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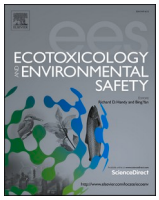

Exposure to phthalate plasticizer compromises normal brain function in an adult vertebrate

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ABSTRACT

Phthalates are key additives in many plastic products and among the most frequently used plasticizers. The release of some of them into the environment has been shown to have serious effects on development and reproduction. Based on such effects, diisononyl phthalate (DINP) has been advocated as a safer alternative to di-2-ethylhexyl phthalate (DEHP). Recently, it has been suggested that DEHP may affect the vertebrate blood-brain barrier. This could have serious consequences not only for the developing, but also for the adult brain. Here we tested for such impact on neuronal function and demonstrate acute exposure effects of both plasticizers on fundamental aspects of brain function in an adult vertebrate. We used the Mauthner neuron in the hindbrain of fish and its diverse inputs from various sensory systems as a model. After exposing intact goldfish to environmentally relevant plasticizer concentration (either 100 μg L^{-1}, or 10 μg L^{-1}), we show from in vivo intracellular recording that one month of environmental exposure to DEHP or DINP affected the sensory input to this central neuron, offset the balance between excitation and inhibition, and reduced its conduction speed by 20 %. The effects of both plasticizers were strong even at the concentration of 10 μg L^{-1}. In an adult vertebrate, our findings thus demonstrate a previously neglected high sensitivity of various crucial brain functions to the acute exposure to phthalates.

1. Introduction

Plasticizers are used in many products of our daily lives (Tickner et al., 2001; Rahman and Brazel, 2004; Heudorf et al., 2007; Akovali, 2012; Ji et al., 2014; Lee and Choi, 2024). They are added, for instance, to polymers to make them flexible and can account for up to 50 % of the weight of the final product (Tickner et al., 2001; Rahman and Brazel, 2004; Walters et al., 2020). Typically, plasticizers are not chemically bound to the polymer, but can leak out over time and thus can affect humans and other organisms.

One of the most commonly used plasticizers in polymeric (e.g., polyvinyl chloride (PVC)) and non-polymeric materials (e.g., paints, varnishes, cosmetics) is di-2-ethylhexyl phthalate (DEHP) (Fig. 1A). DEHP has been so widely used as an industry-standard general-purpose plasticizer (Koo and Lee, 2004; Walters et al., 2020) that it can now easily be detected in the environment as well as in the human body (Gao and Wen, 2016; Bu et al., 2020; Eales et al., 2022). Unfortunately, DEHP is an endocrine disrupting chemical with detrimental effects on developing vertebrates (Latini et al., 2010; Ejaredar et al., 2015; Palanza et al., 2016; Radke et al., 2020; Xu et al., 2020; You and Song, 2021; Ong et al., 2022; Kang et al., 2023). Several countries have therefore restricted the use of DEHP (Gao and Wen, 2016; Bu et al., 2020) and alternative plasticizers have gained importance. Among these a major cost-effective alternative is the widespread diisononyl phthalate (DINP) (Fig. 1A), which is, like DEHP, a high molecular weight ortho-phthalate (Walters et al., 2020). Recently, it has been suggested that DEHP might affect the vertebrate blood-brain barrier (Ahmadpour et al., 2021; Ren et al., 2023), which could have serious consequences also in the mature human brain (Fig. 1B). Here we demonstrate that not only DEHP but also its substitute DINP exerts a direct, acute effect on brain function in an adult vertebrate. We exposed adult goldfish for a comparatively brief period of four weeks to environmentally relevant concentrations (Bergé et al., 2013; Ji et al., 2014; Gao and Wen, 2016; Bu et al., 2020) of either DEHP or DINP (Fig. 1C). The fish were not fed with the phthalate. We either added 10 μg L^{-1} or 100 μg L^{-1} of the respective phthalate (either DEHP or DINP) to the surrounding water. These concentrations both are

Abbreviations: 2-PE, 2-phenoxyethanol; BBB, blood-brain barrier; DEHP, di-2-ethylhexyl phthalate; DINP, diisononyl phthalate; DMSO, dimethyl sulfoxide; MN, Mauthner neuron; PSP, postsynaptic potential.

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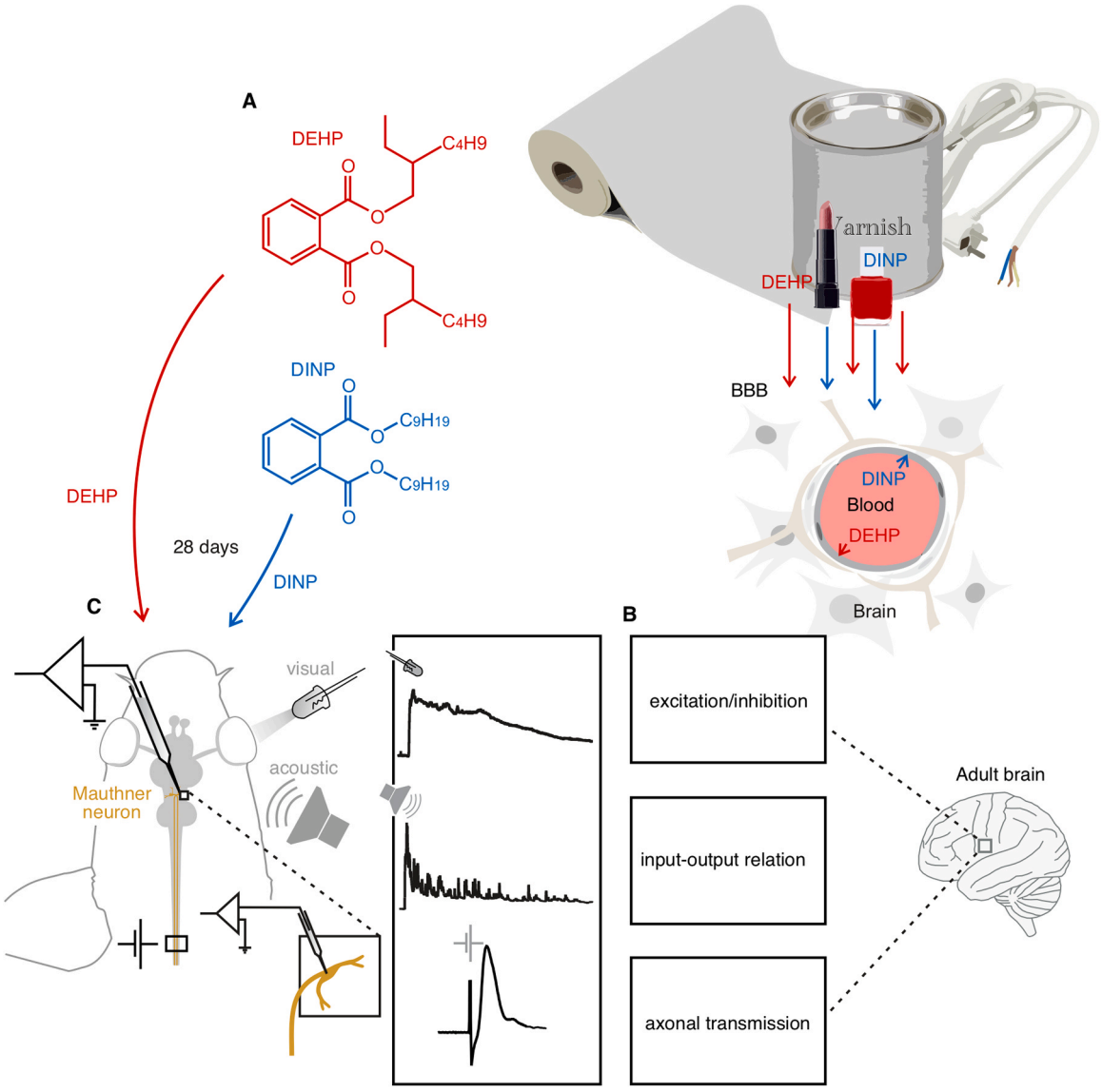


Fig. 1. Studying the in vivo effect of phthalate exposure on neural functionality in the mature CNS of a vertebrate. A High molecular weight ortho-phthalates like DEHP and DINP are additives in many products we get in touch with every day. They are not covalently bound to the final product and therefore leak into the environment. DEHP and maybe other phthalates are assumed to affect the integrity of the blood-brain barrier (BBB), but (B) whether phthalates affect neuronal functionality in the CNS of adult vertebrates is poorly understood. C We exposed adult goldfish for four weeks to environmentally relevant concentrations of either DEHP or DINP. Then we used in vivo intracellular recording in the uniquely suited Mauthner neuron to analyze effects on synaptic transmission, action potential generation and transmission, and on the central processing of sensory information (here acoustic and visual information).

well in the range of concentrations that can be found in our environment. In their review, Bergé et al. (2013) report DEHP concentrations in rainwater up to 39 μg L^{-1}, in surface water up to 64 μg L^{-1}, and in residential wastewater up to 161 μg L^{-1}. Gao and Wen (2016) report DEHP concentrations in freshwater up to 197 μg L^{-1}. Humans in addition take up phthalates from their indoor environment via dust and contact to surfaces and via food (Ji et al., 2014; Bu et al., 2020). In our approach, we used a system that allows a quick and sensitive in vivo assay of the effect of chemicals on diverse aspects of brain function (Machnik et al., 2018a, 2023; Schirmer et al., 2021). By recording intracellularly from the large Mauthner neuron (MN) in the hindbrain of fish, the natural design of this neuron and its connectivity to various sensory centers make it possible to characterize the effects of chemical exposure on action potential generation and propagation, excitatory and inhibitory chemical synaptic transmission and electrical transmission as well as on sensory input into the central nervous system (CNS) (Fig. 1B, C).

Our findings are highly alarming. They demonstrate an acute and unbuffered high sensitivity of an adult vertebrate brain to acute phthalate exposure at low concentration, effects that include strong reductions in axonal conduction speed and changes in the balance between excitatory and inhibitory synaptic inputs.

2. Materials and methods

2.1. Animals and treatment

Experiments were performed in N = 60 goldfish (*Carassius auratus*, Cypriniformes). At the time of the experiments, the fish were at least two years old and had a standard length of 73.8 ± 0.7 mm (range from 63.7 to 90.4 mm) and a weight of 11.3 g (range from 7.5 to 19.8 g). They were taken from a pool of 120 fish obtained from a specialist retailer (Aquarium Glaser GmbH, Germany). In the lab, they had been kept for at least 20 weeks prior to the experiments in glass tanks (250 × 50 × 50 (cm); volume of water: 600 L) in groups of up to 20 individuals per tank.

The tanks were filled with fresh water (temperature: 20 ± 1 °C; conductivity: 0.3 mS cm^{-1}; pH 7.5; $CaCO_3$: 137 mg L^{-1}; NH_3-N < 8 μg L^{-1}; NO_2^- < 5 μg L^{-1}; NO_3^- < 5 mg L^{-1}). Movement and aeration of the water was provided constantly (dissolved oxygen conc.: 9 mg L^{-1}) via motorized filters placed inside the tank. Water changes (30 %) were made once a week and water values checked at least twice a week. The fish were fed with common fish food (sera goldy; sera GmbH, Germany) and thawed mosquito larvae twice daily. The light/dark photoperiod was 12:12 h. Before dividing the fish into experimental groups, they were checked for disorders and responsiveness to visual and acoustic stimuli as used during experiments. We only chose healthy and responsive fish and randomly divided them for the experiments into five groups of N = 12 fish each. One of the groups served as a control. This group was exposed to 0.001 % dimethyl sulfoxide (DMSO) for 28 days, but not to plasticizer. The other groups were exposed to the same concentration of DMSO (used here as vehicle) and nominal concentrations (Bergé et al., 2013; Ji et al., 2014; Gao and Wen, 2016; Bu et al., 2020) of DEHP (di-2-ethylhexyl phthalate; CAS no.: 117–81–7; TCI Chemicals, Germany; l(ow): 10 μg L^{-1} or h(igh): 100 μg L^{-1}), or DINP (diisononyl phthalate; CAS no.: 28553–12–0; l(ow): 10 μg L^{-1} or h(igh): 100 μg L^{-1}) for 28 days. No water changes were carried out after adding DMSO/-plasticizer, but, apart from that, the keeping conditions did not differ from those described above. After the exposure period, the fish were anesthetized and placed in the electrophysiological recording chamber. We then determined the effects of plasticizer exposure on neuronal function and processing of sensory information from in vivo intracellular recording in the MN. We were able to collect robust data in N = 12 fish of the control group (male/female ratio = 5/7), N = 11 fish of the DEHP(h)-group (4/7), N = 12 fish of the DEHP(l)-group (5/7), N = 12 fish of the DINP(h)-group (5/7), and N = 11 fish of the DINP(l)-group (5/6). Two fish of the N = 60 fish (one of the DEHP(h)-group and one of the DINP(l)-group) died during the exposure period. After completion of the measurements, the experimental fish were euthanized. The post-mortem examination of the gonads revealed that all of them were sexually mature. Since sex could be a factor in the way the added phthalate affects the fish, we determined the sex, and tested for differences in outcome between males and females of the same exposure group. However, since there were no significant differences between males and females in any of the tested parameters (Mann-Whitney test or unpaired t test, as required; the lowest P-value was P = 0.0761(unpaired t test (t = 1.978, df = 10) for the delay of the acoustically induced postsynaptic potential in the DINP(h)-group), we concluded similar impact of phthalate exposure here on both sexes, and, therefore, decided that it is justified to pool the sexes for our analyses. All fish were examined post-mortem for conspicuous changes in their internal organs. But none were found in any of the fish. Animal care, surgical procedures and experiments were in accordance with all relevant guidelines of the German Animal Welfare Act and explicitly approved by the Council of State.

2.2. Anesthesia

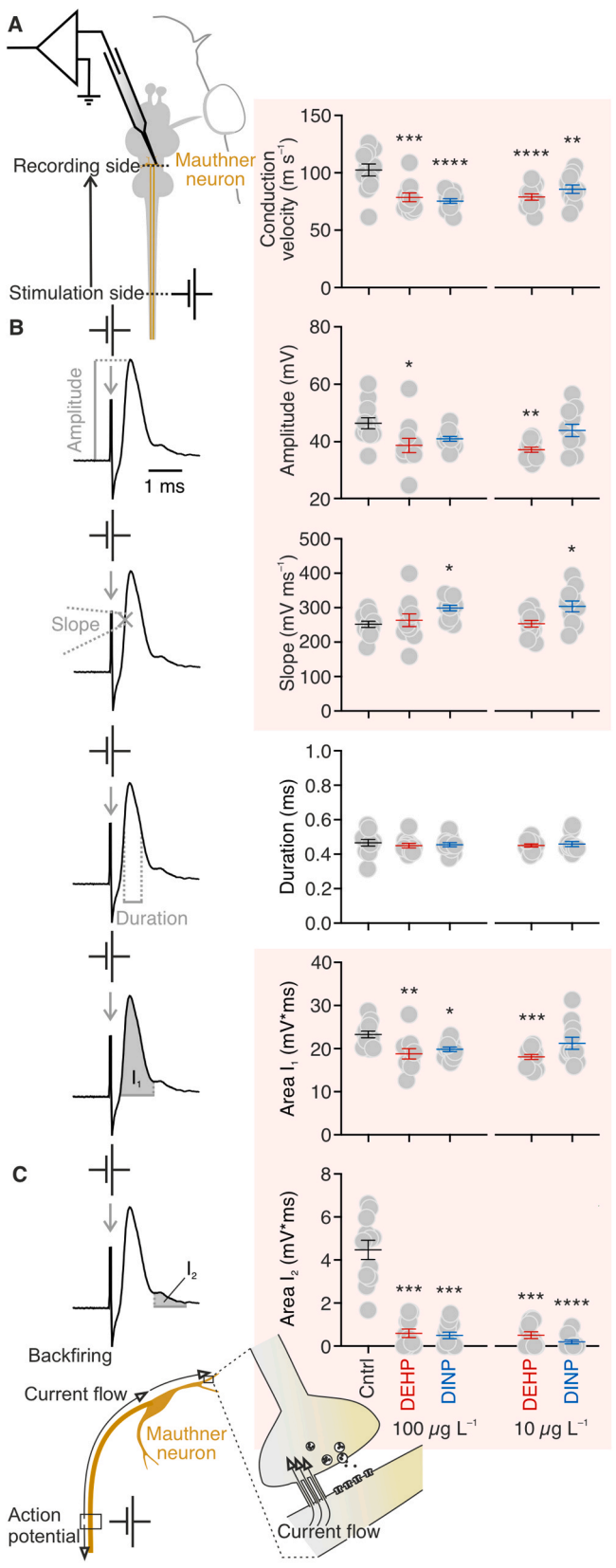
Surgical intervention is required to access the MN for in vivo intracellular recording. This cannot be done without anesthetizing the experimental fish. Since it effectively reduces stress, nociception and the uptake of tactile information, but not the perception and processing of light and acoustic stimuli or the function of central neurons (Machnik et al., 2018a), we used 2-phenoxyethanol (2-PE; 1-hydroxy-2-phenoxy-ethane; CAS no.: 122–99–6; Sigma-Aldrich, Germany; 400 μL L^{-1}) for anesthesia. Prior to surgery, the fish was placed for 15 min in a small tank containing water of its home tank and the 2-PE. Within 10 min, all fish lost equilibrium and responsiveness to touch and handling. Then they were placed in the electrophysiological recording chamber and artificial respiration was established with aerated water via a tube in the fish's mouth and out over the gills at a flow rate of 80 mL min^{-1}. Water of the same quality as in the keeping tanks was used, but during MN recording the temperature was not allowed to vary by more than ± 0.1 °C. The respiration water was delivered to the fish from a tank using a suitably adjusted pump (EHEIM universal 300; EHEIM GmbH & Co. KG, Germany; regular power: 300 L h^{-1}, adjusted to 4.8 L h^{-1}). It also contained the concentration of 400 μL 2-PE per liter respiration water to maintain anesthesia during the surgical procedures and subsequent MN recording.

2.3. Surgery

The MN cell bodies are localized in the hindbrain in the medulla oblongata (Furshpan and Furukawa, 1962; Zottoli, 1978). Their axons cross the midline in the medulla and run down the entire spinal cord to its end. To access the MNs, we opened the skull from above from optic tectum to vagal lobe. The cerebellum was lifted cranially to expose the medulla. Parts of the meninges covering the medulla were removed. To be able to apply electrical pulses to the spinal cord for eliciting action potentials in the MN axons, we additionally exposed a piece of the spinal column (about 5 mm long) from the side in the region of the trunk (22–24 mm caudal to the position of the MN cell bodies; the exact distance was determined after completing MN recording) and positioned a stimulation electrode. To be able to record intracellularly from the MN, it is necessary to prevent the activation of skeletal muscles by MN activation. We did so by injecting d-tubocurarine (CAS no.: 6989–98–6; Sigma-Aldrich; 1 μg g^{-1} body weight) into the core muscles.

2.4. Experimental procedure

We used established techniques to localize and to identify the MN in the medulla (Furshpan and Furukawa, 1962; Machnik et al., 2018b). For recording, we used a bridge-mode amplifier (BA-01X; npi electronic GmbH, Germany) in current clamp-mode. Recording electrodes were pulled from 3 mm-glass capillaries (G-3; Narishige Scientific Instrument Lab, Japan) using a vertical electrode puller (PE-22; Narishige International Limited, UK). To obtain sharp electrodes, the electrode tips were broken. Filled with 5 mol L^{-1} potassium acetate, the electrodes had a resistance between 4 and 7 MΩ. To position the recording electrode, we used a motorized micromanipulator (MP-285; Sutter Instrument, USA) and techniques to determine the recording position in the MN from extracellular space (Furshpan and Furukawa, 1962; Machnik et al., 2018c). Recordings were always taken in the MN soma. The reference electrode was positioned stationary in muscle tissue. Recordings were filtered (Hum Bug Noise Eliminator; Quest Scientific, Canada) and digitized using an analogue-to-digital converter (Micro1401; Cambridge Electronic Design Ltd., UK; sampling rate: 50 kHz) and the acquisition software package Spike2 (version 6; Cambridge Electronic Design Limited, UK). For data analysis, we used custom software written in Python. After positioning the recording electrode in the MN soma, we gave a period of 10 min, in which we did not apply any stimuli. After this period, we applied a set of stereotyped stimuli to elicit MN responses. The set contained repeated antidromic activation of the MN, and repeated acoustic and visual stimulation of the fish. Each of the stereotyped stimuli was consecutively presented to the fish at least 40 times. In total, set presentation took about 10 minutes. To ensure stable recording conditions during this period, we continuously monitored the resting potential of the MN. The termination criterion was set to 5 % deviation of the resting potential. In all cases, however, the deviation was well below this value and the data from all the experimental fish could be included in the analyses. The electrical pulses (pulse duration: 10 μs; stimulation rate: 2 Hz) applied to the spinal cord to elicit action potentials in the MN axons were delivered by a constant-voltage isolated stimulator (DS2A2–Mk.II; Digitimer Ltd., UK). The desired pulse amplitude for the antidromic stimulation was determined by first reducing the amplitude until antidromic stimulation no longer activated the MN. The amplitude was then increased by 5 V above threshold. In the current study, the pulse amplitude for antidromic stimulation ranged



(caption on next column)

Fig. 2. Both DEHP and DINP affect central neurons. A The sketch indicates the experimental setting for eliciting action potentials in the MN axon during recording from the MN soma in the medulla. From that and delay between stimulation in the area of the trunk and detecting the elicited action potential in the MN soma, we determined conduction velocity: In high and in low concentration both plasticizers similarly reduced it. B DEHP in any concentration reduced amplitude and I_1, which is the time-integrated action potential for the first millisecond after action potential onset. DINP in any concentration increased the slope of the rising edge of the action potential, and reduced I_1 in high concentration. Neither DEHP nor DINP affected the duration of the action potential. C Changes in the amplitude of I_2 (time-integrated area in the second millisecond after action potential onset), which is due to backfiring (see text), indicate an impact of phthalate exposure on synaptic transmission. Both phthalates massively decreased I_2. Measurements were taken as indicated. Values are given as mean ± standard error of mean. For the number of independent animal samples tested (N) and the numbers of measurements per animal (n) see Table 2. Values affected by exposure to either DEHP or DINP in any concentration are highlighted by light red background. Significant differences between a certain exposed group and the control are indicated by asterisk(s); one, two, three, four asterisks indicate $P < 0.05$, $P \leq 0.01$, $P \leq 0.001$, or $P \leq 0.0001$, respectively.

from 12 to 30 V. Acoustic stimuli were delivered by a loudspeaker (The box pro Achat 115 MA; Thomann GmbH, Germany), which generated an acoustic pulse (duration: 1 ms; frequency distribution from 25 to 1000 Hz; peak amplitude at 300 Hz) with a sound pressure level (SPL) of 145 dB re 1 µPa. We measured the SPL under water at the position of the fish in the recording chamber with a hydrophone (Type 8106; Brüel & Kjær, Denmark). For visual stimulation, we used a light-emitting diode (LED; RS Components GmbH, Germany), which was positioned directly in front of the ipsilateral eye. The light flash used for visual stimulation had a duration of 7 ms. The LED peak radiation at 569 nm was 700 µW m^{-2} nm^{-1} and the width at 100 µW m^{-2} nm^{-1} was 56 nm (range: 543–599 nm).

2.5. Statistical analysis

Statistical tests were run in the software Prism 8 (version 8.4.3 (471); GraphPad Software, USA) and performed two-tailed with $\alpha = 0.05$. Averages are reported as mean ± standard error of mean. N denotes the number of independent animal samples, n the number of measurements per animal. When data of animals were pooled, we never used the measurement repetition n taken from the individual animals, but a single averaged value for each animal. To test whether the data were distributed normally (Gaussian), we used the Shapiro-Wilk test. When data were normally distributed, we used a parametric test design, otherwise we used a non-parametric test design. To test for differences between the control group and the exposed groups, we either used a one-way ANOVA and in the case of significant differences the Dunnett's (multiple comparisons) test as post-hoc test against control, or a Kruskal-Wallis test and in the case of significant differences the Dunn's (multiple comparisons) test as post-hoc test against control. To test for significant differences between groups exposed to the same plasticizer at different concentration, we used either the unpaired t test or the Mann-Whitney test.

3. Results

3.1. Effects on shape and propagation of the action potential

The MN cell bodies are located in the medulla and the axons of the MNs extend from the hindbrain to the end of the spinal cord (Zottoli, 1978). Electrical pulses applied to the spinal cord at a fixed distance from the soma can easily be used not only to induce action potentials in the MN (Furshpan and Furukawa, 1962), but also to study their propagation speed from the stimulation site to the soma (Fig. 2A). Potential effects of phthalates on the amplitude, shape and propagation of the

Table 1
Spectrum of significant effects on neuronal function after exposure to plasticizer (DEHP or DINP) at any of the examined concentrations (10 μg L^{-1} and 100 μg L^{-1}).

	Test	F	R^2	P	DEHP	DINP
Action potential						
Conduction speed	One-way ANOVA	9.125	0.4078	**< 0.0001**	↓	↓
Amplitude	One-way ANOVA	4.751	0.2639	**0.0024**	↓	
Slope	One-way ANOVA	4.077	0.2353	**0.0059**		↑
Duration	Kruskal-Wallis			0.6520		
Area I_1	One-way ANOVA	5.079	0.2771	**0.0015**	↓	↓
Area I_2	Kruskal-Wallis			**< 0.0001**	↓	↓
Acoustic PSP						
Delay	Kruskal-Wallis			0.2517		
Amplitude	Kruskal-Wallis			**0.0135**	↓	
Slope	One-way ANOVA	3.186	0.1938	**0.0203**	↓	
Duration	Kruskal-Wallis			0.7009		
Area	One-way ANOVA	2.209	0.1429	0.0804		
Visual PSP						
Delay	Kruskal-Wallis			0.6539		
Amplitude	Kruskal-Wallis			**0.0033**	↓	↓
Slope	Kruskal-Wallis			**< 0.0001**	↓	↓
Duration	Kruskal-Wallis			0.7848		
Area	Kruskal-Wallis			**0.0181**	↓	↓

Based on data shown in Figs. 2 and 3. Significant differences are highlighted in bold. ↓ indicates a significant decrease in comparison to control, and ↑ a significant increase; free fields represent values that have not changed in comparison to control.

action potential can in addition be studied in great detail (Fig. 2B). Using these measurements, we discovered that acute exposure to DEHP had multiple and strong effects on action potential generation and on axonal transmission. One of the most remarkable effects (not previously seen with any other agent (Machnik et al., 2018a, 2023; Schirmer et al., 2021)) was that DEHP at both the low (l: 10 μg L^{-1}) and the high (h: 100 μg L^{-1}) concentration used caused a significant and substantial decrease in axonal conduction speed from 100 m s^{-1} to less than 80 m s^{-1} (Fig. 2A; Tables 1, 2; Dunnett's test: Control vs. DEHP(h): $P = 0.0001$, mean diff.: 23.84; Control vs. DEHP(l): $P < 0.0001$, mean diff.: 23.50). Additionally, it reduced the amplitude of the action potential (Fig. 2B; Tables 1, 2; Dunnett's test: Control vs. DEHP(h): $P = 0.0102$, mean diff.: 7.73; Control vs. DEHP(l): $P = 0.0014$, mean diff.: 9.20), and the area I_1 of the time-integrated action potential for the first ms after action potential onset (Dunnett's test: Control vs. DEHP(h): $P = 0.0048$, mean diff.: 4.49; Control vs. DEHP(l): $P = 0.0007$, mean diff.: 5.22). I_1, which is a good measure for the time course of the action potential, was reduced by approximately 20 %. Surprisingly, effects did statistically not differ between the group that was exposed to the lower concentration of DEHP and the group that was exposed to a 10-fold higher concentration (unpaired t test: $P \geq 0.5693$, $t \leq 0.5782$, $df = 21$), indicating that the effects of DEHP exposure were already saturated at the lower dose.

Similar to DEHP, exposure to the high concentration (h: 100 μg L^{-1}) of DINP also reduced the conduction speed in the MN axon by more than 20 % (Fig. 2A; Tables 1, 2; Dunnett's test: $P < 0.0001$, mean diff.: 27.07). Also the time integral I_1 was affected in a similar way (Fig. 2B; Tables 1, 2; Dunnett's test: $P = 0.0342$, mean diff.: 3.46). Different from DEHP exposure, DINP(h) did not affect the amplitude of the MN action potential (Dunnett's test: $P = 0.0938$, mean diff.: 5.42), and increased the slope of its rise (Dunnett's test: $P = 0.0302$, mean diff.: –47.26) – an effect not detected after DEHP exposure (Dunnett's test: $P \geq 0.9117$, mean diff.: ≤ –11.69). At the lower DINP concentration (l: 10 μg L^{-1}) conduction speed was also strongly decreased (>15 %; Fig. 2B; Tables 1, 2; Dunnett's test: $P = 0.0079$, mean diff.: 16.71), and the slope of the action potential increased (Dunnett's test: $P = 0.0175$, mean diff.: –52.05). But no significant effect of DINP(l) could be seen on the amplitude and time integral I_1 of the action potential (Fig. 2B; Tables 1, 2; Dunnett's test: $P \geq 0.3448$).

In summary, the changes we found in the action potential amplitude, the area of the time-integrated action potential I_1 and its slope indicate that both DEHP and DINP exposure have effects on the membrane properties of central neurons and/or on the properties of their voltage-gated ion channels. The changes in conduction speed in addition suggest effects on the myelin sheath of axons.

3.2. Effects on synaptic transmission

The MN receives a wide range of synaptic inputs, including inputs from so-called mixed synapses, in which electrical synapses are immediately adjacent to chemical synapses (Pereda et al., 1995; Cachope et al., 2007; Pereda, 2014). This arrangement allows postsynaptic depolarization to spread to the presynaptic sites and thus to cause further transmitter release (Fig. 2C). For example, an action potential in the soma can spread electrically to the presynaptic site of a mixed synapse and cause transmitter release, causing a postsynaptic potential with a delay of approximately 1 ms after the action potential (Pereda et al., 1995) (delayed potential I_2; Fig. 2C). This so-called backfiring of the action potential can be used to determine the effects of chemicals on synaptic transmission by electrical and chemical synapses (Cachope et al., 2007; Schirmer et al., 2021). This allowed us to demonstrate strong effects of both DEHP and DINP on synaptic transmission. While the time-integrated area I_2, a measure for the delayed potential, was 4.47 ± 0.45 mV*ms in control fish ($N = 12$), it was reduced to less than 15 % in fish exposed to plasticizer (Fig. 2C, Tables 1, 2; Dunn's test: $P \leq 0.0005$, mean rank diff.: ≥ 26.68). This exposure effect on synaptic transmission was not statistically different between DEHP and DINP exposed groups, nor was it statistically different between the groups exposed to the low and that exposed to the high plasticizer concentration (Kruskal-Wallis test: $P = 0.3840$).

3.3. Effects on acoustic inputs

Acoustic pulses cause long-lasting complex postsynaptic potentials (PSPs) in the MN. Changes in properties of the PSP after exposure to a chemical reveal an effect of the chemical anywhere in the chain between transduction, processing or transmission of acoustic information (Fig. 3A). An example of an acoustically induced PSP in the MN of a control fish is shown in Fig. 3B. PSPs occurred between 7.2 and 9.6 ms after the onset of the acoustic pulse (Fig. 3C; Table 2). PSP amplitude was between 4.1 and 16.8 mV and PSP duration (measured at 50 % peak amplitude) up to 77.5 ms. DEHP, but not DINP showed significant effects on the processing chain that underlies an acoustic PSP in the MN (Fig. 3C; Tables 1, 2). DEHP exposure reduced the amplitude of the

Table 2
Values determined in the Mauthner neuron for the action potential and sensory induced postsynaptic potentials in control and exposure groups.

	Control	$DEHP_{(100\ \mu g\ L^{-1})}$	$DEHP_{(10\ \mu g\ L^{-1})}$	$DINP_{(100\ \mu gL^{-1})}$	$DINP_{(10\ \mu g\ L^{-1})}$
Action pot.	N = 12, 45 ≤ n ≤ 64	N = 11, 79 ≤ n ≤ 105	N = 12, 73 ≤ n ≤ 103	N = 12, 74 ≤ n ≤ 99	N = 11, 84 ≤ n ≤ 96
Cond. speed (m s^{-1})	102 ± 5 (61 to 126)	**79 ± 4 (66 to 109)**	**79 ± 3 (61 to 95)**	**75 ± 2 (61 to 87)**	**86 ± 4 (65 to 106)**
Amplitude (mV)	46.4 ± 1.9 (35.0 to 60.12)	**38.7 ± 2.5 (24.8 to 58.4)**	**37.2 ± 0.9 (32.2 to 42.0)**	41.0 ± 0.8 (35.7 to 47.2)	43.9 ± 2.1 (34.4 to 56.6)
Slope (mV ms^{-1})	251.8 ± 9.0 (185.2 to 302.3)	263.5 ± 18.3 (159.1 to 399.3)	253.7 ± 9.5 (195.8 to 305.7)	**299.1 ± 8.3 (249.8 to 340.2)**	**303.9 ± 15.7 (218.4 to 393.4)**
Duration (ms)	0.47 ± 0.02 (0.32 ± 0.57)	0.45 ± 0.01 (0.39 to 0.56)	0.45 ± 0.01 (0.39 to 0.51)	0.46 ± 0.01 (0.38 to 0.54)	0.46 ± 0.01 (0.40 to 0.57)
Area I_1 (mV*ms)	23.29 ± 0.77 (19.90 to 28.73)	**18.80 ± 1.20 (12.62 to 27.91)**	**18.08 ± 0.59 (14.56 to 21.03)**	**19.83 ± 0.51 (16.74 to 23.05)**	21.23 ± 1.39 (15.93 to 31.27)
Area I_2 (mV*ms)	4.47 ± 0.45 (1.67 to 6.61)	**0.59 ± 0.20 (0.00 to 1.60)**	**0.51 ± 0.16 (0.00 to 1.27)**	**0.49 ± 0.15 (0.00 to 1.50)**	**0.20 ± 0.09 (0.00 to 0.91)**
Acoustic PSP	14 ≤ n ≤ 43	20 ≤ n ≤ 51	14 ≤ n ≤ 51	19 ≤ n ≤ 50	23 ≤ n ≤ 52
Delay (ms)	7.50 ± 0.05 (7.32 to 7.93)	7.85 ± 0.21 (7.28 to 9.56)	7.76 ± 0.11 (7.32 to 8.34)	7.64 ± 0.08 (7.42 to 8.34)	7.57 ± 0.10 (7.24 to 8.16)
Amplitude (mV)	10.97 ± 0.83 (5.98 to 16.79)	**7.72 ± 0.83 (4.11 to 12.65)**	**7.90 ± 0.55 (4.67 to 10.03)**	8.78 ± 0.70 (4.98 to 13.69)	10.30 ± 0.59 (6.81 to 13.67)
Slope (mV ms^{-1})	9.10 ± 0.85 (5.06 to 15.50)	**6.08 ± 0.69 (3.02 to 9.91)**	**6.47 ± 0.43 (4.48 to 8.76)**	6.87 ± 0.81 (3.98 to 14.88)	8.29 ± 0.73 (4.21 to 12.91)
Duration (ms)	23.88 ± 6.01 (5.76 to 70.41)	29.54 ± 5.70 (7.69 to 64.13)	20.99 ± 3.94 (5.30 to 41.47)	24.79 ± 4.07 (4.49 to 51.59)	22.23 ± 6.19 (6.88 to 77.53)
Area (mV*ms)	400.9 ± 26.0 (300.2 to 587.7)	332.6 ± 25.9 (197.6 to 485.1)	321.9 ± 20.5 (223.3 to 443.3)	383.1 ± 30.6 (254.6 to 642.8)	400.8 ± 23.2 (249.2 to 497.1)
Visual PSP	13 ≤ n ≤ 41	5 ≤ n ≤ 35	10 ≤ n ≤ 34	3 ≤ n ≤ 32	6 ≤ n ≤ 32
Delay (ms)	27.69 ± 0.53 (24.53 to 30.70)	28.90 ± 1.41 (26.27 to 42.82)	31.37 ± 1.89 (26.16 to 42.05)	27.59 ± 0.44 (26.07 to 31.98)	29.00 ± 0.85 (25.99 to 33.90)
Amplitude (mV)	8.28 ± 0.72 (4.03 to 11.62)	**3.93 ± 1.00 (0.98 to 10.06)**	**3.35 ± 0.91 (0.00 to 8.32)**	**4.32 ± 0.97 (1.11 to 10.62)**	**3.31 ± 1.30 (1.05 to 16.00)**
Slope (mV ms^{-1})	8.68 ± 0.48 (6.19 to 11.48)	**3.53 ± 0.12 (3.02 to 4.31)**	**3.56 ± 0.57 (0.00 to 6.72)**	**3.81 ± 0.20 (3.30 to 5.66)**	**3.92 ± 0.30 (3.26 to 6.61)**
Duration (ms)	112.3 ± 20.4 (20.6 to 207.3)	129.3 ± 18.8 (69.7 to 237.4)	99.1 ± 24.1 (0.0 to 312.7)	105.2 ± 14.8 (58.4 to 233.7)	108.4 ± 13.3 (68.6 to 206.1)
Area (mV*ms)	629.0 ± 84.6 (226.1 to 979.3)	**285.4 ± 75.3 (55.2 to 878.7)**	**287.0 ± 88.0 (0.0 to 880.6)**	**324.5 ± 84.7 (42.1 to 886.4)**	**292.7 ± 132.7 (80.4 to 1460.0)**

Values are given as mean ± standard error of mean; range in parentheses; significant differences from control are highlighted in bold; N = number of independent animal samples; n = measurement repetitions taken from the individual animals.

acoustically induced PSP from 10.97 ± 0.83 mV (N = 12) in controls to 7.90 ± 0.55 mV (N =12) in fish exposed to DEHP(l) (Dunn's test: P = 0.0401, mean rank diff.: 17.75) and to 7.72 ± 0.83 mV (N = 11) in fish exposed to DEHP(h) (Dunn's test: P = 0.0302, mean rank diff.: 18.83). PSP slope was reduced from 9.10 ± 0.85 mV ms^{-1} to 6.47 ± 0.43 mV ms^{-1} in the DEHP(l)-group (Dunnett's test: P = 0.0383, mean diff.: 2.63) and to 6.08 ± 0.69 mV ms^{-1} in the DEHP(h)-group (Dunnett's test: P = 0.0165, mean diff.: 3.02). Again, the effects of plasticizer exposure were not significantly different at the low and the high concentration (amplitude: Mann-Whitney test: P = 0.7399; slope: unpaired t test: P = 0.6263, t = 0.4942, df = 21). Delay (between stimulation onset and PSP onset), PSP duration (measured at 50 % peak amplitude) and the time-integrated PSP for the first 150 ms after PSP onset were neither affected by DEHP exposure nor by DINP exposure (Tables 1, 2).

3.4. Effect on visual processing

Using light flash stimulation of the ipsilateral eye during in vivo intracellular MN recording (Fig. 3A) allowed us to determine the impact of phthalate exposure on the chain from phototransduction, visual processing to transmission to the brain by analyzing visually induced PSPs in the MN (Fig. 3B). In contrast to the processing of acoustic information, both plasticizers caused similar and strong effects here (Fig. 3D; Tables 1, 2). While they did not affect delay and duration of the visually induced PSPs (Fig. 3D; Tables 1, 2), both plasticizers reduced the PSP amplitude (Tables 1, 2; Dunn's test: P ≤ 0.0430, mean rank diff.: ≥ 17.58), the slope of the rising edge of the PSP (Dunn's test: P ≤ 0.0005, mean rank diff.: ≥ 26.89), and the time-integrated PSP for the first 150 ms after PSP onset (Dunn's test: P ≤ 0.0430, mean rank diff.: ≥ 17.67). The reduction caused by the exposure was similar for the plasticizers applied and independent of whether a high or a low concentration was applied: in all exposure groups it ranged between about 50–60 %. The PSP amplitude was reduced from 8.28 ± 0.72 mV in the untreated control group to less than 4.32 ± 0.97 mV in the exposed groups. The slope of the PSP was reduced from 8.68 ± 0.48 mV ms^{-1} to less than 3.92 ± 0.30 mV ms^{-1}, and the time-integrated PSP for the first 150 ms after PSP onset was reduced from 629.0 ± 84.6 mV*ms to less than 324.5 ± 84.7 mV*ms.

4. Discussion

The effects of phthalates on reproductive and developmental processes are well documented (Swan, 2008; Latini et al., 2010; Palanza et al., 2016; You and Song, 2021; Yang et al., 2021; Kirti et al., 2022) and legislation as well as the development of alternative plasticizers are based on our knowledge of such effects (Silano et al., 2019; Ong et al., 2022). While the consequences of developmental changes in the brain caused by phthalate exposure are known (Ejaredar et al., 2015; Radke et al., 2020; Xu et al., 2020), effects of a short-term exposure on the fully developed adult brain have never been quantified. It is also not known how well the homeostatic mechanisms of the intact adult brain might be able to compensate effects of short-term exposure. The results we present here are therefore both striking and alarming: we find that the conventional DEHP and its successor DINP have immediate and strong effects that are fully developed even at the lowest environmentally relevant concentration. The effects include a strong decline in axonal conduction speed, strong and differential influences on synaptic transmission as well as strong effects on the visual pathway. One might argue that we find these effects in a special neuron and in goldfish rather than in a mammal so that it would seem unclear whether similar effects occur in our brains. Here, it is important to recall that basic functions of the brain, such as the generation and propagation of action potentials or synaptic transmission, have been discovered in species such as the squid, *Aplysia*, lobster, and so on, and have later proven to hold also in vertebrates (Martin et al., 2020; Kandel et al., 2021). Therefore, it is very likely that effects found during recording from the MN are relevant in

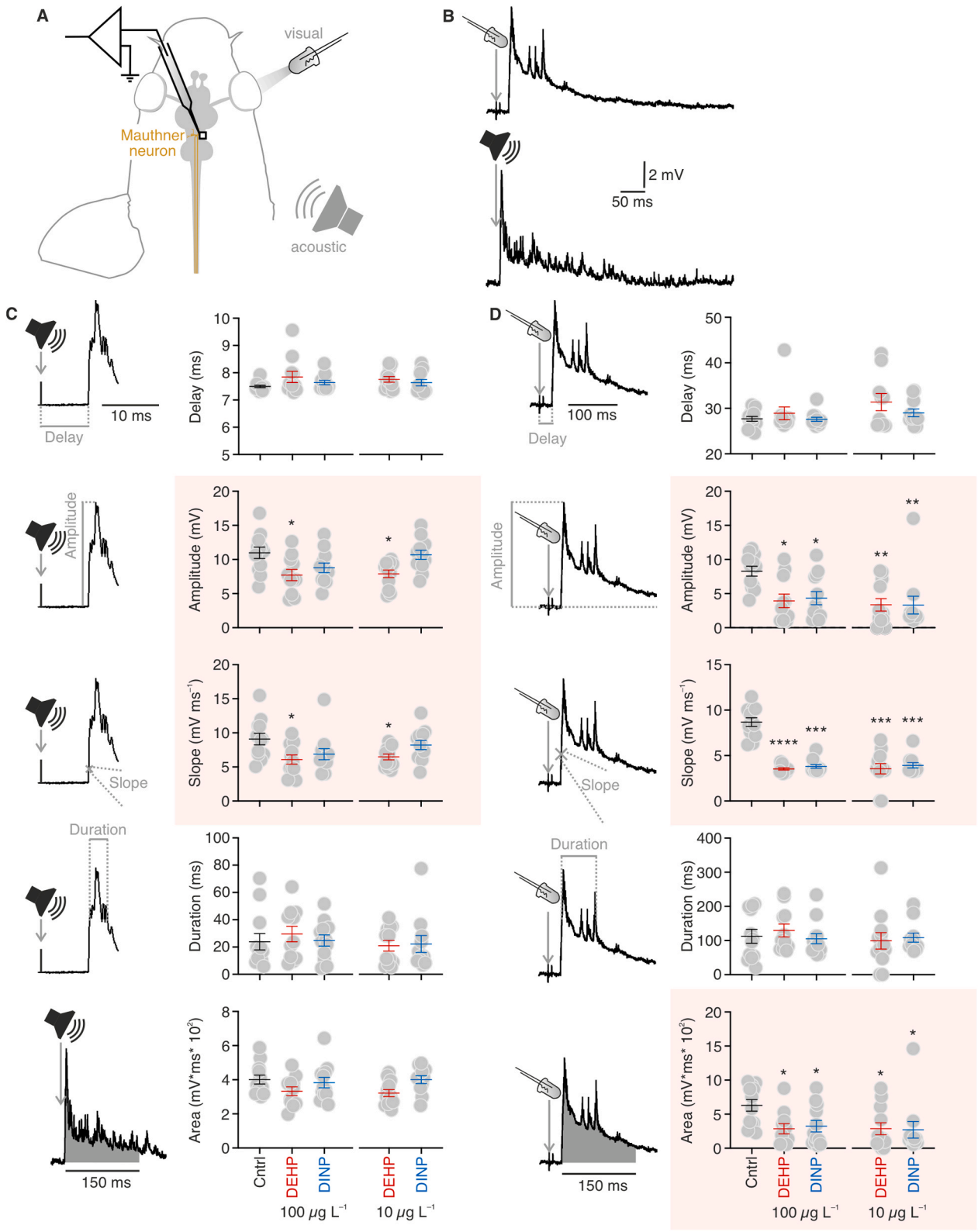


Fig. 3. DEHP and DINP both affect visual information processing, but auditory information processing was only affected by DEHP exposure. A Sketch of experimental setting for visual and acoustic stimulation: only the stimulation has to be changed, while the MN recording is kept (cf. Figs. 1C and 2A). B Examples of PSPs recorded after visual or acoustic stimulation of the fish (as indicated). C PSPs measured in the MN after acoustic stimulation of the fish were reduced in amplitude and slope by DEHP exposure in any concentration, but not by DINP exposure. The delay, duration and the area of the time integrated acoustically induced PSP for the first 150 ms were neither affected by DEHP nor by DINP exposure. D In contrast to that, both phthalates affected the visually in the MN induced PSP. Both reduced amplitude, slope and the area of the time-integrated visually induced PSP for the first 150 ms. Delay and duration were not affected. Measurements were taken as indicated by sketches. Values are given as mean ± standard error of mean. For the number of independent animal samples tested (N) and the numbers of measurements per animal (n) see Table 2. Values affected by exposure to either DEHP or DINP in any concentration are highlighted by a light red background. Significant differences between a certain exposed group and the control are indicated by asterisk(s); one, two, three, four asterisks indicate $P < 0.05$, $P \leq 0.01$, $P \leq 0.001$, or $P \leq 0.0001$, respectively.

other central neurons as well. Furthermore, the view that phthalates might exclusively cross the blood-brain barrier (BBB) in goldfish, but not in humans is unlikely given the similarity of their BBBs (OBrown et al., 2018; Abbott, 2022). It is well possible that the permeability for specific chemicals across the BBB differs across vertebrates, but it is unlikely to have phthalate transmission across the BBB in goldfish and none in humans. The fact that we were able to detect similar effects of phthalate exposure on the function of central neurons for the low and 10-fold higher environmental concentration tested is important in this context: humans get exposed to phthalates mostly through ingestion and their indoor environment, while fish in this study got exposed to phthalates through the surrounding water. These differences in exposure may mean that humans generally take up fewer phthalates from the environment than the fish in the present study. However, even if fewer phthalates are taken up and reach/cross the BBB in humans, it must be assumed on the basis of our results that the effect could still be similar to that observed in our experiments on goldfish. The effects we discovered on axonal conduction speed in the MN would be highly detrimental to the normal function of the MN (Eaton et al., 1977; Hecker et al., 2020). However, similar effects on conduction speed in other axons of the brain would also be detrimental because timing is of major importance in the diverse sensory and motor systems of the brain (cortex (Engel et al., 1992); thalamus (Sillito et al., 1994); cerebellum (Welsh et al., 1995)). The large changes we found in conduction speed are therefore a major concern in the short-term effects of phthalates on adult brains. They may also have an additional consequence: recent studies in mice have provided evidence suggesting that phthalates may affect the integrity of the BBB (Ahmadpour et al., 2021; Ren et al., 2023) which evidently could have massive effects on brain function (Weiss et al., 2009; Obermeier et al., 2013; Xiao et al., 2020; Segarra et al., 2021). While we cannot currently say by which mechanisms phthalates so strongly affect conduction speed, it is highly likely that they do so by affecting the myelin sheath, which is crucial for rapid conductance. Therefore, our findings contribute to the emerging view (Ahmadpour et al., 2021; Ren et al., 2023) that phthalates affect glial cells or their interactions and therefore affect the BBB as well as the glial cells that form the myelin sheath of axons. Given the enormous range of functions of neuroglia, this is an aspect that urgently requires attention. The strong and uncompensated effect of both phthalates on synaptic transmission that we find in the MN already at the lowest environmentally relevant concentration tested suggests that similar effects will also occur in other synapses of the CNS. The consequences of such changes are unknown, but they could contribute to offset the finely maintained balance of excitatory and inhibitory inputs – a renowned cause or negative factor in many neurodegenerative diseases (Ramocki and Zoghbi, 2008; Nelson and Valakh, 2015; Tshala-Katumbay et al., 2015; Ahmadpour et al., 2021; Ren et al., 2023).

5. Conclusion

We demonstrate strong and diverse effects of a relatively brief exposure to phthalates at environmentally relevant concentrations in an adult vertebrate. The absence of significant differences between the low and the high concentration tested, the potentially disruptive effects on neuronal timing as well as on the balance of synaptic inputs (a major factor in several neurodegenerative diseases) and the likelihood that various neuroglial cells are also affected are of fundamental importance. Our results call for immediate attention and definitely for the inclusion of effects on the mature brain in the evaluation of future plasticizers.

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CRediT authorship contribution statement

Benedikt Maric: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Peter Machnik:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stefan Schuster:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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