**Introduction**

- Botulinum toxins act on the neuromuscular junction by inhibition of the acetylcholine release from presynaptic vesicles inducing muscle relaxation. This property is used to treat numerous disorders largely characterized by excessive or inappropriate muscle contraction (e.g. myasthenia, spasticity, dystonia).

- The present study evaluates the effect of botulinum neurotoxin on the neuromuscular junction following a unilateral injection of natural botulinum neurotoxin A1 (BoNT/A1) in the gastrocnemius muscle (Gc) in rats, using three analysis methods for each animal:
  - Pedobarographic behavioural using the Dynamic Weight Bearing test (DWB) (2)
  - Electromyography measurements of the compound muscle action potential (CMAP) (3)
  - Digit abdution score (DAS) (4)

- We also investigated the impact of BoNT/A1 (0.1, 1 and 10 pg/rat) given as 1 vs 2 volumes into Gc.

**Methods**

**Animals**

- Fifty-four adult female Sprague-Dawley rats (Janvier, France) of about 180 g at the injection day (D0) were used. They had free access to water and were fed with pellets complete diet ad libitum.

- Animal procedures of the study were approved by the Animal Care and Use Committee of the French Ministry of National Education, Higher Education and Research.

**Treatment**

- BoNT/A1 (List Biological Labs Inc, USA) diluted in 0.2% Gelatin Phosphate Buffer (GPB) was administered at 0.1, 1 and 10 pg/rat in a total fixed volume of 30 µL.

- In isoflurane-anesthetized rats, BoNT/A1 or GPB were injected into the center of one or two heads of Gc. BoNT/A1 or GPB were administered as a single injection (30µl) in the lateral head of Gc or as a double injection, 15 µL in each of the medial and the lateral heads.

- The right hind paw was injected with BoNT/A1 and the left one with GPB. The control rats received GPB in the right hind paw and no injection into left one at all.

- Investigators were kept blind to the test substance throughout the study.

**Assays**

- **DWB test**
  - Rats were weighed and then individually placed in DWB recording box (24×24×39 cm) with a sensors-inserted floor sensitive to pressure (BioSeb, France). The weight pressure and surface applied on sensor cells by animal’s paws were recorded over 5 min, analyzed and calculated by a software algorithm (DWB13.2b, Bioseb, France).
  - Results were expressed as the ratio of the weight (in surface) of the ipsi/contralateral hind paw, regardless of the postures of the animals (all postures). Other specific postures were also scrutinized, especially the Posture 4 – when 4 paws of rat are in contact with the floor. Posture 3 + 2 = 2 hindpaws and only 1 front paw in contact with the floor and Posture 2 – the rat is standing on its 2 hindpaws.

- **CMAP measurement**
  - CMAP is an electrical signal generated by synchronized activation of a group of muscle fibers following a brief electrical stimulation of motor neuron fibers. It is reduced in muscle injected with BoNT/A1 (5).

- In isoflurane-anesthetized rats, subcutaneous monopolar needle electrodes were used for both stimulation and recording. A ground electrode was placed in the hindpaw pad. The sciatic nerve was stimulated with a single pulse (12.8 mA of 0.2 ms duration) at the sciatic notch. CMAP measured into the center of the lateral Gc was recorded with an electromyograph (Medtronic, France) on the 2 hindpaws.

- Measured parameters of CMAP include amplitude, latency, duration and area, as illustrated in Figure 2.

- **DAS assay**
  - The DAS assay is based on the toe spreading reflex exhibited when rats are grasped lightly around the torso and lifted to the air (6). The reflex is inhibited after administration of BoNT into the Gc measurements. The varying degrees of digit abdution are scored on a five-point scale: 0= normal to 4= maximal reduction in digit abdution.

**Design**

- The study was conducted on 2 cohorts of 27 rats (see tables below).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosis (µL)</th>
<th>Number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1</td>
<td>N=2</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>N=2</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>N=4</td>
</tr>
<tr>
<td>D0 1 µL</td>
<td>0.1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 1 µL</td>
<td>1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 1 µL</td>
<td>10</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 2 µL</td>
<td>0.1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 2 µL</td>
<td>1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 2 µL</td>
<td>10</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 3 µL</td>
<td>0.1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 3 µL</td>
<td>1</td>
<td>N=6</td>
</tr>
<tr>
<td>D0 3 µL</td>
<td>10</td>
<td>N=6</td>
</tr>
</tbody>
</table>

**Results**

**DWB test**

- All postures were affected by BoNT/A1 injection (not shown), but the Posture 2 paws appeared as the most sensitive and only the results in this posture are presented. The effects of BoNT/A1 were similar on the weight and the surface data.

- The vehicle injection did not affect the weight distribution as shown by the ratio weight on the ipsilateral paw / weight applied on the contralateral paw approx. 1.

- The 1st active dose was the mid dose, 1 pg/rat when injected as a double injection only, which decreased the weight ratio by 37%. The highest dose 10 pg/rat led to the same decrease of 60% in weight ratio both for single or double volumes.

**CMAP measurement**

- The vehicle injection did not affect the CMAP parameters which were equivalent in left and right hind paws ( white bars in Fig.3 and 4).

- Compared to the control group, the CMAP parameters of the contratral left paw in rats injected with BoNT/A1 were not affected. A small decrease in amplitude was observed at 1 pg/rat administered as a double volume (Fig.4).

- At the lowest dose of 0.1 pg/rat, BoNT/A1 had an effect only when injected as a double volume, with a decrease in the amplitude by 18 % and an increase in the latency by 30% (Fig.4).

- The doses 1 pg/rat and 10 pg/rat administered as a single volume decreased the amplitude by 62% and 100%, respectively and increased the latency by 30% and 70%, respectively (Fig.3). When BoNT/A was administered as a double volume, the effects were higher to reach 90% and 100% for amplitude and 43% and 129% for latency at 1pg/rat and 10 pg/rat, respectively (Fig.4).

**DAS assay**

- The Control group displayed DAS 0.

- BoNT/A1 induced a dose-response in DAS with 2 and 5 rats reaching DAS1 at 1 pg/rat and 10 pg/rat, respectively. The max DAS value reached was 2 at 10 pg/rat.

- The same response was obtained with the 2 types of BoNT administration.

**Conclusions**

The study conducted showed that:

- Pedobarographic (DWB test) and electromyographic (CMAP) methods allowed a complete assessment of BoNT/A1 neuromuscular activity following injection into gastrocnemius muscle of the rat. These effects observed on digit abdution (DAS assay) were weak and the results too variable.

- CMAP measurement appeared as the most sensitive method as it was able to detect an effect of BoNT/A1 at the lowest dose of 0.1 pg/rat.

- DWB test is an easy test allowing the investigation of functional effect of BoNT/A1 in the free moving rat.

- The study showed that the effect of BoNT/A1 was increased when the volume was administered over 2 sites instead of 1 site.