

**Depressed cardiac contractility and its postnatal development in rats after chemical sympathectomy.**

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Short title: Postnatal cardiac contractility and chemical sympathectomy

## Summary

The contribution of the sympathetic innervation to the postnatal development of cardiac contractility remains unclear. In this study, the postnatal maturation of cardiac contractility was compared in control rats and rats after chemical sympathectomy. The chemical sympathectomy was induced by administration of 6-hydroxydopamine to newborn rats. At days 20, 40 and 60 of postnatal life, the contractile parameters and concentrations of sympathetic neurotransmitters were measured in both right and left ventricles. In rats with chemical sympathectomy, concentrations of norepinephrine were reduced almost completely in both ventricles at all points in time. The contractility of the left ventricle papillary muscles was substantially decreased at all points in time. In contrast, the contractility of the right ventricle papillary muscles was decreased only transiently, showing a recovery at day 60 regardless of the permanently decreased concentration of norepinephrine. The concentration of neuropeptide Y, another neurotransmitter present in sympathetic nerves, showed the same developmental trend as contractility: permanent reduction in the left ventricle, transient reduction with a recovery at day 60 in the right ventricle. The data indicate that the sympathetic nervous system plays an important role in the postnatal development of cardiac contractility and neuropeptide Y may contribute to this effect.

**Keywords:** chemical sympathectomy, contraction, neuropeptide Y, norepinephrine, rat heart

## Introduction

The postnatal development of cardiac contractility is rather complex. In general, the contractile performance of mammalian heart increases and this improvement is due to various processes such as maturation of excitation-contraction coupling with changes in expression of sarcolemmal and reticular  $\text{Ca}^{2+}$  channels and transporters (Tanaka and Shigenobu 1989, Wibó *et al.* 1991), changes at the level of contractile proteins, their isoforms and binding properties (McAuliffe *et al.* 1990, Martin *et al.* 1991) and/or development of autonomic innervation (Robinson 1996). Autonomic (sympathetic or parasympathetic)

innervation influences virtually all aspects of cardiac contractility, on both short-term and long-term basis. The short-term control is usually realised through changes in phosphorylation of  $\text{Ca}^{2+}$  transporters and/or of contractile proteins (e.g. Kameyama 1986), the long-term control involves changes in expression levels of these proteins (e.g. Ogawa *et al.* 1992, Maki *et al.* 1996, Protas and Robinson 1999).

The link between sympathetic innervation and contractile postnatal development was first suggested based on in vitro studies, in which isolated neonatal rat myocytes were either exposed to norepinephrine (Maki *et al.* 1996) or co-cultured with sympathetic neurons (Ogawa *et al.* 1992, Protas and Robinson 1999). In such myocytes the contractile response was increased, which was attributed to the increased number of L-type  $\text{Ca}^{2+}$  current (ICaL) channels. The above studies, however, raise question, what is the actual trophic factor underlying the increase in ICaL. Increase in dihydropyridine receptor expression after exposure to norepinephrine (Maki *et al.* 1996) clearly favours norepinephrine. The study by Protas and Robinson (1999), however, indicates that the sympathetic innervation contributes to the developmental ICaL increase through neurally released neuropeptide Y (NPY), which is stored in most sympathetic nerves as a cotransmitter with norepinephrine (Zukowska *et al.* 2003). The question, whether the sympathetic innervation indeed contributes to contractility improvement in vivo, is less clear and compelling evidence is lacking. Recently, Protas *et al.* (2003) addressed this question using transgenic NPY-deficient mice and their results suggest that also in vivo NPY contributes to the postnatal increase in ICaL.

To elucidate whether norepinephrine and/or NPY contribute to the postnatal development of contractility, we have performed experiments in rats with chemical sympathectomy induced by administration of 6-hydroxydopamine (6-OHDA). The levels of norepinephrine and NPY were measured at 20, 40 and 60 days of postnatal life and correlated with contraction force measurements in both right and left ventricle papillary muscles. The data show a differential

response of the right and left ventricles to the sympathectomy and suggest an important role for NPY in regulation of postnatal contractility development.

## **Methods**

All experiments were conducted in accordance with European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU), the relevant Guidelines of the Czech Ministry of Agriculture for scientific experimentation on animals and were approved by the University Committee for Experiments on Laboratory Animals (Charles University, Czech Republic).

### *Animals*

A total of 180 newborn Wistar rats were used. Pups were randomly divided into two groups on the day of birth, designated as postnatal day 0. The first group (6-OHDA) received subcutaneously 6-hydroxydopamine (6-OHDA; 100 mg/kg) dissolved in 0.9% NaCl solution containing 0.1% ascorbic acid on postnatal days 1-7, 14, 21, and 28. The control group received equal volumes of solvent at the same time points. The mortality in the sympathectomized group was not increased compared to the control group. Before weaning (four weeks after birth), 7-9 pups were kept with one nursing dam and then the animals were housed five *per cage*, fed standard laboratory chow *ad libitum* with free access to drinking water. All rats were used for further experiments at the ages of 20, 40 and 60 postnatal days. The body mass increased with the age and it did not differ between the control and sympathectomized groups:  $42.7 \pm 2.8$  g vs.  $40.1 \pm 1.1$  g at the age of 20 days,  $135.3 \pm 4.1$  g vs.  $128.8 \pm 1.8$  g at the age of 40 days and  $231.1 \pm 7.9$  g vs.  $228 \pm 5.4$  g at the age of 60 days ( $n=10$ ), respectively.

### *Contraction experiments*

Animals were anaesthetized with intraperitoneal injection of urethane (1.5 g/kg body weight) 10 minutes after having received heparin (500 U, i.p.) and their hearts were quickly excised.

The papillary muscles were dissected from the right or the left ventricles and placed in an experimental chamber with Tyrode solution, where they were attached to an isometric force transducer F30 (Hugo Sachs, Germany). The resting tension was set so that the developed twitch tension reached 90-95% of maximum at stimulation frequency of 1 Hz. Double-chamber stimulation (stimulator Pulsemaster A300, WPI, USA) was used. The square-wave voltage pulses had duration of 1 ms and amplitude  $\geq 50\%$  above threshold. Data were recorded using the data acquisition system DiSys (Merlin, Czech Republic). The preparation was perfused with 36°C warm, oxygenated solution at a constant flow rate (6-10 ml/min). After a stabilization period (more than 30 minutes), steady-state contractions at various stimulation frequencies (3, 2, 1, 0.5, 0.3, 0.2 and 0.1 Hz) were recorded. Contractions were measured in arbitrary units (a.u.). Time course of contraction was characterized using time-to-peak (time from resting tension to the peak of contraction, TTP) and half-maximal relaxation time (R/2) parameters. The resting tension was taken as zero.

#### *Extraction of norepinephrine and NPY*

Rats were anaesthetized with urethane (see above) and the hearts were rapidly excised, rinsed with ice-cold saline and freed of connective tissue and fat. Immediately after dissection of free walls of both ventricles, the tissues were frozen on dry ice. The NPY-like material was extracted from pulverized heart compartments by boiling in 10 volumes of 0.1 mol/l acetic acid for 15 min, rapid cooling on ice and homogenization for 30 s using an Ultra-Turrax homogenizer. The homogenates were centrifuged (10,000g, 4°C, 20 min); the supernatants were neutralized with 1 mol/l Tris-base and centrifuged again (5,000g, 4°C, 10 min). The supernatants were stored at -70°C until radioimmunoassay (RIA) for NPY-immunoreactivity measurements.

Norepinephrine was extracted from the heart ventricles by homogenization in 10 volumes of 0.4 mol/l HClO<sub>4</sub> and subsequent centrifugation (10,000g, 4°C, 20 min). Supernatants were diluted 1:10 with distilled water and stored at -70°C until RIA. NPY and norepinephrine

concentrations were assayed in tissue extracts by RIA using commercial kits (Phoenix Pharmaceuticals, CA, USA and IBL Hamburg, FRG).

### *Solutions and chemicals*

The composition of the Tyrode solution was following (in mmol/l): NaCl 137, KCl 4.5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 2, glucose 10, Hepes 5; pH was adjusted to 7.4 with NaOH. Tyramine hydrochloride, propranolol hydrochloride, 6-hydroxydopamine hydrobromide (6-OHDA) and other chemicals were from Sigma (St. Louis, MO, USA) or Lachema (Brno, Czech Republic) if not stated otherwise. All chemicals were of analytical grade.

### *Data analysis*

Results are presented as means  $\pm$  SEM. Statistical differences were tested by unpaired two-tailed Student's t-test or by analysis of variance (ANOVA) followed by post hoc Fisher's Least Significant Difference test, using software package STATISTICA Cz, version 7 (StatSoft, Inc., 2004). Normality of populations and homogeneity of variances were tested before each ANOVA. The results were considered significantly different when  $p < 0.05$ .

## **Results**

### Chemical sympathectomy

To verify the effectivity of the chemical sympathectomy, norepinephrine concentrations and contractile responsiveness of papillary muscles to tyramine and propranolol were determined in the left and right ventricles. As shown in Fig. 1, the absolute values of norepinephrine in control rats did not change with the age of animals. Concentrations of norepinephrine expressed in ng/g tissue wet weight varied between  $628 \pm 81$  (at the age of 20 days) and  $533 \pm 51$  (at the age of 60 days) in the right ventricle,  $458 \pm 54$  (at the age of 20 days) and  $398 \pm 24$  (at the age of 60 days) in the left ventricle. In sympathectomized rats, norepinephrine concentrations reached maximally  $9.42 \pm 4.7$  ng/g in the right ventricle and  $10.53 \pm 5$  ng/g in

the left ventricle at the age of 20 days. The values of norepinephrine concentrations in denervated rats did not change significantly in the whole course of the experiment (Fig.1).

Tyramine stimulates norepinephrine release from the sympathetic nerve terminals (Sulzer *et al.* 2005). In control rats, administration of tyramine significantly increased the contraction force (CF) of papillary muscles from both ventricles independent on age. The maximal increase in CF of the right ventricular papillary muscles in comparison with tyramine-free solution was observed at tyramine concentration of  $10^{-4}$  mol/l in 40-day-old rats ( $162 \pm 8.4\%$ ) and at tyramine concentration of  $10^{-5}$  mol/l in 20- and 60-day-old rats ( $140 \pm 11.6\%$  and  $151 \pm 8.1\%$ , respectively, Fig. 2A). In the left ventricular papillary muscle, the maximum positive inotropic effect of tyramine was found at concentration of  $10^{-5}$  mol/l in all age categories, i.e. compared to tyramine-free solution  $142 \pm 5.8\%$ ,  $160 \pm 4.3\%$  and  $153 \pm 4.5\%$  in 20-, 40- and 60-day-old rats, respectively (Fig. 2B). The values of CF of the right and left ventricular papillary muscles after application of  $\beta$ -adrenergic blocker propranolol ( $10^{-4}$  mol/l) on top of tyramine ( $10^{-4}$  mol/l) were not significantly different from values in tyramine-free solution in any age group (Fig. 2A, 2B). In contrast, both right and left ventricle papillary muscles of sympathectomized rats were insensitive to both tyramine and propranolol in all age groups (Fig. 2C, 2D).

### Contraction experiments

Chemical sympathectomy significantly decreased the contraction force (CF) of the papillary muscle in 20- and 40-day-old rats in the right ventricle and in all age categories in the left ventricle (Fig. 3). CF of the intact rats increased with age in both ventricles and it was  $32.5 \pm 2.8$  a.u.,  $53.2 \pm 4.1$  a.u. and  $58.5 \pm 5.5$  a.u. at stimulation frequency of 1 Hz in the right ventricle at the age of 20, 40 and 60 days, respectively (Fig. 3, the left panel). In the left ventricle of intact rats, CF at 1 Hz was  $27.5 \pm 3.3$  a.u.,  $41 \pm 5.3$  a.u. and  $48.4 \pm 4.04$  a.u. at the ages of 20, 40 and 60 days, respectively (Fig. 3, the right panel). In the right ventricle of denervated rats, CF was significantly lower at the ages of 20 and 40 days being  $10.4 \pm 3.8$

a.u. and  $17.1 \pm 3.5$  a.u. at 1 Hz, respectively. At day 60, however, this reduction disappeared and CF of the right papillary muscle was comparable to control ( $55 \pm 1.4$  a.u. at 1 Hz, Fig. 3, the left panel). In the left ventricle of sympathectomized rats, values of CF were significantly lower compared to control regardless of the age, varying from  $10 \pm 1.5$  a.u. in 20-day-old rats to  $14 \pm 3.6$  a.u. in 40-day-old ones (Fig. 3, the right panel). The effect of the chemical sympathectomy on CF was well expressed at all stimulation frequencies tested (0.1 – 3 Hz), i.e. CF of papillary muscles in denervated rats was always significantly lower than in control rats except the right ventricle papillary muscles at the age of 60 days (Fig. 4).

In both control and denervated rats, time to peak of contraction (TTP) shortened with increasing frequency of stimulation in all age groups. Neither chemical sympathectomy nor age of animals affected TTP. Similarly, half-maximal relaxation time (R/2) shortened with increasing frequency of stimulation and there was no difference between the intact and sympathectomized rats at any age (data at the stimulation frequency of 1 Hz in Table 1).

#### Neuropeptide Y concentrations

To elucidate the impact of chemical denervation on cardiac sympathetic innervation, we measured the ventricular concentrations of NPY that is in addition to norepinephrine located in sympathetic postganglionic fibers. The values of NPY concentrations in controls were not influenced by age and they showed no difference between the right and the left ventricles (Fig. 5). The effect of sympathetic denervation on NPY concentrations was different in the right ventricle compared to the left one. In the right ventricle, the peptide concentrations were significantly lower compared to their respective controls at the ages of 20 and 40 days. At age of 60 days, however, NPY concentrations were markedly increased and were no longer significantly different from the age-matched controls (Fig. 5, upper panel). In the left ventricle, NPY concentrations remained significantly lower than their respective controls in all age categories (Fig. 5, upper panel). Concentrations of NPY showed good correlation with CF



(Fig. 5, lower panel,  $R=0.986$ ) whereas no correlation was found for norepinephrine concentrations and CF ( $R=0.0005$ ).

## Discussion

In our study, administration of 6-OHDA induced an almost complete reduction in norepinephrine concentrations in both right and left ventricles at all points in time measured (20, 40 and 60 days of postnatal life). Tyramine, a stimulator of norepinephrine release from sympathetic nerve terminals, was not able to increase contraction of papillary muscles from sympathectomized rats. These data indicate that the chemical sympathectomy was effective. The contractility was influenced by the chemical sympathectomy differentially in the left and the right ventricle. In the left ventricle, the contraction was substantially depressed at all points in time. In the right ventricle, in contrast to the development of contraction force in the left ventricle and to norepinephrine levels, the contraction was significantly reduced only at 20 and 40 days of postnatal life. At 60 days of postnatal life, the contraction in the right ventricle recovered and was no longer different from control. This differential development of contractility cannot be explained on the basis of norepinephrine tissue concentrations that remained low in both ventricles of sympathectomized rats throughout the whole experiment. The other neurotransmitter present in sympathetic nerves (Zukowska *et al.* 2003), NPY, however, showed a different developmental pattern that exactly matched the development of contractility: full-time suppression in the left ventricle vs. transient (at 20 and 40 days) suppression with recovery (at 60 days) in the right ventricle. This suggests that from the two transmitters stored in sympathetic nerves, NPY, but not norepinephrine, controls the development of contractility. Nevertheless, some contribution of norepinephrine to the development of contractility in the early phases of development cannot be excluded. The mechanisms, by which NPY possibly increases the contractility, are still questionable and were not addressed in this study. Based on recent reports showing that NPY contributes to the developmental increase in I<sub>CaL</sub>, both in vitro (Protas and Robinson 1999) and in vivo (Protas *et al.* 2003), it may be suggested that I<sub>CaL</sub> is the likely target of NPY action.

Nevertheless, other contributing mechanisms such as enhanced synthesis and attenuated degradation of contractile proteins (Nicholl *et al.* 2002) may also be involved.

Another important question is what is the putative source of NPY in hearts of sympathectomized rats. Repeated administration of 6-OHDA to neonatal rats causes permanent destruction of the sympathetic neurons by a two-step process: after its uptake into the sympathetic postganglionic fibers and nerve cell bodies by norepinephrine transporter, 6-OHDA undergoes a series of chemical transformations that lead to reactive oxygen species generation followed by the loss of the cell membrane integrity and the cell death (Picklo 1997). However, beside localization in the sympathetic postganglionic fibers (together with norepinephrine), NPY was also identified in the nonadrenergic intrinsic neurons that are located on the base of the heart above the atrioventricular groove in four distinct groups (Pardini *et al.* 1987). These intrinsic neurons send their processes to adjacent neurons within the intracardiac ganglia, small intensely fluorescent cells, and intramyocardial resistance vessels (Slavikova *et al.* 2003; Corr *et al.* 1990). In addition, NPY was also found extraneuronally in platelets and endocardium (Zukowska-Grojec *et al.* 1993, Jacques *et al.* 2006). The impact of sympathectomy on the myocardial NPY levels might differ depending on the method used. It has been reported that surgical bilateral sympathectomy of the adult rat heart produced similar reductions in concentrations of norepinephrine and NPY in the ventricular tissue (Maccarrone and Jarrott 1987). In contrast, chemical sympathectomy achieved by long-term guanethidine administration to neonatal rats led to differential reductions of norepinephrine and NPY levels in the rat heart: whereas norepinephrine was nearly totally depleted from the myocardium of sympathectomized rats at 6 weeks and 4 months of age, reaching 1-10% of concentrations in untreated animals, levels of NPY were decreased by 46-66% only (Corr *et al.* 1990). In our study, the impact of repeated administration of 6-OHDA on the ventricular NPY levels seemed to be more profound at the ages of 20 and 40 days than that reported for guanethidine sympathectomy, since NPY concentrations were reduced by 81-94% compared to control values. However, at the age of

60 days, right ventricular NPY concentrations were not significantly different from the control values, whereas in the sympathectomized left ventricles, NPY levels remained low. It is thus possible that NPY in the right ventricle recovered from the intrinsic neurons containing NPY that reinnervated relatively thin wall of the right ventricle and that similar reinnervation of much greater mass of the left ventricle could be substantially delayed.

Mammalian heart is known to undergo a significant postnatal development of morphological and functional parameters. Lacking T-tubules, lower density of diads and gap junctions, less cardiomyocyte size, poorly developed sarcoplasmic reticulum and its lower sensitivity to calcium ions are characteristic for immature rat myocardium (Vornanen 1996). The postnatal development of rat cardiomyocytes also includes changes in ion channels expression and function (Wetzel and Klitzner 1996). Because of poor sarcoplasmic reticulum and T-tubules in neonatal heart,  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (CICR) does not play a key role in excitation-contraction coupling, hence the contraction in the developing heart is dependent mainly on transsarcolemmal  $\text{Ca}^{2+}$  influx for contraction generation via  $\text{ICaL}$  and the reverse mode of  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger. CICR from the sarcoplasmic reticulum becomes dominant in cardiac excitation-contraction coupling at the end of third week of postnatal life (Escobar *et al.* 2004). Postnatal maturation of the rat heart is followed by an increase in contraction force of the multicellular preparation that continues till adulthood even if morphological development of the heart is over (Leblanc *et al.* 1998; Capasso *et al.* 1982). Similarly, in our experiments performed on papillary muscles of control rats, the contraction force increased with age to achieve the maximal value at the age of 60 days in both ventricles.

#### Limitations of the study

The contractility was studied using papillary muscles from both ventricles. Papillary muscles represent endocardial portion of the ventricular wall. Given the fact that the endocardial, midmyocardial and epicardial ventricular layers show substantial electrical and contractile differences (Liu *et al.* 1993, Cordeiro *et al.* 2004) as well as differential responses to various

stimuli (McCrossan *et al.* 2004), it is possible that the contractile development in midmyocardial and epicardial layers is different in some aspects. Since the NPY concentrations and contractions in sympathectomized rats show good correlation, a relationship between these two parameters may be suggested. The causality of this relationship, however, remains unclear and new experiments with NPY antagonists and/or antibodies will be necessary.

## Conclusions

The intact sympathetic innervation plays an important role in the development of cardiac contractility. The response of the left ventricle and of the right ventricle to the chemical sympathectomy is different: whereas the left ventricle showed a sustained reduction in contractile performance, in the right ventricle a recovery of contractility was detected. NPY is likely to contribute significantly to the postnatal development of contractility.

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**Tab. 1.** Time to peak of contraction (TTP) and half-maximal relaxation time (R/2) in control and sympathectomized rats at the stimulation frequency of 1 Hz.

RV – the right ventricle, LV – the left ventricle, D = age in days.

	Control rats (n=6)			Sympathectomized rats (n=5)		
	TTP (ms)					
	20D	40D	60D	20D	40D	60D
RV	55 ± 7.6	57 ± 6.1	53 ± 5.5	54 ± 5.1	51 ± 5.9	60 ± 4
LV	58 ± 6.7	59 ± 8.3	55 ± 3.7	59 ± 4.9	55 ± 6.1	57 ± 1.2
	R/2 (ms)					
RV	46 ± 5.9	43 ± 3.8	41 ± 4.7	39 ± 2.7	37 ± 3.8	40 ± 3.3
LV	48 ± 3.2	45 ± 4.8	40 ± 2.1	44 ± 3.8	42 ± 5.3	47 ± 0.7

## Figure legends

**Fig. 1.** Norepinephrine (NE) concentrations in the free walls of the right (RV) and left (LV) ventricles of 20-, 40-, and 60-day-old control and sympathectomized (6-OHDA) rats. Data are expressed in ng/g tissue wet weight as mean ± S.E.M., n = 8 per group and age category, \*p < 0.01, compared to the respective control value.

**Fig. 2.** The effect of tyramine and propranolol on contraction force of the control and sympathectomized rats. A, B. Contraction force (CF) of papillary muscles from the right (RV) and left ventricles (LV) in control rats (n=6). C, D. Contraction force (CF) of papillary muscles from the right (RV) and left (LV) ventricles in sympathectomized rats (n=5). Values were normalized to the CF in tyramine-free solution (taken as 100%). Stimulation frequency was 1

Hz. D = age in days. P = administration of propranolol ( $10^{-4}$  mol/l) on top of tyramine ( $10^{-4}$  mol/l). \*p < 0.01, compared to the value in tyramine-free solution.

**Fig. 3.** The contraction force of the control and sympathectomized (6-OHDA) rats.

Left panel: Contraction force (CF) of papillary muscles from the right ventricles (RV) in control (n=6) and sympathectomized rats (n=5). Right panel: Contraction force (CF) of papillary muscles from the (LV) ventricles in control (n=6) and sympathectomized (n=5) rats. Stimulation frequency was 1 Hz. \*p < 0.01, compared to the respective control value, #P < 0.01, compared to the respective value in the right ventricle.

**Fig. 4.** The contraction force of the control and sympathectomized rats.

A, B. Contraction force (CF) of papillary muscles from the right (RV) and left ventricles (LV) in 20-day-old control (n=6) and sympathectomized (n=5) rats. C, D. Contraction force (CF) of papillary muscles from the right (RV) and left (LV) ventricles in 40-day-old control (n=6) and sympathectomized (n=5) rats. E, F. Contraction force (CF) of papillary muscles from the right (RV) and left ventricles (LV) in 60-day-old control (n=6) and sympathectomized (n=5) rats. \*P < 0.01, compared to the respective control value.

**Fig. 5.** Upper panel, neuropeptide Y (NPY) concentrations in the free walls of the right (RV) and left (LV) ventricles of 20-, 40-, and 60-day-old control and sympathectomized (6-OHDA) rats. Data are expressed in ng/g tissue wet weight as mean  $\pm$  S.E.M., n = 8 per group and age category, \*P < 0.01, compared to the respective control value. Lower panel, correlation of norepinephrine (NE, open symbols) and NPY (closed symbols) concentrations with contraction force (CF) in denervated rats.

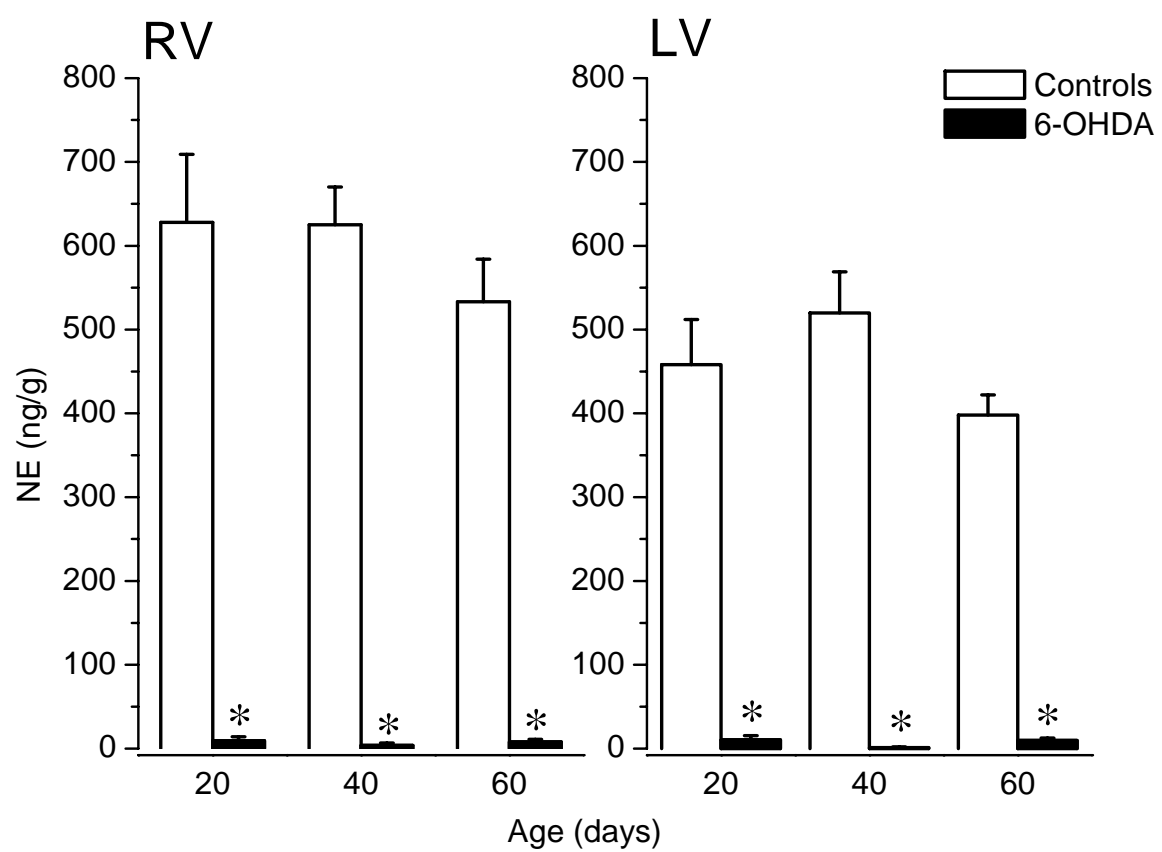


Fig.1



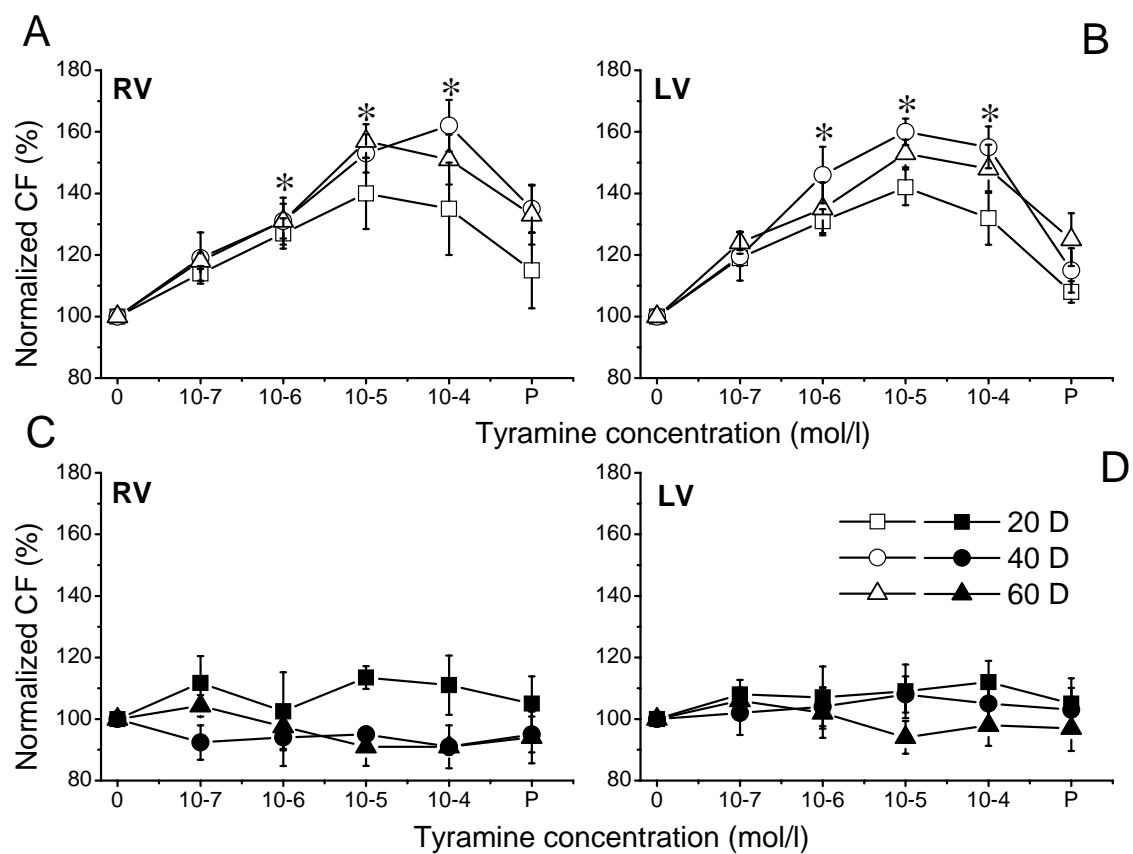


Fig. 2

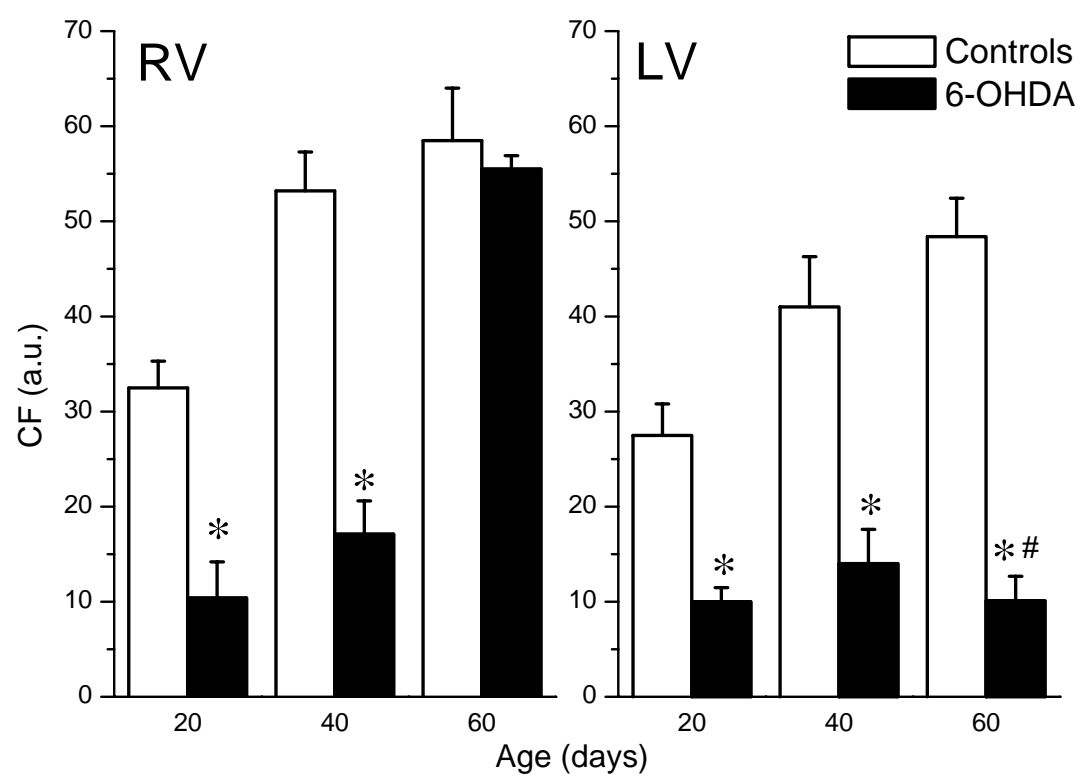


Fig. 3

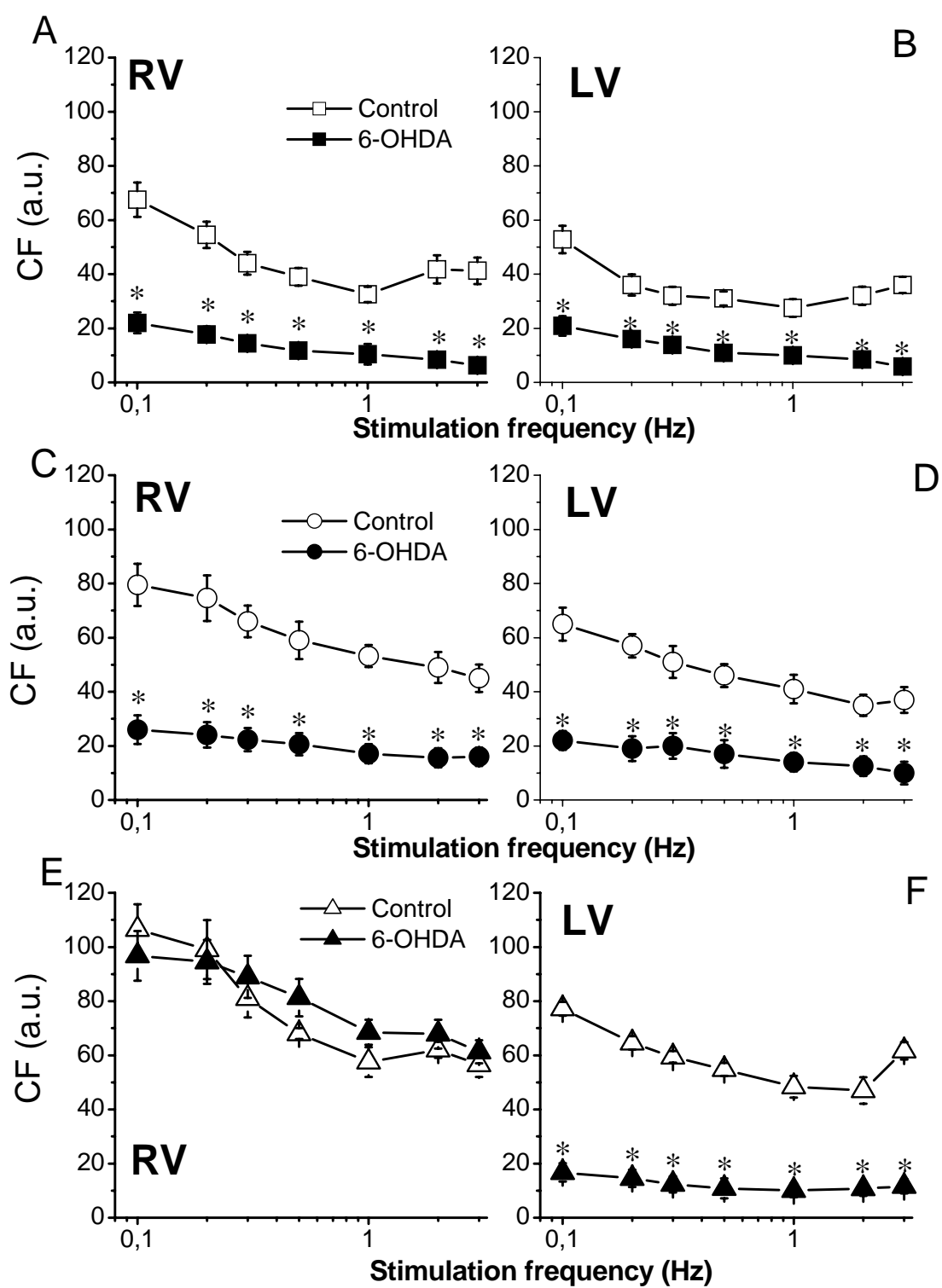


Fig. 4

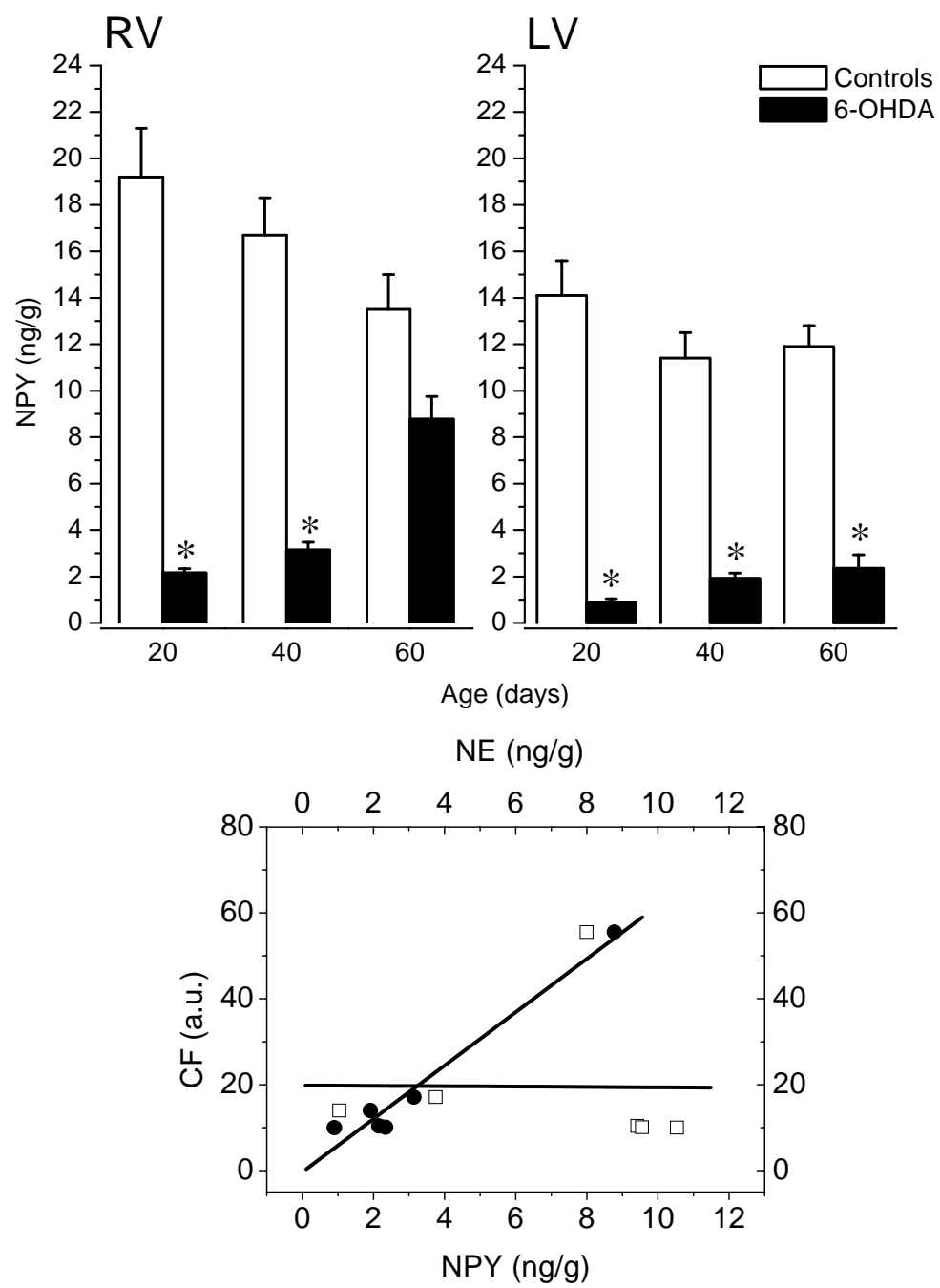


Fig. 5