

Low dose domoic acid influences spontaneous behavior in adult rats

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Running title: Domoic acid and rats behavior

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Abstract:

Domoic acid (DA) is a potent marine neurotoxin present in seafood. Intoxication by DA causes gastrointestinal symptoms like vomiting and diarrhoea and also the so-called amnesic shellfish poisoning (inflicting memory impairment and seizures). Since exposure to non-convulsive doses is relevant to human health, we investigated the effect of low dose DA administration in adult Wistar rats. Rats were administered with DA at the dose 1.0 mg/kg and their behavior was monitored for one hour in three sessions. The first session started immediately after DA administration. The second and third sessions started one and two weeks later. After the third session, the histochemical analysis of the hippocampi of the animals was conducted (Fluoro-Jade B, bis-benzimide). DA increased time spent by locomotion and distance travelled in the second half of the first session and this effect was pronounced during the second and third sessions. Exploratory rearing was decreased by DA administration in the first half of the first session. DA influenced grooming in a biphasic manner (decrease followed by an increase of time spent by grooming). This biphasic trend was observed even two weeks after the DA administration. Histochemistry of DA treated rats did not confirm the presence of apoptotic bodies, Fluoro-Jade B positive cells were not found neither in CA1 nor CA3 area of the hippocampi. Our study revealed that a low dose of DA affects short and long-term the spontaneous behaviour of rats without inducing neuronal damage.

Key words: domoic acid, rat's behavior, Fluoro-Jade B, hippocampus

Introduction:

Domoic acid (DA) is a cyclic tricarboxylic amino acid sharing many structural and functional similarities with kainic acid, an analogue to glutamic acid. In nature, DA is produced mostly by marine diatoms of the genus *Pseudonitzschia* (Perl *et al.* 1990). These algae, as consumed by shellfish and anchovies lead to final consumers that are endangered by DA intoxication: sea mammals, birds and, finally, humans. In late eighties of 20th century there were 250 reports of intoxications caused by consumption of mussels contaminated by DA followed by various gastrointestinal symptoms like vomiting, abdominal cramps, diarrhea and other symptoms like severe headache, coma, seizures and also memory deficit (Perl *et al.* 1990, Pulido *et al.* 2008, Kumar *et al.* 2009, Lefebvre *et al.* 2010, Todd 1993). DA was identified as the responsible neurotoxin and the name amnesic shellfish poisoning was given to the acute state of DA intoxication (Wright *et al.* 1989, Todd 1993, Jeffery *et al.* 2004).

DA binds with glutamate receptors in central nervous system. Prior research showed that DA prefers kainate receptors and forms a bond 3 – 20 times more powerful than kainic acid itself (Stewart *et al.* 1990). More recent studies confirm that DA also binds with 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptors (AMPA) (Larm *et al.* 1997) and indirect activation of N-methyl-D-aspartate receptors (NMDA) may occur at high DA concentrations. Over-stimulation of mentioned glutamate receptors causes intracellular accumulation of Ca^{2+} and leads to production of reactive oxygen species and eventually causes cell death (Coyle and Puttfarcken 1993, Costa *et al.* 2010). One of the most susceptible regions of CNS is hippocampal formation (Giordano *et al.* 2006), namely areas CA1 and CA3

(Cendes *et al.* 1995). DA, when administered to rats causes broad spectrum of behavioral patterns like scratching, tremor, seizures, wet dog shakes and influences the locomotor activity (Baron *et al.* 2013, Levin *et al.* 2005).

In this experiment we aimed to examine and quantify long-term effect of DA injection in subconvulsive dose. Secondly we tested the effect of DA on the morphological changes of hippocampal region using the methods of histochemistry. We hypothesized that a) locomotor activity of experimental animals will be increased by subconvulsive dose of DA and that this effect remains persistent over weeks b) hippocampal regions, namely areas CA1 and CA3 will not exhibit cells undergoing neuronal degeneration.

Materials and methods

Animals and experiment design

Twelve naive male Wistar albino rats were used in this experiment (six treated with DA, six were injected with corresponding volume of saline). The average weight of animal was 155 g (test day 1). All animals (breeding of the Institute of Physiology 1st Faculty of Medicine, Charles University in Prague) were housed in standard 12 h light/dark cycles (with lights on at 06:00 hr.) in temperature-controlled environment (22-23°C). All experiments took place between 08:00 and 15:00 in a room with lights on (light intensity between 150 and 200 lx at the level of cages). During the tests animals had no access to the food and water. Immediately after placing animals inside experimental room, they were randomly assigned into two experimental groups, weighted prior each session and marked. First group was treated intraperitoneally with 1mg/kg of DA (Sigma), dissolved in saline recalculated volume 1mg of DA per 1ml of saline. Second group was treated with saline (intraperitoneally)

in equal volume. Animals were then placed and tested in Laboras apparatus (Metris B.V., Netherlands) to monitor their behavior for one hour (session 1). Laboras™ is automated system for continuous behavior tracking and analysis. Mechanical vibrations generated by animal (locomotion, rearing etc.) are transformed into electrical signal. Such signals are processed, classified and compared with the predetermined characteristic patterns of Laboras software (Van de Weerd *et al.* 2001). During each session the animals were left undisturbed. After each session, animals were returned to their home cages and housed for another seven days (food and water ad libitum). After this period all animals were placed in Laboras apparatus again and their behavioral pattern was observed during one hour period (experimental day 8, session 2). One week later, the last session took place (experimental day 15, session 3). Day after the third session animals were transcardially perfused under deep thiopental anaesthesia, brains were removed from the skull and processed for histological examination. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee and are in agreement with the Czech Government Requirements and Requirements of European Communities Council Directive (86/609/EEC).

Apoptosis detection and neuronal degeneration analysis

Combination of two fluorescein dyes is used in our laboratory and was described elsewhere (Riljak *et al.* 2007, Riljak *et al.* 2010). Briefly: animals were perfused under deep thiopental anaesthesia with 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Brains were removed, postfixed for one hour in 4 % buffered paraformaldehyde and then submerged for 1 h into 20 % sucrose for cryoprotection. Each brain was sliced in the frontal plane into 40 µm thick sections with a cryostat at

-20 °C. Then two different histochemical methods were used: Fluoro-Jade B (Histo-Chem Inc.) which is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration and Hoechst 33342 (bis-benzimide, Sigma-Aldrich) staining was used as an apoptotic marker, which detects apoptotic nuclei with condensed and/or fragmented DNA. Tissue sections were then mounted onto gelatinized slides and allowed to dry at room temperature. Slides were then immersed in ethanol, distilled water and potassium permanganate (KMnO₄, Sigma-Aldrich). Staining proceeded in a dim place by immersing slides into 0.001% Fluoro-Jade B solution for 30 min with occasional gentle shaking, then immersed in 0.01% Hoechst staining solution for 10 min and dehydrated (in ethanol series), cover slipped using D.P.X. neutral mounting medium and allowed to dry. The tissue was examined using an epifluorescent microscope OLYMPUS AX 70 PROVIS with blue (450-490 nm) excitation light. Following regions in the hippocampal area were analyzed for possible signs of degeneration or apoptosis: CA1 area of the hippocampus, CA3 area of the hippocampus, the hilus of the dentate gyrus, the dorsal blade of the dentate gyrus and the ventral blade of the dentate gyrus.

Statistical analysis

Spontaneous behavior (three one hour sessions at experimental day 1, 8 and 15) were analyzed over ten minutes successive intervals (0-10min, 10-20min, 20-30 min, 30-40 min, 40-50min, 50-60min). Each measured behavioural parameter was analyzed separately. Data were subjected to non-parametric tests (because of non-Gaussian data distribution). To compare the differences between particular groups over ten minutes intervals, Kruskal-Wallis and Mann-Whitney test were used, if

$p < 0.05$, results were considered as significant. Micrographs were analyzed qualitatively by two experimentators, no quantitative analysis was made.

Results

All animals treated with DA (or saline) survived. Weight of control animals and animals treated with DA did not significantly differ (after one week: 215 g controls rats vs. 211 g DA rats; after two weeks: 259 g controls rats vs. 271 g DA rats). Results regarding particular behavioral category are listed below:

Immediate DA effects on animal's behavior

Horizontal activity – time spent by locomotion and distance travelled (Fig. 1)

Statistical analysis showed that the acute effect of DA-treatment became significant twenty minutes since the administration of DA (3rd 10 minutes interval). Locomotion was significantly increased in 3rd ($p < 0.05$), 4th ($p < 0.05$) and 5th ($p < 0.05$) time interval (Fig. 1). Average distance travelled by DA treated rats was shorter after DA injection (within the first ten minutes), but did not reach statistical significance. As rats' activity increased, DA treated rats travelled longer distance in 5th interval ($p < 0.05$).

Vertical activity – rearing (Fig. 2)

Exploratory rearing was decreased in first half of session, namely in 1st ($p < 0.05$) and 2nd ($p < 0.05$) time interval.

Grooming (Fig. 3)

In the first session, the DA had a biphasic effect on grooming behavior. In the first half of session, the grooming activity was lowered (significantly in 2nd time interval,

p<0.05) and increased in the second half of the session (significant difference in 4th time interval, p<0.05).

Delayed DA effect on animals behavior (experimental day 8 and 15)

Horizontal activity – time spent by locomotion and distance travelled (Fig. 1)

Analyzed data showed that the behavior of DA treated rats was affected even two weeks after the DA administration. In DA treated animals the both measured variables that are the time spent by locomotion, and travelled distance were increased in comparison with control animals. Specifically, we observed significant difference in 4th time interval, experimental day 15 in both mentioned variables. Decreased locomotion over the course of an hour-long session in both control and DA animals reflects habituation; this phenomenon was more expressed in control than in DA treated animals suggesting an impairment of habituation in DA treated animals.

Vertical activity- rearing (Fig. 2)

Time spent by rearing significantly differed in 4th (30-40 min, p<0.05). DA treated rats spent longer time by rearing mainly in second half of session (experimental day 8 and 15). Exploratory rearing was decreased in first half of both sessions, but such decrease did not reach the level of statistical significance.

Grooming (Fig. 3)

As for grooming, the biphasic effect persisted. In the first half of session, the grooming activity was lowered (significantly in 2nd time interval, p<0.05, test day 8)

and it increased in the second half of the session (significant difference in 4th time interval, $p < 0.05$, test days 8 and 15).

Apoptosis detection and analysis of neuronal degeneration (Fig. 4 and 5)

The hippocampi of the animals were analyzed two weeks after the DA administration. The pyramidal cell layer was unaffected and left intact. CA1 and CA3 areas of DA treated rats did not differ from controls. Amount of neuronal cells stained by fluorescein dye was minimal and cannot be (Fig. 5). As well as bis-benzimide staining did not confirm presence of apoptotic bodies (Fig. 4).

Discussion

The purpose of this study was to investigate the acute and long-lasting effects of low dose of DA on spontaneous behavior in rats. It was followed up during the time period of three weeks to reveal lasting effect of DA on the behavior of adult male rats. After that, rat brains were examined histologically to evaluate possible morphological changes.

Behavioral changes

Because rats are around 20 to 40 times less sensitive to DA administered orally than humans (due to their poor DA gastro-intestinal absorption and faster elimination), we decided to use intraperitoneal route of administration - the toxicity of DA via this route of administration is more than thirty times higher (Iverson *et al.* 1989), so i.p. administration should (at least partially) mimic oral administration of DA in humans. Second important aspect regarding the DA was its dosage – we tested the long-lasting effect of subconvulsive dose (not introducing partial seizures or status epilepticus) on spontaneous behavior of rats (Tryphonas *et al.* 1990).

Immediate DA effects

Saline treated (and as well naïve) rats placed in Laboras chamber are very active during the first half hour (novel environment). After this period they tend to decrease their locomotor activity and are commonly immobile until the end of session (habituation phenomenon). The DA animals immediately after the DA injection traveled the same distance as control animals during the first twenty minutes. The DA animals spent less time by rearing and grooming in that interval. Similar observation was made by Baron and coworkers (Baron *et al.* 2013). Interestingly, we described the very same pattern of behavior after administration of another stimulatory drug – nicotine (Jandová *et al.*, 2013). Therefore, it is possible, that decreased tendency to explore was not necessarily caused by DA but by e.g. gastrointestinal distress. As that decreased exploration disappeared, the DA treated rats became more active, groomed more, spent more time by locomotion and travelled longer distance. This pattern reflects very probably the absorption and distribution of DA. Baron *et al.* explained the biphasic pattern of grooming and locomotor activity (decrease followed by an increase in that type of behavior) by activation of kainic acid receptors in gastrointestinal tract (Baron *et al.* 2013, Kirchgessner *et al.* 1997). Another possible explanation of increased locomotor activity in the last twenty minutes of observation could be possible anxiolytic-like effect of DA. Such effect was described for kainic acid in the past (Mikulecká *et al.* 1999) and because these two compounds act on the same receptors, the DA could influence the level of anxiety (e.g. in novel environment) in the similar way. We observed that the DA influenced the process of habituation – while the rats treated with saline were resting and immobile, the DA treated rats were still in motion and

exploring. Moreover, this altered process of habituation could be observed even two weeks later in DA rats.

Long term DA effects

In addition to the first observation session evaluated immediately after the DA administration, the animals were observed also on 8th and 15th day after DA administration. After placing animals in Laboras chamber, saline treated animals became inactive faster compared to first session, and in forty minutes their locomotor and exploratory activity was nearly zero. DA treated rats exhibited certain level of locomotion during the whole session, and such activity was statistically higher at least in some time intervals (see figure). Most interesting finding was that grooming pattern preserved its biphasic shape. The grooming activity in DA rats was lowered after placing in Laboras chamber – then increased, and peaked after forty minutes. Grooming behavior is believed to be influenced strongly by emotional stress such as placing animals into new environment (some parameters regarding the grooming are useful for evaluation and scoring of anxious behavior in rats; van Erp et al., 1994, Estanislau et al., 2013). Intensity of grooming in DA treated rats increased in second half of open field tests – it can indicate, that the process of deactivation from stress caused by novelty is shifted in time if compared with control. It is possible, that the decreased vertical activity immediately after the DA administration and the increased grooming behavior in the second half of observation period support the hypothesis that DA could influence the anxiety level by activation of kainate receptors via their anxiolytic effect – and that effect could be also persistent in time. Proposed hypothesis should be confirmed in future experiments by longer behavioral tests and by autoradiographic study evaluating possible changes in density of glutamate receptors.

Histochemical correlations

Hippocampal regions CA1 and CA3 are very sensitive to DA administration (Cendes *et al.* 1995). Areas CA1 and CA3 of hippocampus were examined to evaluate histochemical changes using Fluoro-Jade B dye and stained by bis-benzimide. No significant neuronal damage was observed, Fluoro-Jade B dye did not reveal degenerating neurons in any area of the hippocampus. Possibly, convulsive doses would be needed to trigger cell death. This finding is in line with experiment of Kubova and coworkers, where subconvulsive doses of another excitotoxine – kainic acid – did not lead to the morphological damage of hippocampal area (Kubova *et al.* 2001).

To conclude, subconvulsive dose of DA affects the behavioral pattern of adult rats, influencing the process of habituation. Such effects are observable weeks after the DA administration. DA influences grooming pattern and such change is also persistent in time. Analysis with Fluoro-Jade B dye did not reveal degenerating neurons neither in CA1 nor CA3 area of the hippocampus.

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Figure legends

Figure 1

DA-induced effects on horizontal activity: locomotion (duration), and distance traveled. Whole one hour session divided into six 10-minutes intervals. Particular graphs represent 1st, 8th and 15th experimental day. Red lines represent DA treated rats, black lines saline treated rats. * Results significant at $p < 0.05$, error bars were calculated as \pm SEM.

Figure 2

DA-induced effects on rearing (vertical activity). Whole one hour session divided into six 10-minutes intervals. Particular graphs represent 1st, 8th and 15th experimental day. Red lines represent DA treated rats, black lines saline treated rats. * Results significant at $p < 0.05$, error bars were calculated as \pm SEM.

Figure 3

DA-induced effects on grooming behavior. Whole one hour session divided into six 10-minutes intervals. Particular graphs represent 1st, 8th and 15th experimental day. Red lines represent DA treated rats, black lines saline treated rats. * Results significant at $p < 0.05$, error bars were calculated as \pm SEM.

Figure 4

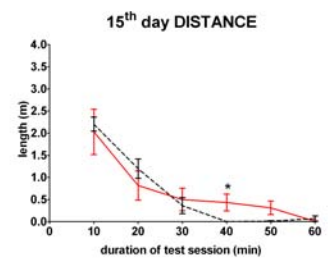
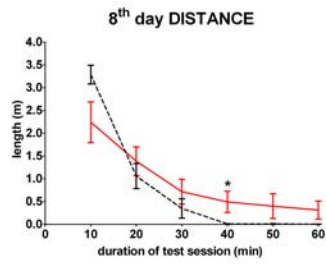
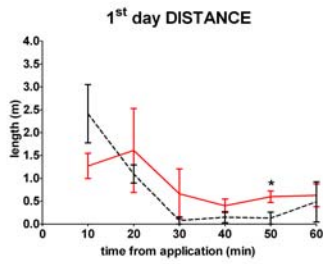
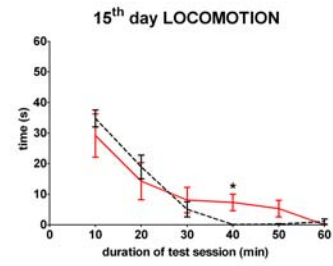
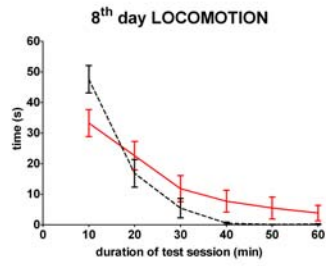
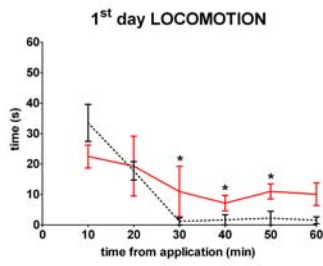
CA1 area of the hippocampus. Bis-benzimide, Hoechst 33342 staining. DA treated rat (1 mg/kg). Direct magnification 100x. Scale bar in the right corner represents 200 μm .

Figure 5

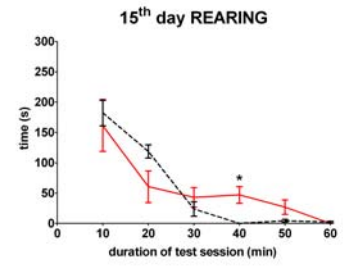
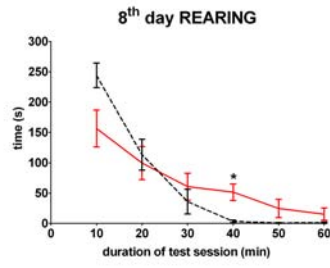
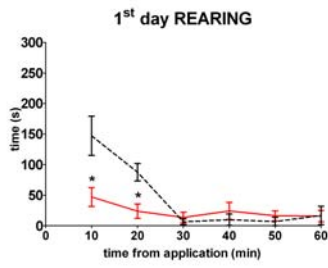
CA1 area of the hippocampus. Fluoro-Jade B staining. DA treated rat (1 mg/kg).

Direct magnification 100x. Scale bar in the right corner represents 200 μm .

Horizontal activity



Vertical activity



Grooming

