A novel in vivo approach to study circadian rhythmicity of glucocorticoids using adeno-associated virus

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Introduction

The hypothalamic-pituitary-adrenal (HPA) axis regulates glucocorticoid release in a circadian rhythm as well in response to stress. There are two independent pathways that regulate circadian glucocorticoid production, both potentially mediated by the adrenal molecular clock:

1. Adrenal innervation by sympathetic preganglionic fibers which are carried in the thoracic splanchnic nerve.
2. Diurnal rhythms in plasma adrenocorticotropic hormone (ACTH).

Two distinct tissues make up the adrenal gland: the cortex (mesoderm-derived), and the medulla (neural crest). The medulla is thought to modulate cortical function, but the precise mechanism remains unknown.

An experimental approach that genetically manipulates adrenal activity could potentially delineate the interplay between medullary and cortical function. One way to do this is to infect adrenal tissue with recombinant adeno-associated virus (AAV) capable of silencing a gene of interest.

This is a novel approach in the adrenal gland, so we must first develop a protocol that will optimize methodological variables such as AAV serotype, surgical procedure, injection volume, and infection time-course.

Methods

Experiment 1

**Injections**: In rats under general anesthesia, a midline incision opened the intraperitoneum allowing access to adrenal glands by retraction (same procedure used in all experiments)

**AAV Serotypes**: AAV2 (UMN Core; 1.925e10 vg/ml); AAV5 (UMN Core; 1.8e12 vg/ml)

**Injection**: 5 µl of neat or 10 µl of 0.2 dilution of each serotype, injected at a rate of 1 µl/minute using an automated infusion pump

**Time-course**: Adrenal glands were collected after 1 or 2 weeks

**Immunohistochemistry**: Adrenal glands were sectioned at 10 µm using a cryostat and immunostained using polyclonal rabbit anti-GFP (qG) and donkey anti-rabbit (Rb) Cy3 conjugate (same procedure used in all experiments)

Experiment 2

**AAV Serotypes**: AAV2 (UMN Core; 1.925e10 vg/ml); AAV5 (UMN Core; 1.8e12 vg/ml); AAV2 (Powell Gene Therapy; 1.8e12 vg/ml)

**Injection**: 10 µl uninfected, injected at a rate of 1 µl/minute using an automated infusion pump

**Time-course**: 6 weeks

**Functional testing**: after 2 weeks, rats were subjected to a 15-minute restraint stress. Blood samples were collected by tail snap at 0, 15, 30 and 60 minutes. Plasma ACTH and corticosterone levels were assessed using radioimmunoassay. AAV injected animals were compared to controls that underwent a sham surgery (incision and exposure of adrenal glands, but no injection).

Experiment 3

**AAV Serotypes**: AAV5 (UMN Core; 1.8e12 vg/ml); AAV2 (Powell Gene Therapy; 1.8e12 vg/ml); AAV8 (Dr. Murray Blackmore; 2012 vg/ml & 2013 vg/ml)

**Injection**: 10 or 25 µl delivered as a bolus, re-injection of mannitol (5 µl) with AAV5

**Time-course**: 4 weeks

**Functional testing**: same as described in Experiment 2, but without sham surgery controls.

Experiment 1

![Fig 1. Adrenal infection by AAV5-GFP after 1 week (A) and 2 weeks (B). Both images are of cortical cells only, as none of the injected adrenals showed any GFP expression in the medulla region. AAV2-GFP did not infect any adrenals. There was no apparent relationship between injection volume and degree of infection. Scale bar = 100 µm.]

Experiment 2

![Fig 2. AAV2-GFP (A) from Powell Gene Therapy yielded infection comparable to that by AAV5-GFP (B). AAV2-GFP from the UMN Core still did not successfully infect any adrenals. A higher objective revealed differential labeling of cell components with immunostaining of AAV2-GFP (C). GFP appeared to be localized to the nucleus, while antibody labeling of GFP appeared only in the cytoplasm. Injection volume did not affect the degree of infection. Scale bars = 100 µm.]

Experiment 3

![Fig 3. The intensity of AAV2-GFP was low but its signal became greatly amplified with immunostaining (A). AAV5-GFP produced a bright signal, but infected less of the adrenal than AAV2 and AAV8 (B). AAV8-GFP infected much of the cortex, and also appeared to infect medullary cells (C). There was no apparent relationship between the injection volume and infection. Injection of AAVs did not appear to affect adrenal function. Red – DAPI; scale bar = 200 µm.]

Conclusions

AAVs infect the rat adrenal gland to varying degrees

Serpotype

- AAV2 and AAV5 may selectively target the cortex. AAV8 yields greater infection overall, and has the most potential for medullary infection

Volume

- Volume of AAV delivered does not correlate to infection. AAV titer (concentration of viral genomes) is more important

Injection

- Bolus injection is more efficient than slow infusion. Coinjection of mannitol, an osmotic agent, does not increase infection.

Injection of AAV-GFP does not appear to alter adrenal function, making AAVs viable tools for functional genetic manipulations of the adrenal gland in the future.

Future experiments will address the following variables:

- A longer time course following delivery of AAV (3-4 weeks) to determine if infection can increase with time.
- Attempt a dorsal surgical approach, which involves clamping off the adrenal gland along with surrounding fat. This method may minimize outflow of AAV and allow for more accurate, consistent infections.
- Double-label for tyrosine hydroxylase (medulla-specific) and GFP to verify that medullary cells are being infected.

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References