Experimental Autoimmune Autonomic Neuropathy

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Abbreviated title: EAAN

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ABSTRACT

Antibodies specific for the neuronal ganglionic nicotinic acetylcholine receptor (nAChR) are found in high titer in serum of patients with subacute autonomic failure. This clinical disorder is known as autoimmune autonomic neuropathy (AAN). Rabbits immunized with a neuronal nAChR α3 subunit fusion protein produce ganglionic nAChR antibodies and develop autonomic failure (experimental AAN, or EAAN). We used quantitative measures of autonomic function to demonstrate that this animal model of neuronal nAChR autoimmunity recapitulates the cardinal autonomic features of AAN in man. Signs of dysautonomia in the rabbit range from isolated cardiovagal impairment to severe panautonomic failure with fixed mydriasis, gastroparesis, dry eyes, impaired heart rate variability, hypotension and low plasma catecholamines. The severity of autonomic failure correlates with serum antibody levels. Immunohistochemical staining of superior cervical ganglia and myenteric plexus neurons demonstrates intact presynaptic nerve terminals and intact postsynaptic neurons containing cytoplasmic nAChR, but lacking surface nAChR. These findings define the autonomic physiology and histopathology of this novel animal model and support the concept that AAN in man is a disorder of ganglionic cholinergic synaptic transmission caused by ganglionic nAChR antibodies.
INTRODUCTION

Animal models play a critical role in the study of human neurological disorders. Studies of animals with experimental autoimmune myasthenia gravis (EAMG), for example, provided definitive evidence that antibodies against the muscle nAChR are the cause of myasthenia gravis (Patrick and Lindstrom 1973; Lennon et al. 1975; Lennon and Lambert 1980). EAMG is a universally accepted model for evaluating treatment strategies for myasthenia gravis and other human autoimmune disorders.

Neuronal nAChRs play a critical role in mediating fast synaptic transmission through autonomic ganglia. The ganglionic nAChR is a pentameric ion channel receptor that is structurally and antigenically similar to the muscle nAChR (Patrick and Stallcup 1977) and is typically composed of two $\alpha 3$ subunits in combination with $\beta 4$ subunits (Schoepfer et al. 1989; Patrick et al. 1993). Transgenic mice that are homozygous for null mutations in the $\alpha 3$ gene lack ganglionic nAChRs and have profound autonomic dysfunction (Xu et al. 1999).

The autonomic nervous system is critical for maintenance of cardiovascular, thermoregulatory, gastrointestinal, and neuroendocrine function. Autonomic failure can result from a variety of pathophysiological mechanisms and often has devastating consequences. Autoimmune autonomic neuropathy (AAN) is a severe, subacute form of autonomic failure that affects sympathetic, parasympathetic, and enteric autonomic function. Patients typically present with orthostatic hypotension, gastrointestinal dysmotility (commonly gastroparesis and severe constipation), anhidrosis, impaired pupillary light responses, dry eyes and dry mouth (sicca symptoms), and bladder dysfunction (Suarez et al. 1994; Klein et al. 2003). Most patients with this syndrome have serum autoantibodies specific for the neuronal ganglionic nAChR. High antibody levels are significantly correlated with severe dysautonomia (Vernino et al. 2000) and
with a predominance of “cholinergic” symptoms (impaired bowel and bladder motility, impaired pupillary function and sicca symptoms) (Klein et al. 2003).

We have previously reported an animal model of ganglionic nAChR autoimmunity produced by immunizing rabbits with a recombinant α3 neuronal nAChR subunit fusion protein (Lennon et al. 2003). In response to a single immunization, rabbits develop a chronic dysautonomic syndrome that we named experimental autoimmune autonomic neuropathy (EAAN). We previously described the overt signs of autonomic failure in these rabbits (Lennon et al. 2003). Using quantitative measures of autonomic function, we now demonstrate that EAAN in the rabbit reproduces the phenotype of AAN in man, namely a generalized dysautonomia with prominent “cholinergic” failure. We also demonstrate that chronic EAAN is characterized by a marked loss of postsynaptic nAChR on autonomic neurons.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Mayo Clinic Animal Care and Use Committee. Female outbred New Zealand white rabbits (Harlan, Indianapolis, IN), 8-10 weeks of age, were housed in individual cages. They were gently restrained in a “Bunny-Snuggle” (Lomir Biomedical, Malone, NY) for all procedures and were acclimated to handling in the research environment for 4 weeks prior to immunization. All autonomic tests were performed in awake animals.
Immunization

Details of antigen preparation and immunization have been described previously (Lennon et al. 2003). On experimental day 0, eighteen rabbits received intradermal immunization (divided among multiple dorsal sites) with 800 µg of recombinant GST-fusion protein (human neuronal nAChR α3 subunit, N-terminal extracellular domain, residues 1-205) emulsified in complete Freund’s adjuvant (CFA). Six control rabbits received saline in CFA. Twelve control rabbits were immunized with an unrelated fusion protein in CFA. Food intake was restricted from day 16 in six of these rabbits who served as weight-matched controls.

Laboratory studies

Blood (4-6 mL) was collected weekly from the central ear artery of resting rabbits (after completion of autonomic testing). Ganglionic nAChR binding antibodies were quantitated by a previously described radioimmunoprecipitation assay (Vernino et al. 1998, 2002). Concentrations of norepinephrine and dihydroxyphenylglycol (DHPG, the deaminated metabolite of norepinephrine) were determined from plasma samples (collected in EDTA and reduced glutathione, and stored at -80°C) using high-pressure liquid chromatography analysis.

Quantitation of heart rate and heart rate variability

Electrocardiograms (ECG) were recorded weekly from rabbits resting in a quiet room. Surface electrodes were applied to shaved sites over the dorsal midline and left sternal border with a ground electrode on the left ear. The ECG waveform was digitized at 2 kHz for off-line analysis. Stable 2-minute epochs of the ECG were analyzed using customized software to detect R-R intervals. Mean R-R interval was used to calculate resting heart rate. From differences
between successive R-R intervals, we calculated the root-mean-square of successive differences (rMSSD) as a simple time-domain measure of heart rate variability that is relatively unaffected by changes in heart rate (Tuininga et al. 1995). High frequency (0.2 to 1 Hz) and low frequency (0.02 to 0.15 Hz) components of heart rate variability were determined from spectral analysis of R-R intervals in 15-minute ECG recordings using a modified Wigner distribution (sampling frequency of 4 Hz, Gaussian window with alpha = 2.5)(Novak and Novak 1993). To confirm that the high frequency peak in spectral power coincides with the respiratory frequency, and therefore reflects respiratory modulation of heart rate via parasympathetic cardiovagal afferents, we recorded respiration simultaneously using a thermistor in front of the nares.

Quantitation of lacrimation

Basal and reflex tear production were assessed in resting unanesthetized rabbits, on at least four separate occasions for each rabbit, using a standard Schirmer tear test. A sterile calibrated strip of filter paper (ColorBar™ strips, Eagle Vision, Memphis, TN) was placed in the lower conjunctival sac. The migration of moisture on the paper strip was recorded after 5 minutes.

Measurement of systolic blood pressure

Systolic blood pressure was measured in resting rabbits using non-invasive cuff plethysmography. The rabbit’s right foreleg was shaved, and a clinical neonatal blood pressure cuff was applied. An optical pulse sensor (modified from a commercially available system, Harvard Apparatus, Holliston, MA) was placed on the foreleg distal to the cuff. Cuff pressure and pulse waveform were digitized and recorded simultaneously for off-line analysis. The cuff
was inflated to 140 mmHg and allowed to deflate slowly. The pressure at which the pulse waveform first appeared was recorded as the systolic pressure. Several determinations were made at each testing session. In six rabbits, a blood pressure telemetry device (Data Sciences International, St. Paul, MN) was implanted surgically in the abdominal aorta. After recovery from surgery, systolic blood pressure was determined using the non-invasive cuff method while simultaneously monitoring intraaortic pressure. The blood pressure values determined by the two methods were highly concordant. To assess orthostatic changes, rabbits were restrained in a fabric harness and held upright for 5 minutes while blood pressure and heart rate were recorded.

**Histopathological studies**

Rabbits were euthanized after day 110 by cardiac exsanguination while anesthetized (intraperitoneal ketamine and xylazine). At autopsy, we collected multiple fresh tissue samples including distal ileum and superior cervical ganglia. Tissues were fixed in 4% paraformaldehyde for 24 hours, equilibrated in 30% sucrose, and frozen as tissue blocks in embedding medium (Tissue-Tek®, O.C.T. compound). Sections (8 μm) were stained with hematoxylin and eosin, or double-labeled using monoclonal antibodies specific for synaptophysin (mouse IgG1, Chemicon International, Temecula, CA) and nAChR (MAb4, rat IgG specific for muscle nAChR and neuronal, α-bungarotoxin-insensitive, nAChRs, produced in our laboratory)(Lennon and Lambert 1981; V.A. Lennon and T.J. Kryzer, unpublished data). Human serum containing anti-neuronal nuclear autoantibody, type 1 (also known as “anti-Hu”) was used to label neuronal nuclei and perikaryon for quantitation of neuronal density and number (Fairman et al. 1995). Bound IgG was visualized using secondary antibodies conjugated with fluorescein or Cy3 (Chemicon). Fluorescence images (1 μm optical sections) were obtained using a laser scanning confocal
microscope (Ziess LSM 510).

**Statistical analysis**

Levels of ganglionic nAChR antibody in responder rabbits reached a plateau by day 40 after immunization (Lennon et al. 2003). We analyzed pooled autonomic data collected between days 40 and 110. Data from rabbits with high antibody levels (n=10) and from those with low antibody levels (n=4) were compared with data from control rabbits (n=12) and from food-restricted control rabbits (n=6). We used the two-tailed t-test to compare mean values for continuous autonomic variables. When an autonomic variable was assessed repeatedly over time, we also performed repeated measures analysis of variance (ANOVA). The distributions of skewed variables (rMSSD and antibody level) were normalized using a log transformation prior to analysis. The non-parametric Spearman correlation was used to evaluate the relationship between antibody level and autonomic data. Statistical analyses were performed using JMP Software (Version 4, SAS Institute, Inc.).

**RESULTS**

From day 20, ganglionic nAChR antibody was detected in 16 of 18 rabbits challenged with nAChR α3 fusion protein. Antibody levels were high in 11 (peak values 4.0 to 143.0 nmol/L) and low in five (peak values 0.2 to 1.9 nmol/L). As reported previously, rabbits with high ganglionic nAChR antibody levels fail to gain weight and have impaired pupillary response to light, gastroparesis and enlarged bladder at autopsy (Lennon et al. 2003). The two rabbits that did not produce antibodies in response to antigenic challenge did not differ from control rabbits in any autonomic variable. To control for the physiological effects of failure to
gain weight in animals with high antibody levels, food intake was restricted in 6 control rabbits to produce weight changes similar to those seen in EAAN rabbits.

Two of the 16 rabbits producing antibodies were euthanized before completing autonomic evaluation; one succumbed to severe intestinal pseudoobstruction, and another aspirated barium during a gastrointestinal motility study. The Table summarizes results of detailed quantitative autonomic studies for 14 seropositive rabbits and 18 control rabbits. Autonomic function in the 6 food-restricted control rabbits did not differ from the other controls.

*Rabbits with EAAN have impaired parasympathetic function*

The resting heart rate of rabbits with low antibody levels did not differ significantly from that of control rabbits. However, the resting heart rate of rabbits with high antibody levels was significantly higher (Fig. 1A). Administration of atropine (0.4 mg/kg i.v.) caused a marked increase in heart rate in control rabbits but did not significantly increase the heart rate of EAAN rabbits (Fig. 1B) indicating markedly reduced resting cardiovagal tone.

To further assess parasympathetic cardiovagal function, we measured beat-to-beat heart rate variability and performed spectral analysis. Resting heart rate variability was significantly reduced in 13 of 14 rabbits with EAAN (Fig. 1C), and the variability (rMSSD) was inversely correlated with antibody level (Fig. 1D). In rabbits with high antibody levels, spectral analysis of heart period variability revealed a complete loss of high frequency power as well as a marked reduction in low frequency power (90% decrease in mean low frequency power compared to controls) (Figs. 1E, F). The ratio of high frequency power to low frequency power (Hf/Lf, a measure of the relative strength of the cardiovagal parasympathetic influence on heart rate) was also reduced. The mean Hf/Lf in EAAN rabbits with high antibody levels was 0.19, compared to
Parasympathetic secretomotor function was assessed by measuring tear production. Lacrimation was significantly reduced in 10 of 14 rabbits with EAAN (Fig. 2A), and tear output was inversely correlated with antibody level (Fig. 2B). In five EAAN rabbits with dry eyes, intravenous administration of an acetylcholinesterase inhibitor (pyridostigmine, 0.1 mg/kg) transiently restored reflex tear production to normal levels.

**Rabbits with EAAN have impaired sympathetic function**

General sympathetic tone was assessed by measuring plasma catecholamine concentrations at rest. Plasma norepinephrine and DHPG levels were significantly lower in EAAN rabbits than in controls (Fig. 3A), and plasma norepinephrine concentrations showed a weak inverse correlation with antibody level (Spearman $\rho = -0.51$, $P=0.06$).

Resting systolic blood pressure was significantly lower in EAAN rabbits than in controls (Fig. 3B) consistent with an impairment of peripheral vascular tone. This resting hypotension could not be explained as a result of weight differences seen in EAAN rabbits with high antibody levels since resting systolic blood pressure in food-restricted control rabbits was normal. Resting blood pressure was inversely correlated with antibody level (Figs. 3C). Orthostatic hypotension, however, was not demonstrated in any rabbit.

**Surface nAChR is reduced on autonomic neurons of rabbits with EAAN**

At autopsy, most EAAN rabbits had dilated bowel and marked bladder distention (Lennon et al. 2003). Other tissues (brain, spinal cord, heart, adrenal gland, and cervical autonomic ganglia) were grossly normal. Superior cervical ganglia (SCG) and myenteric plexus
ganglia from distal ileum were examined histologically and immunohistochemically. There was no evidence of inflammation in these tissues. The density of neurons in cross-sections of SCG of EAAN rabbits did not differ from controls. However, the numbers of neurons in myenteric plexus ganglia were significantly reduced in EAAN rabbits with high antibody levels, 54.3 ± 10.1 neurons per cross-section of ileum (mean ± S.D.), compared to 77.2 ± 1.6 in controls (t-test; P=0.02).

Presynaptic terminals were readily identified in the myenteric ganglia and SCG of both EAAN and control rabbits as punctate synaptophysin-immunoreactivity in perineuronal areas (Fig. 4). Neurons in control rabbit ganglia exhibited nAChR immunoreactivity (MAb4) on the surface of the cell body, within the perikaryon and in the synaptic perineuronal areas. In all control ganglia, MAb4 immunoreactivity colocalized with synaptophysin indicating close proximity of presynaptic terminals to postsynaptic nAChRs. In ganglia from rabbits with high ganglionic nAChR antibody levels, however, MAb4 immunoreactivity was reduced and did not colocalize with synaptophysin (Fig. 4) indicating a loss of synaptic nAChR. Remaining MAb4 immunoreactivity was restricted to the cytoplasm of the perikaryon, presumably representing newly synthesized nAChR within endoplasmic reticulum or recently internalized nAChR. Immunohistochemical abnormalities were more variable in rabbits with low antibody levels; synaptic nAChR was present but qualitatively reduced compared to controls.

DISCUSSION

Patients with autoimmune autonomic neuropathy (AAN) characteristically have widespread autonomic failure affecting parasympathetic, sympathetic and enteric functions. This report demonstrates that experimental autoimmune autonomic neuropathy (EAAN), induced in
the rabbit by immunization with a neuronal nAChR α3 subunit protein, reproduces the important clinical features of AAN. The constellation of autonomic signs including the prominence of parasympathetic and enteric abnormalities is typical of the dysautonomia seen in patients with AAN (Klein et al. 2003). In both the human disorder and EAAN, the overall severity of dysautonomia correlates with the serum level of ganglionic nAChR antibody. In the rabbit, abnormalities in individual quantitative measures of autonomic function also correlate with antibody level.

Although postganglionic neurons may be either cholinergic or adrenergic, fast synaptic transmission through autonomic ganglia is mediated predominantly by nAChRs containing α3 subunits. Thus, the ganglionic lesion in EAAN impairs both cholinergic (gastrointestinal, secretomotor, cardiac chronotropic) and adrenergic functions. To characterize dysautonomia in rabbits with EAAN, we adapted non-invasive measures that are widely used to assess autonomic function in the clinical setting. As anticipated, reduction in the plasma concentration of DHPG proved to be a more sensitive indicator of mild adrenergic failure than the plasma norepinephrine concentration. Only 20% of peripherally released norepinephrine spills over into the bloodstream while 80% is taken up and metabolized to DHPG. Hence, plasma DHPG is a better index of postganglionic neuronal adrenergic function (Goldstein et al. 1988). The lack of orthostatic hypotension in EAAN rabbits is not surprising because of their compact body, small limbs and the short column of blood between the heart and brain.

Autonomic failure in rabbits with high antibody levels was uniformly generalized and severe. In rabbits with low antibody levels (< 2.0 nmol/L), however, manifestations of autonomic failure were mild and restricted in distribution. The two rabbits with lowest antibody levels had subtle dysautonomia that was not grossly apparent but was readily revealed by
quantitative autonomic testing. Both showed a mild reduction in plasma DHPG; one had dry eyes and the other had mild cardiovagal impairment (reduced heart rate variability). Similarly, patients with low levels of ganglionic nAChR antibody may present with limited manifestations of AAN, such as isolated gastrointestinal dysmotility or orthostatic intolerance (Vernino et al. 2000).

Immunohistochemical studies revealed a marked deficiency of surface and synaptic ganglionic nAChR on neurons in both sympathetic and enteric ganglia of rabbits with chronic EAAN. These results explain our previously reported electrophysiological findings which demonstrated that autonomic neurons in EAAN are viable, but have impaired responses to cholinergic synaptic input (Lennon et al. 2003). A plausible mechanism for loss of surface nAChR is cross-linking of surface nAChR by ganglionic nAChR-specific antibody leading to accelerated internalization and degradation, as has been demonstrated for muscle nAChR in myasthenia gravis (Lennon 1977; Drachman et al. 1978). In chronic EAAN, the density of neurons in paravertebral sympathetic ganglia appeared to be normal, but there was a reduction in the number of neurons in enteric plexus suggesting that some autonomic neurons not only lose synaptic nAChR but also sustain immunocytotoxic or apoptotic cell death. Inflammation and loss of ganglionic neurons have been documented in biopsied bowel of patients with paraneoplastic autoimmune autonomic neuropathy (Lennon et al. 1991; Condom et al. 1993; Anderson et al. 1996). Enteric neurons may be particularly sensitive to an immunological attack directed against neuronal ganglionic nAChR because fast cholinergic transmission is important in both the extrinsic and intrinsic neural regulation of gut motility, and in both the motor and mechanosensory limbs of the enteric nervous system.

EAAN represents the first animal model of neurological disease caused by autoimmunity
against neuronal nAChR. The reduction of synaptic nAChR in EAAN is reminiscent of the loss of muscle endplate nAChR seen in MG and in experimental autoimmune MG. The implications of an antibody-mediated channelopathy at a neuronal synapse, however, may be quite different from a similar process at the neuromuscular junction. Unlike the neuromuscular junction, neuronal synapses do not typically have a high safety margin for synaptic transmission. Also, an immunological attack at a neuronal synapse has a greater potential to cause irreversible damage to the postsynaptic cell. It is conceivable that compensatory utilization of alternative neurotransmitters (including neuropeptides and nitric oxide) may allow ganglionic autonomic synapses to continue to function in the face of cholinergic transmission failure.

The relative preservation of autonomic neurons suggests that EAAN (and by extension AAN in man) may be reversible. Strategies to improve ganglionic synaptic transmission or repopulate the synapse with nAChR would be expected to improve autonomic function. Consistent with this hypothesis, we found that one autonomic parameter (lacrimation) consistently improved after administration of an acetylcholinesterase inhibitor.

Our data define characteristic changes in autonomic physiology in EAAN which presumably result from an immune-mediated defect in ganglionic synaptic transmission. These findings implicate a specific loss of synaptic ganglionic AChR in this disorder. EAAN replicates the phenotype of AAN in man, and in the future, this animal model will allow development and rigorous preclinical testing of novel therapeutic strategies.
ACKNOWLEDGEMENTS/GRANT SUPPORT

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References


Lennon VA, and Lambert EH. Myasthenia gravis induced by monoclonal antibodies to


FIGURE LEGENDS

FIG. 1. Heart Rate Variability. A: Resting heart rate (HR) was significantly higher in rabbits with high levels of ganglionic nAChR antibodies compared to control rabbits. The resting heart rate in rabbits with low antibody levels was not different from controls. B: After intravenous injection of atropine (0.4 mg/kg), controls rabbits \( (n = 5) \) show a marked increase in HR while rabbits with high antibody levels \( (n = 5) \) show a negligible HR increase. C: Resting heart rate variability (quantified by the root-mean-square of successive differences, rMSSD, in heart period) was impaired in EAAN rabbits compared to controls. D: Heart rate variability was inversely correlated with antibody level (Spearman \( \rho = -0.75; p = 0.002 \)). The rabbit with the lowest antibody level had normal HR variability. E: Short segment of R-R interval data from a control rabbit (upper trace) and an EAAN rabbit (lower trace). The EAAN rabbit has lower R-R interval (higher HR) and marked loss of variability. F: Power spectral analysis of HR variability in a control rabbit (upper trace) reveals large high frequency (arrow) and low frequency peaks. In a rabbit with high antibody level (lower trace), there is a complete loss of high frequency variability and an attenuation of the low frequency peak. * \( P < 0.01 \); ** \( P < 0.0001 \).

FIG. 2. Lacrimation. A: Tear output, measured by Schirmer test, is significantly reduced in rabbits with high antibody levels compared to controls. ** \( P < 0.0001 \)

B: The reduction in tearing correlated with antibody level (Spearman \( \rho = -0.62; p = 0.018 \)).
FIG. 3. Sympathetic Function. A: Plasma norepinephrine (NE) in rabbits with high antibody levels and DHPG concentrations in all EAAN rabbits were significantly lower than controls. B: Resting systolic blood pressure was reduced in rabbits with high antibody levels. C: Mean resting systolic blood pressure was inversely correlated with antibody level (Spearman $\rho = -0.54$, $p = 0.05$). * $P < 0.01$; ** $P < 0.0001$

FIG. 4. Immunohistochemical studies of autonomic ganglia. Immunofluorescence confocal photomicrographs (400 x, original magnification) of autonomic ganglia labeled with nAChR monoclonal antibody MAb4 (red) and anti-synaptophysin monoclonal antibody (green). The overlay image shows areas of synaptophysin and nAChR colocalization (yellow). A: Superior cervical ganglia. Control ganglia show abundant MAb4 staining and colocalization of nAChR with synaptophysin in perineuronal synaptic areas. In EAAN rabbits, MAb4 staining is reduced and confined to the neuronal cell body. B: Myenteric plexus (from distal ileum). Control ganglia show abundant perineuronal MAb4 staining which colocalizes with synaptophysin. In myenteric ganglia of rabbits with high autoantibody levels, nAChR is confined to the neuronal cytoplasm; no colocalization of nAChR with synaptophysin is observed.
### TABLE 1: Summary of autonomic findings in EAAN rabbits and controls

<table>
<thead>
<tr>
<th>Autonomic Parameter</th>
<th>EAAN High Ab Level</th>
<th>EAAN Low Ab Level</th>
<th>Controls</th>
<th>Weight Loss Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 4)</td>
<td>(n = 12)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Weight change (Day 60, grams)</td>
<td>- 96 ± 78</td>
<td>+ 270 ± 198</td>
<td>+ 851 ± 95</td>
<td>+ 71 ± 65</td>
<td>---</td>
</tr>
<tr>
<td>Gastroparesis (by fluoroscopy)</td>
<td>5 / 5</td>
<td>0 / 4</td>
<td>0 / 6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Impaired pupil light response</td>
<td>10 / 10</td>
<td>0 / 4</td>
<td>0 / 12</td>
<td>0 / 6</td>
<td>---</td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>207 ± 3.8</td>
<td>195 ± 7.4</td>
<td>194 ± 2.5</td>
<td>192 ± 5.0</td>
<td>0.04†</td>
</tr>
<tr>
<td>rMSSD* (msec)</td>
<td>2.3 ± 0.14</td>
<td>3.6 ± 0.82</td>
<td>8.1 ± 0.95</td>
<td>8.7 ± 1.80</td>
<td>0.0002†</td>
</tr>
<tr>
<td>Tear output (mm / 5 min)</td>
<td>9.4 ± 0.6</td>
<td>16.3 ± 2.7</td>
<td>17.5 ± 0.5</td>
<td>16.6 ± 1.9</td>
<td>&lt; 0.0001†</td>
</tr>
<tr>
<td>Resting systolic BP (mm Hg)</td>
<td>87 ± 1.8</td>
<td>99 ± 3.5</td>
<td>104 ± 1.7</td>
<td>100 ± 1.3</td>
<td>&lt; 0.0001†</td>
</tr>
<tr>
<td>Resting NE (pg/mL)</td>
<td>164 ± 19</td>
<td>236 ± 34</td>
<td>265 ± 29</td>
<td>291 ± 47</td>
<td>0.01†</td>
</tr>
<tr>
<td>Resting DHPG (pg/mL)</td>
<td>890 ± 27</td>
<td>914 ± 17</td>
<td>1384 ± 100</td>
<td>1200 ± 48</td>
<td>&lt; 0.0001†</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. or as number of rabbits / number tested.

* rMSSD = root-mean-square of successive differences of R-R interval (a measure of heart rate variability primarily reflecting the strength of cardiovagal innervation)

† Repeated measures ANOVA; all EAAN rabbits (n = 14) compared to all controls (n = 18). Values shown are from data collected between days 40–110 after immunization.
A. MAb4 (AChR)  synaptophysin  overlay
   Control  
   EAAN

B. MAb4 (AChR)  synaptophysin  overlay
   Control  
   EAAN