAG	TTGTTTCTCCTCTGGCCTGGTG	100	Seo et al., 2009
mAChR-M3	CTGGCGCATTTACAAGGAGACCC	CTACCAGTTCCCGACATGTGCTC	200	
mAChR-M4	TTTCTACCTACCTGTGGCCATCATG	AGTGCCTGAGGTCTTCCTGCTG	100	
mAChR-M5A	CCAGGAGCAACGCGGTCAAATC	TGGAAGCCTCCTGGAGACACTG	130	
EFL-1α	CAAGGAAATCCGTCGTGGATAC	ACGGCGAAACGACCGAGGGG	327	*NM 001279647

mAChR-M2: muscarinic acetylcholine receptor subtype M2; mAChR-M3 muscarinic acetylcholine receptor subtype M3; mAChR-M4: muscarinic acetylcholine receptor subtype M4; mAChR-M5A: muscarinic acetylcholine receptor subtype M5A; EFL-1α: elongation factor 1-alpha; * for EFL-1α mRNA sequence (GenBank accession number).

performed by real-time PCR using a 7500 Fast Real-Time PCR thermocycler (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems). Expression levels of mAChR subtypes were normalized with elongation factor-1 alpha (EFL-1α). The oligonucleotides used in this research are listed in Table 1. Mean ± SD was determined for each subtype of mAChR in brain and SMNC, and expression levels between tissues were compared with a *Student's t-test*. The statistical significance was determined when $p < .05$.

3. Results

The results show that the brain and SMNC of Nile tilapia fish express four subtypes of mAChR (M2-M5A). There were significant differences in mAChR subtypes expression between immune cells and brain tissue. In brief: the M2 expression on brain ($\overline{X} = 6.45$, SD = 1.6) and SMNC ($\overline{X} = 10.92$, SD = 1.96), $p < .001$. While the M3 expression on brain ($\overline{X} = 8.85$, SD = 2.1) and SMNC ($\overline{X} = 13.84$, SD = 1.92), $p < .001$. Additionally, the M4 expression on brain ($\overline{X} = 8.30$, SD = 1.89) and SMNC ($\overline{X} = 11.71$, SD = 2.0), $p < .004$. Finally, the M5A expression on brain ($\overline{X} = 9.27$, SD = 2.01) and SMNC ($\overline{X} = 12.27$, SD = 1.43), $p < .004$. This indicates that all mAChR subtypes are more abundant in SMNC than in brain (Fig. 1).

4. Discussion

Neuronal presence of mAChR is well characterized in mammals; nonetheless, there are few extensive studies concerning non-mammalian vertebrate species (Williams and Messer Jr, 2004). While the presence of mAChR has already been reported in fish brain (Beauvais et al., 2001; Williams and Messer Jr, 2004; Arenzana et al., 2005; Seo et al., 2009; Toscano-Márquez et al., 2013), this research confirms the expression of mAChR subtypes M2, M3, M4, and M5A Neuronal mAChRs

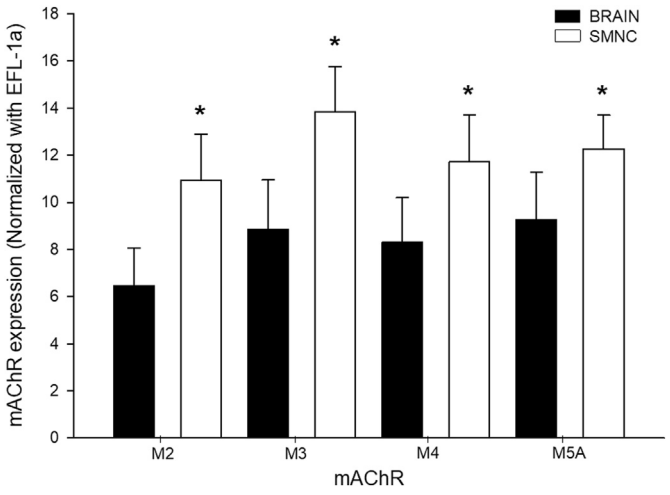

Fig. 1. Expression of mAChR subtypes (M2, M3, M4, and M5A) in brain (black) and SMNC (white) of Nile tilapia ($n = 7$), *: Student's *t*-test; $p < .05$.

have important functions associated with proliferation, differentiation, and survival of nervous system cells (Carruthers et al., 2015). In the central nervous system, mAChRs are involved in a variety of vegetative, sensory, behavioral, cognitive, and motor functions (Eglen, 2005). Additionally, mAChRs regulate a wide range of physiological activities such as heart rate, smooth muscle contraction, and glandular secretions (Eglen, 2005; Wess et al., 2007).

The expression of mAChR has been confirmed in monocytes and macrophages as well as lymphocytes isolated from blood, lymph nodes, spleen, and thymus from mammal models and immune cell lines (Kawashima and Fujii, 2003; de la Torre et al., 2005; Razani-Boroujerdi et al., 2008; Kawashima et al., 2012; Koarai et al., 2012). In mammals five subtypes of mAChR have been characterized (Kawashima and Fujii, 2004). In respect to M1 mAChR subtype, Seo et al., 2009 suggested that M1 receptor was probably lost in a common ancestor of teleost fish. Particularly in Nile tilapia, the presence of mAChR has been reported in lymphoid organs (spleen, kidney) (Seo et al., 2009). Additionally, our research group has proven the presence of cholinergic system components (ACh, AChE, and cholinergic receptors) in immune cells isolated from this species (Toledo-Ibarra et al., 2014; Toledo-Ibarra et al., 2016). Notwithstanding, this is the first report of mRNA mAChR subtypes (M2, M3, M4, and M5A) in SMNC isolated from a teleost fish (Fig. 1).

This evidence reinforces the notion of a close communication between immune system cells and the central nervous system (Romero et al., 2004). Furthermore, research groups strongly suggest that immune system cells express receptors for and synthesize neurotransmitters. Together with cytokines, these molecules are key for the immunoregulation, homeostasis, and maintenance of organisms (Pavlov et al., 2003; Kawashima et al., 2012; Toledo-Ibarra et al., 2016; Fujii et al., 2017; Torrealba et al., 2018).

The cholinergic system (neuronal and non-neuronal) could play an important role in immunomodulation. For example, nicotinic acetylcholine receptor (nAChR) agonists, particularly subunit α7, unmistakably downregulate TNF-α, IL-1β, and IL-6 production. On the other hand, a pro-inflammatory role of mAChRs is suggested given that mAChR agonist administration induces inflammatory processes while antagonists inhibit them (Bos et al., 2007; Razani-Boroujerdi et al., 2008).

As previously described, mAChR stimulation increases intracellular Ca^{2+} flux, up-regulates *c-fos* expression, and affects cell proliferation (Kawashima and Fujii, 2004). The optimal development, proliferation, differentiation, and activation of immune cells demands mAChR pathways (de la Torre et al., 2005; Zimring et al., 2005; Qian et al., 2011; Jinno et al., 2017). Besides, mAChRs are related with immune defense given that antigenic stimuli enhance the cholinergic machinery (Kawashima and Fujii, 2004). Additionally, it has been described that mAChRs, and specifically M3 subtype, play a pivotal role in immune defense against parasitic and bacterial infections (Darby et al., 2015; McLean et al., 2016). Additionally, mAChRs modulate the antibody class switching (Fujii et al., 2007). These studies have established the importance of mAChR signaling during innate and adaptive immune responses.

Research on the non-neuronal cholinergic system of non-

mammalian model immune cells is scarce. This work detects neuronal cholinergic components (mAChR M2-M5A) expressed in spleen mononuclear cells of Nile tilapia (*O. niloticus)*. The data obtained in this research provide evidence of bidirectional communication between the neuronal and immune systems, demonstrating that this axis is a conserved mechanism in vertebrate evolution. In addition, studies on the lymphocyte cholinergic system could have important biomedical implications, since ACh and cholinergic agents are relevant immunomodulators that surely will impact on the therapeutic strategies for chronic inflammatory disease, such as asthma, chronic obstructive pulmonary disease (COPD), palmoplantar pustulosis, amount others; even implications in cases of poisoning by anticholinesterase agents, such as organophosphorus and carbamate pesticides.

Disclosure of interest

None.

Funding

This work was funded by a grant from the financial resources of SEP-CONACyT-Mexico for Basic Research [Project no. 2012-179508] to M.I. Girón-Pérez. Covantes-Rosales CE is a PhD student from the "Doctorado en Ciencias Biológico-Agropecuarias" graduate program at the Universidad Autónoma de Nayarit (México). Toledo-Ibarra GA is a doctoral student from "Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM)".

Acknowledgments

Thanks to Nerea Roher PhD, (Institute of Biotechnology and Biomedicine (IBB), Autonomous University of Barcelona, Spain), for supplying EFL-1α oligonucleotides sequences.

References

Arenzana, F.J., Clemente, D., Sánchez-González, R., Porteros, Á., Aijón, J., Arévalo, R., 2005. Development of the cholinergic system in the brain and retina of the zebrafish. Brain Res. Bull. 66 (4–6), 421–425.

Beauvais, S.L., Jones, S.B., Parris, J.T., Brewer, S.K., Little, E.E., 2001. Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (Oncorhynchus mykiss). Ecotoxicol. Environ. Saf. 49 (1), 84–90.

Bos, I.S.T., Gosens, R., Zuidhof, A.B., Schaafsma, D., Halayko, A.J., Meurs, J., Zaagsma, J., 2007. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. Eur. Respir. J. 30, 653–661.

Carruthers, S.P., Gurvich, C.T., Rossell, S.L., 2015. The muscarinic system, cognition and schizophrenia. Neurosci. Biobehav. Rev. 55, 393–402.

Darby, M., Schnoeller, C., Vira, A., Culley, F., Bobat, S., Logan, E., Cunningham, A.F., Brombacher, F., Selkirk, M.E., Horsnell, W.G.C., 2015. The M3 muscarinic receptor is required for optimal adaptive immunity to helminth and bacterial infection. PLoS Pathog. 11 (1), e1004636.

Díaz-Resendiz, K.J.G., Toledo-Ibarra, G.A., Girón-Pérez, M.I., 2015. Modulation of immune response by organophosphorus pesticides: fishes as a potential model in immunotoxicology. J Immunol Res 2015, 213836.

Eglen, R.M., 2005. Muscarinic receptor subtype pharmacology and physiology. Prog. Med. Chem. 43, 105–136.

Fujii, Y.X., Tashiro, A., Arimoto, K., Fujigaya, H., Moriwaki, Y., Misawa, H., Fujii, T., Matsui, M., Kasahara, T., Kawashima, K., 2007. Diminished antigen-specific IgG 1 and interleukin-6 production and acetylcholinesterase expression in combined M 1 and M 5 muscarinic acetylcholine receptor knockout mice. J. Neuroimmunol. 188 (1), 80–85.

Fujii, T., Mashimo, M., Moriwaki, Y., Misawa, H., Ono, S., Horiguchi, K., Kawashima, K., 2017. Expression and function of the cholinergic system in immune cells. Front. Immunol. 8, 1085.

Jinno, M., Ohta, S., Tanaka, A., Satou, H., Uno, T., Fujiwara, A., Uchida, Y., Manabe, R., Kuwahara, N., Hirai, K., Miyata, Y., 2017. Muscarinic M3 receptor blockage inhibits the development of M2 macrophages in allergic inflammation. In: C35. Asthma and Allergy Cellular Investigations. American Thoracic Society, pp. A5282.

Kawashima, K., Fujii, T., 2003. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci. 74 (6), 675–696.

Kawashima, K., Fujii, T., 2004. Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front. Biosci. 9 (1–3), 2063–2085.

Kawashima, K., Fujii, T., Moriwaki, Y., Misawa, H., 2012. Critical roles of acetylcholine and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune function. Life Sci. 91 (21 – 22), 1027–1032.

Kepka, M., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2013. Neuroendocrine modulation of the inflammatory response in common carp: adrenaline regulates leukocyte profile and activity. Gen. Comp. Endocrinol. 188, 102–109.

Koarai, A., Traves, S.L., Fenwick, P.S., Brown, S.M., Chana, K.K., Russell, R.E., Nicholson, A.G., Barnes, P.J., Donnelly, L.E., 2012. Expression of muscarinic receptors by human macrophages. Eur. Respir. J. erj01367–2010.

de la Torre, E., Davel, L., Jasnis, M.A., Gotoh, T., de Lustig, E.S., Sales, M.E., 2005. Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice. Breast Cancer Res. 7 (3), R345–R352.

McLean, L.P., Smith, A., Cheung, L., Urban Jr., J.F., Sun, R., Grinchuk, V., Desai, N., Zhao, A., Raufman, J.P., Shea-Donohue, T., 2016. Type 3 muscarinic receptors contribute to intestinal mucosal homeostasis and clearance of Nippostrongylus brasiliensis through induction of TH2 cytokines. Am. J. Physiol. Gastrointest. Liver Physiol. 311 (1), G130–G141.

Pavlov, V.A., Wang, H., Czura, C.J., Friedman, S.G., Tracey, K.J., 2003. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol. Med. 9 (5–8), 125.

Qian, J., Galitovskiy, V., Chernyavsky, A.I., Marchenko, S., Grando, S.A., 2011. Plasticity of the murine spleen T-cell cholinergic receptors and their role in in vitro differentiation of naive CD4 T cells toward the Th1, Th2 and Th17 lineages. Genes Immun. 12, 222–230.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol. Lett. 148 (1), 23–33.

Razani-Boroujerdi, S., Behl, M., Hahn, F.F., Pena-Philippides, J.C., Hutt, J., Sopori, M.L., 2008. Role of muscarinic receptors in the regulation of immune and inflammatory responses. J. Neuroimmunol. 194 (1–2), 83–88 (Research, 7(3), R345).

Rio, D.C., Ares, M., Hannon, G.J., Nilsen, T.W., 2010. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc 2010 (6), pdb–prot5439.

Romero, L.P., Hernández, M.E., Salinas, F.L., López, G.S., 2004. Interacciones neuroendocrinoinmunológicas. Salud Ment. 27 (3), 19–25.

Seo, J.S., Kim, M.S., Park, E.M., Ahn, S.J., Kim, N.Y., Jung, S.H., Kim, J.W., Lee, H.H., Chung, J.K., 2009. Cloning and characterization of muscarinic receptor genes from the nile tilapia (Oreochromis niloticus). Mol. Cell 27 (3), 383–390. https://doi.org/10.1007/s10059-009-0048-5.

Toledo-Ibarra, G.A., Rojas-Mayorquín, A.E., Girón-Pérez, M.I., 2013. Influence of the cholinergic system on the immune response of teleost fishes: potential model in biomedical research. Clin. Dev. Immunol. 2013.

Toledo-Ibarra, G.A., Díaz-Resendiz, K.J.G., Pavon, L., Girón-Pérez, M.I., 2014. Cholinergic activity in mononuclear cells of nile tilapia (Oreochromis niloticus) fish. Adv. Neuroimmune Biol. 5 (4), 229–234.

Toledo-Ibarra, G.A., Díaz-Resendiz, K.J.G., Pavón-Romero, L., Rojas-García, A.E., Medina-Díaz, I.M., Girón-Pérez, M.I., 2016. Effects of diazinon on the lymphocytic cholinergic system of Nile tilapia fish (Oreochromis niloticus). Vet. Immunol. Immunopathol. 176, 58–63.

Torrealba, D., Balasch, J.C., Criado, M., Tort, L., Mackenzie, S., Roher, N., 2018. Functional evidence for the inflammatory reflex in teleosts: a novel α7 nicotinic acetylcholine receptor modulates the macrophage response to dsRNA. Dev. Comp. Immunol. 84, 279–291.

Toscano-Márquez, B., Dunn, R.J., Krahe, R., 2013. Distribution of muscarinic acetylcholine receptor mRNA in the brain of the weakly electric fish Apteronotus leptorhynchus. J. Comp. Neurol. 521 (5), 1054–1072.

Verburg-van Kemenade, B.M.L., Van der Aa, L.M., Chadzinska, M., 2013. Neuroendocrine–immune interaction: regulation of inflammation via G-protein coupled receptors. Gen. Comp. Endocrinol. 188, 94–101.

Wess, J., Eglen, R.M., Gautam, D., 2007. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat. Rev. Drug Discov. 6 (9), 721–733. https://doi.org/10.1038/nrd2379.

Williams, F.E., Messer Jr., W.S., 2004. Muscarinic acetylcholine receptors in the brain of the zebrafish (Danio rerio) measured by radioligand binding techniques. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 137 (4), 349–353.

Zimring, J.C., Kapp, L.M., Yamada, M., Wess, J., Kapp, J.A., 2005. Regulation of CD8+ cytolytic T lymphocyte differentiation by a cholinergic pathway. J. Neuroimmunol. 164, 66–75.