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Changes in cytochrome oxidase activity following spatial working memory learning in rats treated with tacrine

Magdalena Méndez-López, Marta Méndez, Laudino López and Jorge L. Arias
Universidad de Oviedo

We evaluated change in cytochrome oxidase (COx) activity of the hippocampus and related structures of the limbic system following spatial working memory learning in rats after treatment with tacrine (8.0mg/kg). Control groups treated with saline and tacrine and an untreated group were added. Acetylcholinesterase optical density levels were also measured. The tacrine and saline groups showed similar behavioral results, but a decrease in COx activity was found in the tacrine group in the prefrontal cortex, nucleus accumbens, anterior thalamus, hippocampus and nucleus basalis of Meynert. Similarly, acetylcholinesterase levels of the tacrine group were lower in most of the regions. Learning-related increase in COx activity was found in the prefrontal cortex and dentate gyrus in the saline group. The tacrine group presented the same increase in the anterodorsal thalamus, dentate gyrus, CA3 and mammillary nuclei. These results suggest that inhibition of the acetylcholinesterase produces a different pattern of learning-related neuronal activity in the limbic system of the rat.

Cambios en la actividad citocromo oxidasa tras el aprendizaje de memoria de trabajo espacial en ratas tratadas con tacrina. Se evaluaron los cambios en la actividad citocromo oxidasa (COx) del hipocampo y estructuras relacionadas del sistema límbico tras el aprendizaje de memoria de trabajo espacial en ratas tratadas con tacrina (8,0 mg/Kg). Se añadieron al estudio grupos control tratados con solución salina y tacrina, y un grupo sin tratamiento. También se midieron los niveles de densidad óptica de la acetilcolinesterasa. Los grupos salino y tacrina mostraron resultados conductuales similares, pero se encontró una disminución en la actividad COx en la corteza prefrontal, núcleo accumbens, tálamo anterior, hipocampo y núcleo basal de Meynert del grupo tacrina. Del mismo modo, los niveles de la acetilcolinesterasa del grupo tacrina fueron más bajos en la mayoría de las regiones. Incrementos en la actividad COx relacionados con el aprendizaje fueron encontrados en la corteza prefrontal y el giro dentado en el grupo salino. El grupo tacrina presentó este tipo de incrementos en el tálamo anterodorsal, giro dentado, CA3 y núcleos mamilares. Estos resultados sugieren que la inhibición de la acetilcolinesterasa produce un patrón diferente de actividad neuronal asociada al aprendizaje en el sistema límbico de la rata.

Cholinergic neurotransmission is known for its role in cognitive functioning in the mammalian brain. The cholinergic basal forebrain neurons send projections to several areas that mediate learning and memory including, among other regions, the hippocampus and neocortex (Woolf, 1991).

The termination of acetylcholine (ACh) neurotransmission is carried out by a metabolizing enzyme called acetylcholinesterase (AChE; Silman & Sussman, 2008). However, agents can restore ACh neurotransmission by preventing the action of AChE. The first studied was tacrine, which caused short term improvement of cognitive symptoms in Alzheimer's disease patients (Irizarry & Hyman, 2001). Besides positive effects in humans, studies with animals also found that tacrine enhanced spatial memory in maze tasks (Murphy, Foley, O'Connel, & Regan, 2006; Schildein, Huston, & Schwarting, 2000).

Cytochrome oxidase (COx) is a mitochondrial enzyme that participates in the oxidative phosphorylation process in which energy is produced in the form of ATP. Therefore, COx activity reflects changes in the neuronal metabolic capacity induced by energy requirements and is an indicator of neuronal activity of brain regions in response to learning processes including spatial learning (Conejo, González-Pardo, López, Cantora, & Arias, 2007; González-Lima & Cada, 1994; Méndez-López, Méndez, López, & Arias, 2009).

In spite of the large number of studies on tacrine, there are no studies that assess spatial learning-related changes in brain COx activity as a result of tacrine treatment in rats that are submitted to an easy memory task performed in the Morris water maze (MWM). Therefore, the aim of the present work was to study the changes in COx and AChE activities induced by tacrine after training rats in a spatial working memory (WM) task with a short retention interval. Thus, we aim to compare the changes in brain activity induced by tacrine with those of saline-treated animals with similar behavioral performance.

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Correspondencia: Magdalena Méndez-López
Facultad de Psicología
Universidad de Oviedo
33003 Oviedo (Spain)
e-mail: magdamndez@gmail.com

The functional contribution of brain regions that have been known for their role in spatial learning was assessed. Thus, we explored the nucleus basalis of Meynert (Rispoli et al., 2008), the dorsal hippocampus (Cimadevilla, López, Nieto, Aguirre, & Fernández, 2009; Jung, Wiener, & McNaughton, 1994) and associated regions (medial prefrontal cortex, nucleus accumbens, anterior thalamus, mammillary bodies and ventral tegmental area), which are involved in the performance of spatial tasks in rats (Delatour & Gisquet-Verrier, 2000; Levin, Briggs, Christopher, & Auman, 1994; Romanides, Duffy, & Kalivas, 1999; Vann & Aggleton, 2003; Warburton, Baird, Morgan, Muir, & Aggleton, 2001).

Method

Animals

We used 40 male Wistar rats (225-260 g) from the vivarium of Oviedo University that were maintained under standard laboratory conditions (21 ºC temperature, 65-70% relative humidity, and 12 hr light/dark cycle). The procedures were carried out according to the Directive 86/609/EEC of the European Communities Committee.

Procedure

Animals were divided into the following groups (n= 8): A tacrine group (TA) that was submitted to the spatial task following tacrine injection, a saline group (SAL) that was also submitted to the same task following saline injection and an untreated control group (CO). Yoked swimming control groups that received tacrine injection (SC-TA) and saline injection (SC-SAL) were added. These groups swam in the maze during a time equal to mean of the escape latencies recorded for the TA and SAL groups, respectively, receiving an identical number of trials but without the escape platform and cues used.

Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine; Sigma, St. Louis, MO, USA) was freshly dissolved in 0.9% (w/v) phosphate-buffered physiological saline. TA group was injected with tacrine (8 mg/Kg, i.p.) and SAL group received same injection of the vehicle solution. The dose of tacrine used has been shown to be effective previously (Jackson & Soliman, 1996). Substances were administered 30 min before each daily training session.

Spatial WM was evaluated in the MWM (Morris, 1984). The maze and the training procedure were detailed by Méndez-López et al., (2009). Briefly, a habituation session was performed one day prior to the training schedule. The WM training involved a paired sample task. Each daily session consisted of two identical trials (sample and retention). During both trials, the platform was hidden 2 cm below the level of the water. The sample consisted of releasing the animal from one of the four starting points of the pool and letting it swim until it reached the hidden platform or 60 sec had elapsed. If the animal had not reached the hidden platform in this time, it was placed on the platform and kept there for 15 sec. The intertrial interval was 5 s. The task demands a recall of the position occupied by the hidden platform during the sample. The locations of the platform and of the start positions varied on the different days in a pseudorandom order. Training finished when the group achieved the learning criteria established as statistically significant lower retention latency compared to sample latency within one session, and also compared to retention latency of the first session.

Brains were removed 90 min after the end of the task and frozen in isopentane (Sigma-Aldrich, Germany). Coronal sections (30 μm) containing prelimbic and infralimbic cortex (PLc and ILc, respectively), nucleus accumbens core (NACc), nucleus accumbens shell (NACs), anterodorsal thalamus (ADT), anteroventral thalamus (AVT), anteromedial thalamus (AMT), nucleus basalis of Meynert (NBM), CA1, CA3, dentate gyrus (DG), medial mammillary nucleus (MM), lateral mammillary nucleus (LM) and ventral tegmental area (VTA) were studied. These regions were anatomically defined according to Paxinos & Watson's atlas (2005). The distance in mm of the regions, from anterior to posterior, corresponded to Bregma levels 3.0 for PLc and ILc; 2.28 for NACc and NACs; -1.72 for ADT, AVT and AMT; -1.8 for NBM; -3.24 for DG, CA3 and CA1 and -4.8 for MM, LM and VTA.

Sections containing the regions of interest were processed using a modified version of the acetylcholinesterase histochemistry described by Slattery, Morrow, Hudson, Hill, Nutt, & Henry (2005). Sections were immersed in a solution of 0.256 g cupric sulfate (Sigma, St. Louis, MO, USA), 3.2 mg ethopropazine, 0.92 g acetylthiocholine iodide, and 0.6 g glycine in 800 ml of sodium acetate buffer (0.05 M, pH 5.0). Then, the sections were washed, immersed in a 1.25% (w/v) ammonium sulfide solution and fixed in formalin. Also, another set of sections was processed using the quantitative COx histochemistry described by González-Lima & Cada (1994). The procedure was detailed by Méndez-López et al., (2009).

Regarding densitometric analysis, optical density (OD) measurements of COx and AChE histochemistry were taken as described earlier (Méndez-López et al., 2009). To control staining variability across baths, sets of tissue homogenate standards of different thickness from Wistar rat brain were included with each bath in both AChE and COx histochemistries (González-Lima & Jones, 1994). In AChE histochemistry, regression curves and coefficients between section thickness and AChE OD measured from each set of standards were calculated for each bath. OD values measured for the brain regions selected were put on the same level using the OD differences calculated from the regression plots of the brain standards. Acetylcholinesterase values are expressed as arbitrary units of OD. In COx histochemistry, the brain standards were used to convert tissue OD measures to COx activity units via a regression equation based on their OD and spectrophotometrically determined enzymatic activity (González-Lima & Jones, 1994). OD values measured were converted into COx activity units (μmol cytochrome c oxidized/min g wet tissue at 23 ºC) using the regression curves.

Data analysis

With regard to the learning criteria, a paired *T*-test was used to evaluate the differences between escape latencies in sample and retention trials within a training group (TA, SAL). The escape latencies measures of the trained groups were compared. A two-way repeated-measures ANOVA with *group* and *session* as independent variables and repeated measures in the last factor was performed separately for the sample and retention trials. A one-way ANOVA was carried out separately for saline and tacrine groups to assess differences between control, swimming control and training groups in AChE and COx values in each brain region. Tukey's tests were applied as post hoc test and significance was

accepted when $p<0.050$. Finally, *T*-tests for independent samples were performed to assess differences between trained groups (TA, SAL) in AChE and COx values.

Results

Spatial working memory

The learning criterion was established in the third session in the tacrine (TA) and saline (SAL) groups. A lower escape latency was found in the retention trial compared with the sample trial within this session [TA: t (7)= 3.236, p= 0.014 and SAL: t (7)= 4.674, p= 0.002], and compared with the first retention trial [TA: t (7)= 2.560, p= 0.038 and SAL: t (7)= 3.072, p= 0.018]. Also, in retention trials, the ANOVA showed an effect of session [F (2,28)= 5.723, p= 0.008], a lower escape latency was found in the third session compared to the first (p= 0.006) (Figure 1).

AChE histochemistry

Table 1 shows the results. Significant differences between TA, SC-TA and CO groups were found in the NACc [F (2,21)= 61.974, $p<0.001$], NACs [F (2,21)= 44.109, $p<0.001$] and VTA [F (2,21)= 62.374, $p<0.001$], where TA presented higher OD levels than control groups ($p<0.01$). Also, differences were found in the AVT [F (2,21)= 9.007, p= 0.001] and the LM [F (2,21)= 3.998, p= 0.034], CO group presented lower levels compared with TA ($p<0.05$). In the AVT, CO group had also lower levels than SC-TA (p= 0.008).

The comparisons of AChE OD levels obtained from the SAL, SC-SAL and CO groups revealed that these levels were different in the NACc [F (2,21)= 96.076, $p<0.001$], NACs [F (2,21)= 47.131, $p<0.001$], ADT [F (2,21)= 24.048, $p<0.001$], AVT [F (2,21)= 35.795, $p<0.001$] and LM [F (2,21)= 7.419, p= 0.004]. Similarly, differences between groups were found in the VTA [F (2,21)= 137.529, $p<0.001$] and NBM [F (2,21)= 21.652, $p<0.001$]. Post hoc tests revealed that SAL group showed higher OD levels than CO and SC-SAL ($p<0.05$).

The *T*-tests performed to compare AChE OD levels between trained groups showed lower levels in TA group compared to SAL in most of the regions ($p<0.05$, Table 1).

COx histochemistry

Table 2 illustrates the results. Significant differences between CO, SC-TA and TA groups were found in the NACc [F (2,21)= 14.559, $p<0.001$], NACs [F (2,21)= 10.066, $p<0.001$] and AVT [F(2,21)= 14.384, $p<0.001$], where CO presented lower COx activity than SC-TA and TA groups ($p<0.05$). Similarly, COx activity was different between groups in the ADT [F (2,21)= 14.086, $p<0.001$], the DG [F (2,21)= 25.079, $p<0.001$], the CA3 subfield [F (2,21)= 9.176, p= 0.001] and the mammillary nuclei [MM: F (2,21)= 25.146, $p<0.001$ and LM: F (2,21)= 15.748, $p<0.001$]. TA group had higher COx activity compared to CO and SC-TA ($p<0.05$).

The comparisons of COx activity measures obtained from the SAL, SC-SAL and CO groups revealed differences between groups in the PLc [F (2,21)= 95.818, $p<0.001$], ILc [F (2,21)= 38.578, $p<0.001$] and DG [F (2,21)= 63.149, $p<0.001$]. In these regions, SAL group presented higher COx activity than SC-SAL and CO ($p<0.001$). Differences between groups were also found in the NACc [F (2,21)= 108.693, $p<0.001$], NACs [F (2,21)= 34.258, $p<0.001$], anterior thalamic nuclei [ADT: F (2,21)= 80.457, $p<0.001$; AVT F (2,21)= 112.733, $p<0.001$ and AMT: F (2,21)= 25.286, $p<0.001$] and mammillary nuclei [MM: F (2,21)= 30.867, $p<0.001$ and LM: F (2,21)= 31.296, $p<0.001$]. CO group showed lower COx activity than the rest of groups ($p<0.05$). Finally,

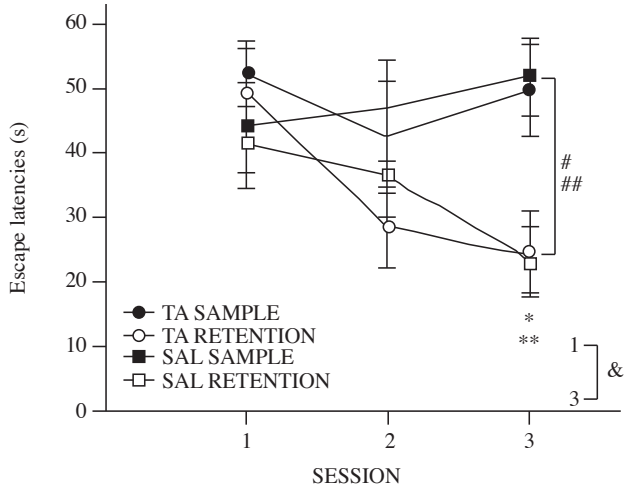

Figure 1. *Escape latencies of the TA and SAL groups in the WM task during the 3 training sessions. Data are expressed as the mean ± S.E.M. Significance of differences between the sample and retention trials within the third session in TA (#p= 0.014) and SAL (##p= 0.002), paired T-test. Significance of differences between retention trial (session 1) and retention trial (session 3) in TA (*p= 0.038) and SAL (**p= 0.018), paired T-test. Significant reduction of escape latency in the retention trial 3 compared to the retention trial 1: &p= 0.006, ANOVA (group × session (1-3), repeated measures: session) and Tukey's test*

Table 1
Comparisons between the studied groups in AChE optical density (OD) levels in each brain region. Mean±S.E.M.

	CO N= 8	SC-SAL n= 8	SAL n= 8	SC-TA n= 8	TA n= 8	TA vs SAL *t(14), p
PLc	5.09±0.43	5.24±0.24	4.72±0.38	5.18±0.43	4.96±0.43	0.42, n.s.
ILc	5.27±0.73	6.95±0.50	7.12±0.71	6.28±0.52	6.31±0.60	-0.88, n.s.
NACc	60.57±2.09	78.62±6.32	160.3±6.61 [a,d]	107.2±1.67 [a]	127.6±7.07 [a,g]	-3.38, .004
NACs	103.2±1.37	114.1±10.24	224.5±13.42 [a,d]	152.1±5.46 [a]	178.4±8.20 [a,g]	-2.93, .011
ADT	24.09±0.61	27.71±0.39 [b]	31.16±1.02 [a,e]	26.63±1.17	24.74±1.53	-3.49, .004
AVT	44.54±0.97	54.34±1.49 [b]	65.32±2.43 [a,d]	52.91±1.27 [b]	54.24±2.58 [b]	-3.13, .007
AMT	16.86±0.66	18.65±0.65	17.58±0.81	17.01±0.89	13.80±1.28	-2.49, .026
DG	7.26±0.53	6.56±0.37	8.41±0.83	6.48±0.43	5.58±0.50	-2.93, .011
CA3	7.55±0.28	7.33±0.45	8.64±0.74	7.77±0.30	6.42±0.55	-2.41, .030
CA1	4.38±0.28	5.65±0.34	5.28±0.61	4.99±0.72	3.55±0.32	-2.49, .026
MM	9.60±0.69	12.34±1.37	11.09±0.52	7.80±0.38	8.69±0.69	-2.77, .015
LM	18.40±1.80	20.18±1.04	25.93±1.39 [b,e]	23.62±1.46	24.18±1.50 [c]	-0.86, n.s.
VTA	29.33±0.75	37.46±1.76 [b]	66.97±2.21 [a,d]	27.59±2.69	56.08±2.11 [a,f]	-3.56, .003
NBM	113.1±6.59	139.9±6.89	184.0±9.32 [a,e]	110.7±12.03	111.1±11.48	-4.93, <.00

Note: CO= control cage. SC-SAL= yoked swimming control saline. SAL= saline. SC-TA= yoked swimming control tacrine. TA= tacrine.
[a] $p<.001$, [b] $p<.01$ and [c] $p<.05$ increased levels compared to CO; [d] $p<.001$ and [e] $p<.05$ increased levels compared to SC-SAL; [f] $p<.001$ and [g] $p<.05$ increased levels compared to SC-TA (One-way ANOVA, post hoc Tukey's test).
* Values of *T*-test to asses differences between trained groups (TA and SAL). n.s.= not significant

there were significant differences between groups in CA3 and CA1 [F (2,21)= 21.518, p<0.001 and F (2,21)= 7.896, p= 0.003, respectively], where SAL group showed higher COx activity only in relation to CO group (p<0.01).

The T-tests performed to compare COx activity between trained groups showed lower COx values in TA group compared to SAL in most of the regions (p<0.05, Table 2).

Discussion

Our behavioral results agree with other studies where similar escape latencies of tacrine and saline groups were found in the MWM, using the same dose and route of administration (Jackson & Soliman, 1996). In addition, other behavioral parameters were not different between groups (data not shown).

Decrease of the AChE OD levels in the tacrine group was found in most of the regions. Previously, an inhibitory effect of tacrine on AChE activity was reported after spatial training (Jackson & Soliman, 1996). However, these OD levels increased in the nucleus accumbens and VTA of trained animals, independently of the pharmacological manipulations. This could reflect a learning-related increase in brain ACh levels, as was found previously (Hata, Kumai, & Okaichi, 2007; Pych, Chang, Colon-Rivera, & Gold, 2005). In this regard, the injection of ACh receptor antagonists into these regions impaired learning in maze tasks (Levin et al., 1994; Schildein et al., 2000). The involvement of the VTA in the spatial WM was also found, revealing the relevance of this area in the regulation of the dopamine levels in the prefrontal cortex (Romanides et al., 1999).

In addition, the ADT, AVT, LM and NBM showed a learning-related increase in AChE OD levels after treatment with saline. The absence of AChE inhibition in SAL group could explain this result. The LM and anterior thalamic nuclei have been found to be involved in the spatial navigation (Stackman & Taube, 1998; Vann & Aggleton, 2003). Likewise, the NBM provides synaptic projections to the prefrontal cortex involved in spatial WM (Rispoli et al., 2008).

With regard to COx activity, the oxidative metabolism increased following learning in some regions showing different pattern of brain activation between groups. Overall, lower levels of COx activity were found in several regions of rats treated with TA in comparison with rats injected with saline. This could suggest that inhibition of AChE facilitates brain activity, reducing the levels of oxidative metabolism required to solve the task. In relation to this, it was shown that AChE inhibition reduces the activity of regions involved in a WM task in humans (Furey, Ricciardi, Schapiro, Rapoport, & Pietrini, 2008).

Learning-related changes in COx activity were found in the prefrontal cortex and DG of the SAL group, whereas the ADT, dorsal hippocampus and mammillary nuclei were activated in the TA group. An increase in COx activity was previously reported in the DG, ADT and mammillary nuclei of rats that performed the same task (Méndez, Méndez-López, López, Aller, Arias, & Arias, 2009; Méndez-López et al., 2009). In line with this, it was shown an involvement of the DG and CA3 in the acquisition of a spatial WM task with short retention interval (Lee & Kesner, 2003).

As regards COx activity changes found in the prefrontal cortex, the involvement of this region in the performance of similar MWM learning tasks was also shown using COx histochemistry (Méndez et al., 2009). However, it must be pointed out that no changes in COx activity were found in this region following learning in rats treated with tacrine. This indicates that the trained animals with normal levels of AChE activity show prefrontal cortex and DG activation, whereas AChE inhibition leads to learning-related activation of the hippocampus, mammillary nuclei and anterior thalamus. The role of cortical ACh has been related with spatial WM performance in tasks that require attentional processes (Sarter, Bruno, & Givens, 2003). In this study, the metabolic activation of cortical regions is showed in trained animals without pharmacological manipulations. However, ACh enhancement is not accompanied by an increase in energy metabolic costs of the prefrontal cortex during the performance of the task. This might suggest that increased ACh levels make it possible to solve the task without increasing cortical energy demands. Thus, the COx activity changes showed by TA group reflect a main contribution of the hippocampal extended system to the spatial WM.

Table 2
Comparisons between the studied groups in COx activity units (μmol cytochrome c oxidized/min g wet tissue at 23 ºC) in each brain region. Mean±S.E.M.

	CO n= 8	SC-SAL n= 8	SAL n= 8	SC-TA n= 8	TA n= 8	TA vs SAL *t(14), p
PLc	21.68±0.33	24.24±0.79	34.95±0.90 [a,d]	23.64±0.97	25.18±1.70	-5.07, <.00
ILc	20.69±0.29	23.74±0.92	31.64±1.24 [a,d]	24.89±0.63	24.11±2.02	-3.18, .007
NACc	23.79±0.50	34.72±0.80 [a]	37.00±0.70 [a]	30.42±0.50 [a]	28.82±1.40 [b]	-5.22, .001
NACs	29.28±0.80	36.85±0.33 [a]	40.25±1.42 [a]	34.45±0.41 [a]	32.85±1.13 [c]	-4.08, .001
ADT	31.91±0.85	44.96±0.87 [a]	48.33±1.15 [a]	38.13±0.58	45.16±2.88 [a,f]	-1.02, n.s.
AVT	25.58±0.47	35.71±0.75 [a]	37.91±0.60 [a]	30.12±0.32 [c]	32.24±1.44 [a]	-3.63, .003
AMT	20.29±0.62	24.51±0.68 [a]	26.75±0.65 [a]	19.51±0.80	20.35±1.23	-4.58, .001
DG	25.99±0.50	32.41±0.57 [a]	37.19±0.96 [a,d]	28.26±0.44	33.78±1.21 [a,e]	-2.20, .045
CA3	18.02±0.57	21.74±0.54 [b]	23.76±0.75 [a]	18.66±0.39	20.92±0.53 [b,f]	-3.10, .008
CA1	19.56±0.46	21.08±0.66	22.72±0.55 [b]	20.63±0.46	20.10±0.91	-2.47, .027
MM	27.64±1.10	35.35±0.52 [a]	38.82±1.31 [a]	28.23±0.82	38.51±1.61 [a,e]	-0.15, n.s.
LM	26.23±0.81	32.17±0.75 [a]	34.82±0.80 [a]	29.39±0.78	36.06±1.88 [a,f]	-0.61, n.s.
VTA	15.75±0.87	17.23±0.32	17.45±0.20	16.50±0.54	17.15±0.94	-0.32, n.s.
NBM	11.62±0.38	11.94±0.44	12.91±0.41	11.28±0.41	11.45±0.47	-2.34, .035

Note: CO= control cage. SC-SAL= yoked swimming control saline. SAL= saline. SC-TA= yoked swimming control tacrine. TA= tacrine.
[a] p<.001, [b] p<.005 and [c] p<.05 increased activity compared to CO; [d] p<.001 increased activity compared to SC-SAL; [e] p<.001 and [f] p<.05 increased activity compared to SC-TA (One-way ANOVA, post hoc Tukey's test).
* Values of T-test to asses differences between trained groups (TA and SAL). n.s.= not significant

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