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Comparative analysis of pharmacological treatments with N-acetyl-DL-leucine (Tanganil) and its two isomers (N-acetyl-L-leucine and N-acetyl-D-leucine) on vestibular compensation: Behavioral investigation in the cat

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ABSTRACT

Head roll tilt, postural imbalance and spontaneous nystagmus are the main static vestibular deficits observed after an acute unilateral vestibular loss (UVL). In the UVL cat model, these deficits are fully compensated over 6 weeks as the result of central vestibular compensation. N-Acetyl-DL-leucine is a drug prescribed in clinical practice for the symptomatic treatment of acute UVL patients. The present study investigated the effects of N-acetyl-DL-leucine on the behavioral recovery after unilateral vestibular neurectomy (UVN) in the cat, and compared the effects of each of its two isomers N-acetyl-L-leucine and N-acetyl-D-leucine. Efficacy of these three drug treatments has been evaluated with respect to a placebo group (UVN + saline water) on the global sensorimotor activity (observation grids), the posture control (support surface measurement), the locomotor balance (maximum performance at the rotating beam test), and the spontaneous vestibular nystagmus (recorded in the light). Whatever the parameters tested, the behavioral recovery was strongly and significantly accelerated under pharmacological treatments with N-acetyl-DL-leucine and N-acetyl-L-leucine. In contrast, the N-acetyl-D-leucine isomer had no effect at all on the behavioral recovery, and animals of this group showed the same recovery profile as those receiving a placebo. It is concluded that the N-acetyl-L-leucine isomer is the active part of the racemate component since it induces a significant acceleration of the vestibular compensation process similar (and even better) to that observed under treatment with the racemate component only.

1. Introduction

Acute unilateral lesion of the peripheral labyrinth induces in most of the species a typical syndrome made of static (at rest) and dynamic (when moving the head or whole body) deficits. The static syndrome includes oculomotor (spontaneous vestibular nystagmus) and postural (head tilt towards the lesioned side, support surface enhancement, circling and falls to the lesioned side) deficits. This syndrome results from the strong imbalance between the spontaneous resting activity of the vestibular nuclei (VN) neurons on both sides, characterized by a strong shut-down of activity on the ipsilesional side (Ris & Godaux, 1998; Ris et al., 1995; Zennou-Azogui et al., 1993; Lacour et al., 1989; Smith and Curthoys, 1989; Curthoys, 1989; Lacour and Tighilet 2010; Dutia 2010 for reviews). The dynamic syndrome includes a deep alteration of the vestibulo-ocular reflex, responsible for the poor gaze stabilization observed during head motion to the lesion side, which induces oscillopsia and fall in vestibular loss patients. The equilibrium function is also strongly impaired due to the alteration of the vestibulo-spinal reflexes controlling whole body orientation and stabilization in space.

The vestibular deficits are totally or partially compensated over time in a process called «vestibular compensation» (reviewed in Smith and Darlington, 1991; Dieringer, 1995; Vidal et al., 1998; Lacour and Tighilet, 2010). The functional recovery includes both plastic events within the VN, which rebalance the activity on both sides, responsible for the recovery of the static functions (Lacour and Tighilet, 2010), and sensory and behavioral substitution processes mainly involved in the recovery of the dynamic functions. The real nature of the mechanisms restoring the spontaneous resting discharge of the deafferented vestibular cells is very complex. Neurochemical (Darlington and Smith, 2000) and

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structural (Tighilet et al., 2007a) reorganizations in the VN are very likely involved, and modifications of the membrane properties of the VN neurons (increased intrinsic excitability, decreased sensitivity to inhibitory neurotransmitters) have been described (see Vidal et al., 2015; Lacour and Bernard-Demanze, 2015).

The neuropharmacology of the vestibular system is relatively well documented (see Soto and Vega, 2010), but the miraculous drug to treat vestibular loss patients remains to be discovered. Many papers showed in animal models that the vestibular compensation process could be improved with different pharmacological substances (Peppard, 1986; Darlington et al., 1991; Smith and Darlington, 1991; Lacour and Sterkers, 2001), many of them inducing a shortening of the time constant of the functional recovery. Particularly interesting are drugs behaving as agonizts or antagonists of the histaminergic and GABAergic systems, or as blockers of the calcium channel blockers (Rascol et al., 1995; Lacour and Sterkers, 2001; Hain and Uddin, 2003; Soto and Vega, 2010, Soto et al., 2013; Chabbert, 2013; Lin and Aligene, 2013). N-acetyl-DL-leucine (Tanganil: Laboratoires Pierre Fabre) is used in clinical practice for the symptomatic treatment of vestibular patients with acute vertigo and neurovegetative signs of peripheral vestibular origin (Neuzil et al., 2002). This drug improved postural compensation in patients after unilateral vestibular neurotomy and labyrinthectomy (Ferber-Viart et al., 2009), a result we had already demonstrated in experimental preliminary investigations conducted in our UVN cat model (Pascalis, 1990; Lacour, 1995). In the mice animal model, N-acetyl-DL-leucine reduced vertigo (Leau and Ducrot, 1957), and electrophysiological data recorded in vitro in a guinea-pig model suggested that N-acetyl-DL-leucine might rebalance the abnormal membrane potential of the VN neurons (Vibert and Vidal, 2001).

The present study summarizes experimental data we have collected in the UVN cat model between 2000 and 2011. It was aimed at (1) confirming our preliminary data on the beneficial effects of N-acetyl-DL-leucine on postural compensation, and (2) comparing the effects of the racemate to those of each of its two isomers (N-acetyl-L-leucine and N-acetyl-D-leucine). The efficacy of these three drug treatments was evaluated on the postural control (observation scales, support surface measurements), the locomotor balance (maximum performance on a rotating beam), and the spontaneous nystagmus (in the light). It was assessed by comparison with a placebo group (UVN animals receiving saline water).

2. Materials and methods

2.1. Ethics statement

All experiments were carried out in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication no. 80-23) revised 1996 for the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the Policy on Ethics approved by the Society for Neuroscience in November 1989, and amended in November 1993. Every attempt was made to minimize both the number and the suffering of animals used in this experiment. Animals were housed in a large confined space with normal diurnal light variations and free access to water and food.

2.2. Study design and drug protocol

The experiments were conducted on a population of 17 cats (ISOQUIMEN, Barcelona, Spain). The animals were subdivided into four groups, each of them being submitted to a unilateral vestibular neurectomy (UVN) performed on the left side.

- Control group: 5 UVN cats receiving no drug treatment but saline water,
- First experimental group: 4 UVN cats treated with the racemate component (N-acetyl-DL-leucine),
- Second experimental group: 4 UVN cats treated with the N-acetyl-L-leucine isomer,
- Third experimental group: 4 UVN cats treated with the N-acetyl-D-leucine isomer.

Concerning the treated groups, the pharmacological treatment was started just after the end of the surgery and administrated until complete functional recovery. The intravenous (IV) route of administration was performed with the cat under local anesthesia. The animals received the IV injection during the first three postlesion days. Thereafter, a per os (p.o.) treatment was administrated by adding the drug in the food. For the racemate, the doses were 30 mg/kg/day for the i.v. route, and then 60 mg/kg/day for the p.o. route. For the isomers, the doses were 15 mg/kg/day, and then 30 mg/kg/day, for the i.v. and p.o. routes, respectively. This protocol mimics the dosage and routes of administration used for the acute and chronic treatment of vertigo in vestibular defective patients. It takes into account the absolute bioavailability (45%) observed for the p.o. route compared to the i.v. one. For the control group, saline water was administered by using the i.v. route and covered the first three postlesion days.

2.3. Vestibular neurectomy

Adult male cats weighing 4–5 kg were anesthetized with ketamine (20 mg/kg, i.m.; Rhône Poulenc, Mérieux, France), received an analgesic (Tolfedine, 0.5 ml, i.m.; Vetoquinol, Lure, France) and were kept at physiological body temperature using a blanket. The vestibular nerve was sectioned on the left-side at the post-ganglion level in order to leave the auditory division intact after mastoidectomy, partial destruction of the bony labyrinth, and surgical exposure of the internal auditory canal (Xerri and Lacour, 1980). Animals were maintained under antibiotics for 7 days and analgesics for 3 days.

The classical postural, locomotor, and oculomotor syndrome seen just after surgery was used to assess the effectiveness of the lesion. Completeness of vestibular nerve section had been assessed by histological procedures in previous studies (Lacour and Roll, 1976). A strong spontaneous horizontal vestibular nystagmus with its slow phase directed to the lesioned side is observed, associated to a vertical eye deviation. The postural symptom consisted of strong hypotonia of the limb extensors ipsilateral to the lesion and strong hypertonia of the contralateral extensor muscles. The muscular tone asymmetry prevented the animal from holding itself upright and, consequently, all the cats remained lying on their lesioned side after UVN. In addition, a strong head tilt toward the lesioned side associated with a rotation of the chin to the intact side was seen just after surgery. When the animal is capable to stand erect, it shows an enhanced support surface and falls on its lesioned side. As soon as it can walk, its locomotion trajectory is deviated toward the side of the lesion and it falls many times.

2.4. Quantification of the behavioral deficits

2.4.1. Static postural deficits

The posture deficits and recovery were evaluated by measuring the surface delimited by the four legs of the cat while standing erect at rest, without walking. Support surface is considered a good estimate of postural control since it reflects the cat's behavioral adaptation compensating the static vestibulospinal deficits induced by the vestibular lesion (Tighilet et al., 1995). As a rule, the surface was very small in the normal cat (about 50–100 cm^2) and

greatly increased in the days following unilateral vestibular lesion. To quantify the support surface, cats were placed in a device with a graduated transparent floor that allowed them to be photographed from underneath. Five repeated measurements were done for each cat tested at each postoperative time, and an average was calculated for each experimental session. The support surface was measured as the surface delimited by the four legs by an image analysis system (Canvas, 9^{TM}, Deneba software, Miami, FL). Postlesion data were compared to prelesion values by using individual references, each animal being its own control. Recovery of static posture function was assessed by the changes and development of the support surface over time. The recovery was considered as total when the support surface returned to the preoperative value (unity, i.e.1).

2.4.2. Static vestibulo-ocular deficits

The spontaneous horizontal vestibular nystagmus induced by the UVN was recorded by videotracking of the eye movement (SIMI device). One day after surgery, the cat was placed on an apparatus with its head fixed, thus maintaining the horizontal semi-circular canals in the horizontal plane. The frequency of the horizontal spontaneous nystagmus was measured in the light as the number of quick phase beats towards the contralateral side relative to UVN in 10 s (five repeated measures per animal per sampling time). Each recording session (duration = 15 min) was located at the same period of the day (in the morning) in order to counteract possible variations due to the alertness state of the animals. Full recovery was achieved when the vestibular nystagmus totally disappeared in the light. (Tighilet et al., 2006).

2.4.3. Sensorimotor activity

The cats were placed in an open-field and their behavior was evaluated by computing the displacement of virtual markers on their head and body (SIMI device). Each recording session was placed also at the same period of the day, and the recordings were made one hour after the drug was administered. As a rule, four recordings of three mins each were done, with an interval of 15 min between the sessions. In addition, observation scales were used to qualitatively describe the behavior of the animal (walk, jump…), to evaluate the motivation and the neurovegetative signs (vomiting), etc.

Finally, a sensorimotor activity score was calculated by mixing the following criteria:

Prostration (0 or 1)
Try to move but cannot (0 or 1)
Walking (0–4, depending on the number of steps/min)
Circling: (0–2)
Tumbling: (0–2)
Head nystagmus (0 or 1)
Deviations of the locomotor trajectory and falls (0–2)
Active exploration, climbing and jumping (0–4)
Neurovegetative signs (0–2)
Motivation (0 or 1)

The curves evaluating the sensorimotor activity as a function of the postlesion time were elaborated from this 20 points scale. The analysis was performed for each group of cats during the first five postlesion days only. Results close to zero point to a strong impairment of the sensorimotor activity of the animals while those close to 20 indicate a nearly complete recovery.

2.4.4. Locomotor balance recovery

The rotating beam test was used to evaluate the deficits and the recovery over time of the dynamic equilibrium function in the UVN cats. Two compartments were connected by a horizontal beam (length: 2 m; diameter: 0.12 m) placed 1.2 m from the ground. The beam could be rotated along its longitudinal axis with a constant angular velocity ranging from 0° to 750°/s (linear tangential speed: 0–37 m/min). Animals were conditioned to cross over the beam when a light signal was turned on in the opposite compartment. First crossings were made on the immobile beam and, thereafter, on the rotating beam. Rotation velocity of the beam was progressively increased and we measured the highest speed of beam rotation that did not induce a fall in four consecutive trials. This constituted the maximal locomotor balance performance (MaxP) of the cat before surgery. The preoperative training necessitated 8–12 training periods of 1 h per day. Very few inter-animal differences were noticed (range: 27–37 m/min; mean: 33 m/min; SD: 2.08 m/min). The MaxP values recorded after UVN were expressed for each cat in percent of its preoperative MaxP.

2.4.5. Data analysis

Statistical analysis was carried out using repeated-measures analyses of variance (ANOVAs) with group (treatments, controls) or functions (nystagmus, posture, locomotor balance) as the between-animals factors and postlesion sessions as the within-animals factors. Global evaluation was done with the Fisher PLSD multicomparison test, while comparisons for groups were evaluated with the Newman–Keul test. Results were considered significant for $P < 0.05$.

The sensorimotor activity was evaluated separately by plotting the global scores obtained from the observation scales during the first five postlesion days. No particular statistical analysis was done on these data since they reflect qualitative more than quantitative observations.

3. Results

The general ANOVA showed significant inter-group differences for each of the three parameters analyzed in this study: spontaneous vestibular nystagmus [$F(3,120) = 147.9$; $P < 0.0001$], support surface [$F(3,78) = 1098$; $P < 0.0001$], and Maximal Performance on the rotating beam [$F(3,50789) = 69$; $P < 0.0001$]. Interestingly, no significant differences were found between the animals within each group, suggesting that groups were homogeneous and that drug treatments did not differently affect the animals composing each group (see Table 1).

The Anovas with repeated-measures also showed significant differences depending on the postlesion time for the spontaneous vestibular nystagmus [$F(9,372) = 999.3$; $P < 0.0001$], support surface [$F(24,14) = 1428.2$; $P < 0.0001$], and Maximal Performance on the rotating beam [$F(23,18507) = 222.1$; $P < 0.0001$] (Table 1). The interaction between these two factors (group and postlesion time) was also significant for the nystagmus [$F(27,11) = 31.3$; $P < 0.0001$], posture [$F(72,0.78) = 78.2$; $P < 0.0001$], and Maximal Performance on the rotating beam [$F(69,1272) = 15.3$; $P < 0.0001$] (see Table 2).

Complementary ANOVAs indicated that the control group (lesioned, saline water) and the lesioned group treated with the isomer D (N-acetyl-D-leucine) behaved similarly (non significant differences). In the same way, the two lesioned groups treated with either the racemate component (N-acetyl-DL-leucine) or the isomer L (N-acetyl-L-leucine) showed similar recovery profiles (NS). By contrast, the two former groups (placebo, isomer D) differed significantly from the two latter ones (racemate, isomer L) ($P < 0.0001$).

Taken together, the data demonstrate that the vestibular compensation process is strongly accelerated in the groups treated with the racemate component or the isomer *L*, while isomer *D* has no effect at all on the recovery process. This latter group behaved

Table 1
Statistical analysis of saline (NaCl), N-acetyl-DL-leucine, N-acetyl-D-leucine and N-acetyl-L-leucine treatments on the horizontal spontaneous nystagmus, posture, and equilibrium function in unilateral vestibular neurectomized cats.

Source of variation	df	*F*	*P*
Nystagmus			
Inter-group differences	3	147.9	0.0001
Within-group differences	16	12.1	NS
ANOVA (repeated measures)	9	999.3	0.0001
Posture			
Inter-group differences	3	1097.7	0.0001
Within-group differences	16	11.6	NS
ANOVA (repeated measures)	24	1428.2	0.0001
Locomotor balance function			
Inter-group differences	3	69.0	0.0001
Within-group differences	16	8.4	NS
ANOVA (repeated measures)	23	222.1	0.0001

Repeated-measure analysis of variance (ANOVA) of the horizontal spontaneous nystagmus, posture recovery, and equilibrium function recovery. Groups (unilateral vestibular neurectomized cats treated with N-acetyl-DL-leucine, N-acetyl-D-leucine, N-acetyl-L-leucine or NaCl) are the main fixed effects providing the sources of variation among cats. df: degree of freedom; *F*: Scheffé's test; *P*: probability level; NS: non significant.

Table 2
Statistical analysis of saline (NaCl), N-acetyl-DL-leucine, N-acetyl-D-leucine and N-acetyl-L-leucine treatments on the horizontal spontaneous nystagmus, posture, and equilibrium function in unilateral vestibular neurectomized cats.

Source of variation	df	*F*	*P*
Nystagmus			
Interaction group × time delay	27	31.3	0.0001
Posture			
Interaction group × time delay	72	78.2	0.0001
Locomotor balance function			
Interaction group × time delay	69	15.3	0.0001

Repeated-measure analysis of variance (ANOVA) of the horizontal spontaneous nystagmus, posture recovery, and equilibrium function recovery. Group (unilateral vestibular neurectomized cats treated with N-acetyl-DL-leucine, N-acetyl-D-leucine, N-acetyl-L-leucine or NaCl) and postoperative treatment period are the main fixed effects providing the sources of variation among cats. Significant interaction between these two variables are illustrated. df: degree of freedom; *F*: Scheffé's test; *P*: probability level.

like the placebo group receiving saline water. These differential effects of the treatments are seen for all the parameters analyzed (static posture, vestibular nystagmus, locomotor balance recovery).

3.1. Compensation of the horizontal spontaneous nystagmus

Fig. 1 shows the recovery time-course of the spontaneous nystagmus recorded in the light after UVN, as a function of the postoperative time. As already reported in previous studies (Tighilet et al., 2006, 2007b; Dutheil et al., 2013) the vestibular nystagmus totally disappeared within one week in the light in the lesioned untreated cats. The same recovery profile is shown here for the group receiving only isomer D. In both groups, the linear regression curves were similar, with slopes of 2.1 and 2.6 for the control and isomer D groups, respectively, and similar time delays for total suppression of the nystagmus (7 and 8 days, respectively).

By contrast, the groups of cats treated with either the racemate component or the isomer L showed a strong acceleration of the nystagmus compensation. This faster recovery is shown by the linear regression curves exhibiting higher regression slopes (3.6 and 3.9 for the racemate and isomer L, respectively; $P<0.001$) and the earlier time delays for nystagmus suppression (4 days: $P<0.0001$). One can see significant differences between these two groups and the two previous ones (controls and isomer D) as early as the first postlesion day. The mean number of beats/10s was

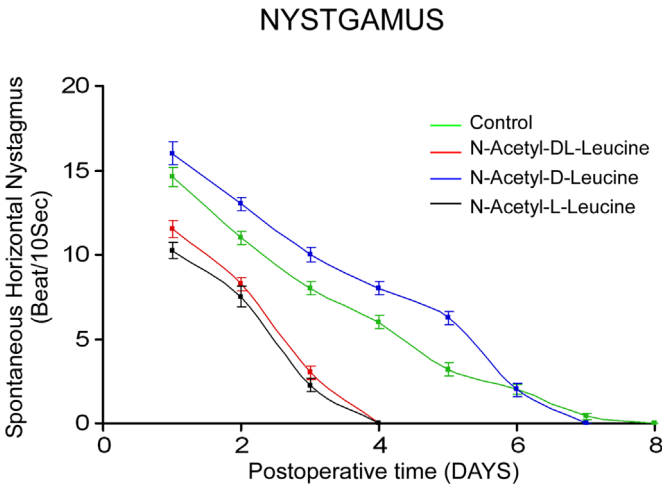

Fig. 1. Comparison of the effects of the three pharmacological treatments with respect to the controls on the compensation of the spontaneous horizontal vestibular nystagmus recorded in the light.Time-course of the number of beats in the vestibular nystagmus (per 10 s; ordinates) as a function of the postoperative time (days; abscissae) for the four groups of cats ($N=4$ per group but $=5$ for the controls): the UVN untreated cats (controls, saline water), the UVN cats receiving the racemate component (N-acetyl-DL-leucine), the group of UVN cats receiving isomer L only (N-acetyl-L-leucine) and the group receiving isomer D only (N-acetyl-D-leucine). Each data point represents the mean number of horizontal vestibular nystagmus (quick phase eye movements in 10 s) recorded in the four groups of UVN cats. S.E.Ms. are reported as vertical lines. Recovery tested in the light is faster under N-acetyl-L-leucine and N-acetyl-DL-leucine treatments than for controls and N-acetyl-D-leucine treatment.

significantly higher ($P<0.05$) at this time delay in the controls and the group of cats receiving the isomer D (14.6 ± 1.5 and 16.0 ± 1.4 respectively: NS) compared to those animals receiving the racemate or the isomer L (11.5 ± 1 and 10.25 ± 0.5 respectively: NS). By the third postlesion day, the mean number of nystagmus beats was strongly reduced in these two latter groups (3 ± 0.8 and 2.25 ± 0.9 for the racemate and the isomer L, respectively), while high values are still observed in the controls and the group of cats receiving the isomer D (8 ± 0.7 and 10 ± 0.8 respectively).

3.2. Compensation of the static postural deficits

Fig. 2 illustrates the mean recovery profiles of the static postural deficits as evaluated by the measures of the support surface performed in the four groups of cats.

As previously described for the vestibular nystagmus compensation, the support surface returned to control values very much faster in the groups isomer L (N-acetyl-L-leucine) and racemate DL compared to the controls and to the group isomer D (N-acetyl-D-leucine) ($P<0.0001$). The fastest recovery was found in the group isomer L, which recovered normal values ($=1$) as early as 16 days postlesion. At that time delay, the racemate group showed a mean support surface of 1.3 ± 0.15 (NS) while controls and groups isomer D still exhibited support surfaces three times higher ($3.2\pm.11$ and 3.3 ± 0.14, respectively; $P<0.05$). In addition, the kinetics of the recovery profile differed according to the groups. Recovery was achieved almost linearly for the controls and group isomer D (slopes around 0.1), and normal values of the support surface were seen 40 days postlesion on the average. By contrast, groups isomer L and racemate DL exhibited an exponential time course with a reduction of the support surface between the 5th and the 15th postlesion days, which was significantly higher than for the other groups, and significantly higher in group isomer L with respect to group racemate DL.

As early as the second postlesion day, the values recorded in groups isomer L and racemate DL were significantly lower than in

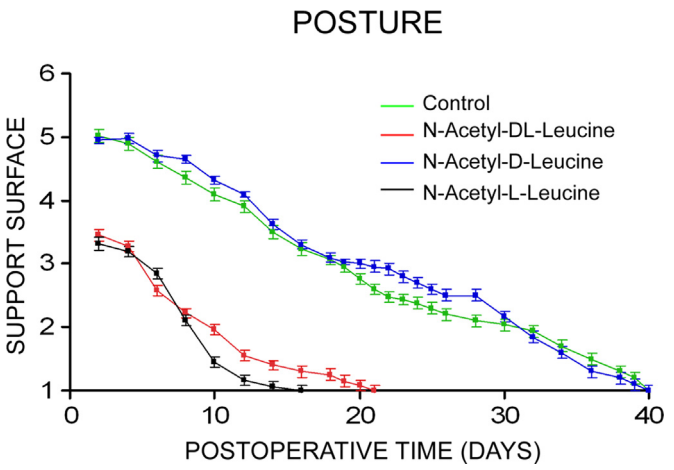

Fig. 2. Comparison of the effects of the three pharmacological treatments with respect to the controls on the compensation of the static postural deficits.Curves indicating the mean postoperative recovery of the support surface in the four experimental groups of cats : UVN untreated cats (controls, saline water), UVN cats receiving the racemate component (N-acetyl-DL-leucine), UVN cats receiving isomer L only (N-acetyl-L-leucine) and the group receiving isomer D only (N-acetyl-D-leucine). Data recorded after vestibular deafferentation were related to individual references and normalized with respect to the preoperative values referred to unity (one being close to 50 cm^2). Error bars represent S.E.M. Note the strong increase in support surface acutely after UVN and again the faster recovery in the groups of cats treated with N-acetyl-L-leucine and N-acetyl-DL-leucine compared to controls and N-acetyl-D-leucine treatment.

the controls and in group isomer D ($P<0.0001$). At this time delay, the mean values were 3.3 ± 0.15 in group isomer L, 3.4 ± 0.3 in group racemate DL (NS), 4.95 ± 0.1 in group isomer D ($P<0.05$), and 5.0 ± 0.4 in the control group ($P<0.05$). Moreover, return-to-normal support surface was observed within the first two weeks in group isomer L and within the first three weeks in group racemate DL ($P<0.05$)

3.3. Locomotor balance recovery

Fig. 3 illustrates the recovery profiles of the equilibrium function in the same four groups of cats. Again, strong and significant differences were observed between the controls and group isomer

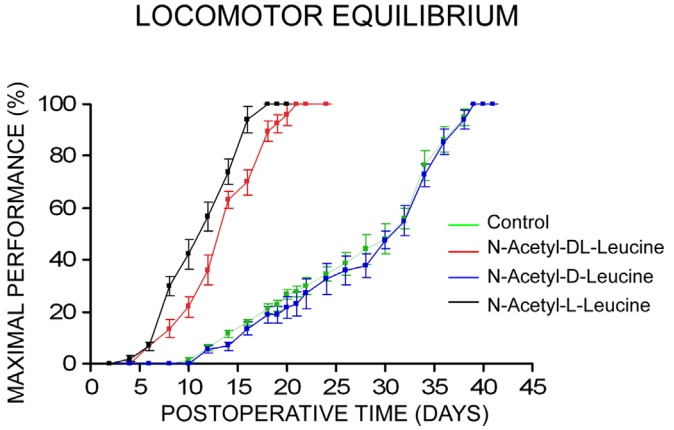

Fig. 3. Comparison of the effects of the three pharmacological treatment with respect to the controls on the locomotor balance recovery. Time course of the Maximal Performance (MaxP) recorded on the rotating beam test (ordinates: in percent of the preoperative MaxP) as a function of the postlesion time (abscissa: in days). Mean results ($\pm$ S.E.M.) recorded in the four groups of UVN cats. Same conventions as for Figs. 1 and 2. Note again the strong acceleration of the recovery time under N-acetyl-L-leucine and N-acetyl-DL-leucine treatments, illustrated by the significantly shorter time required to achieve full compensation (3 weeks instead of 6 weeks for controls and N-acetyl-D-leucine; $P<0.0001$).

D on one hand, and between groups isomer L and racemate DL on the other hand. The differences were shown for both the time course of recovery of the animals' Maximal Performance (MaxP), and the earliest effects concerned the racemate group and the isomer L group.

Concerning the kinetics, effects on the locomotor balance recovery were similar to those reported for the support surface recovery (Fig. 2) and for the nystagmus compensation in the light (Fig. 1). In the controls and the group isomer D, the animals could walk again on the unrotating beam (MaxP=0) 8–10 days after their vestibular lesion. This time delay was strongly shortened in the racemate and isomer L groups (2.5 days on average: $P<0.001$). Note that these time delays roughly correspond to those required for nystagmus compensation in these corresponding groups.

The time constant of the locomotor balance recovery process was significantly shortened for the animals of groups isomer L and racemate DL compared to those of the controls and the group isomer D. MaxP was restored significantly earlier ($P<0.001$) in the former groups (18 days and 21 days for the groups isomer L and racemate DL, respectively) compared to the two other groups (42 days on the average). That means that functional recovery was achieved twice earlier under N-acetyl-L-leucine and N-acetyl-DL-leucine treatment than under N-acetyl-D-leucine or placebo treatment.

In addition, the recovery time course was still almost linear in the controls and in the group isomer D (slopes of 2.85 and 2.84, respectively) while it exhibited once again an exponential aspect for groups isomer L and racemate DL (slopes of 6.5 and 5.7, respectively), as described previously. For instance, the MaxP recorded 8 days postlesion reached $30\pm27\%$ in group isomer L and $13.5\pm6.8\%$ in group racemate DL while it remained at 0% in the controls and in group isomer D. Fourteen days postlesion, the MaxP was $74\pm17.9\%$ and $63.1\pm26.9\%$ in groups isomer L and racemate DL, respectively, and it was still as low as $11.6\pm2.9\%$ and $6.7\pm3.1\%$ in the controls and in the group isomer D, respectively.

3.4. Global sensorimotor activity

The behavioral scores reflecting the global sensorimotor activity for each cat of the different groups have been evaluated using observation scales (see methods). The mean curves showed very clearly a behavioral advantage as early as the first postlesion day for the cats treated with the isomer L (mean score=14) and the racemate (mean score=11.5), compared to the animals receiving the isomer D or no treatment at all, whose sensorimotor activity was dramatically reduced (mean score=0) (Fig. 4).

These gross observations did not point to differences between groups racemate DL and isomer L, whose recovery was achieved very fast with respect to the other two groups. By the second postlesion day, the UVN cats from groups L and DL were not so different from those of normal unlesioned cats. Most of their postlesion deficits had disappeared: their head tilt was strongly reduced, they exhibited no circling, no tumbling, and they could walk nearly normally without showing deviations of their locomotor trajectory. In addition, they were able to climb and to jump without apparent difficulties. By contrast, the animals from groups C and D displayed the whole vestibular syndrome as classically reported in the literature. And their global score developed very slowly during the first postlesion week.

The strong beneficial effects of N-acetyl-L-leucine and N-acetyl-DL-leucine were also evidenced on their neurovegetative syndrome. Salivation and vomiting were not seen in these animals in the first days following their vestibular lesion, and they did not show any sign of prostration. They looked very motivated, active, and alert, a behavior that contrasted drastically with that of the animals in the two other groups.

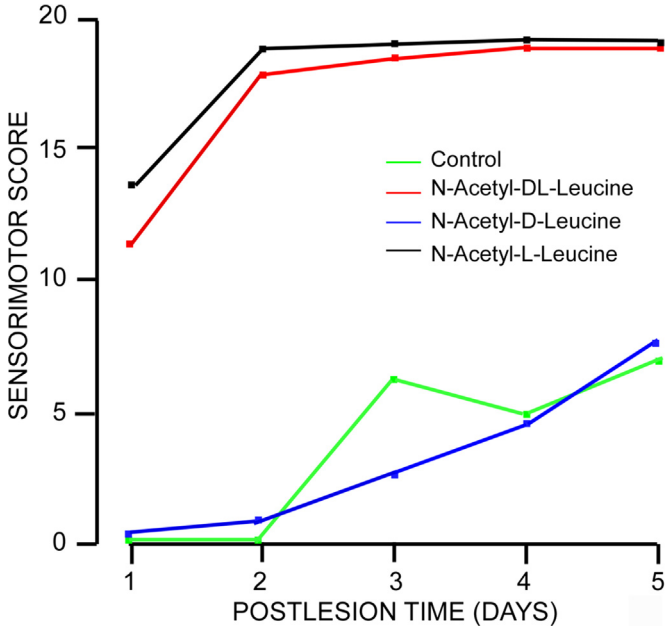

Fig. 4. Effects of the three pharmacological treatments on the global sensorimotor activity. Behavioral sensorimotor scores (ordinates) as a function of the postlesion time (abscissa: days) in the four groups of UVN cats. Same conventions as for Figs 1–3. One can observe that animals receiving the racemate or isomer L were very active as early as the first and second postlesion days compared to the cats of the control and isomer D groups, which did not show any sign of sensorimotor activity at these time delays. The global sensorimotor score improved much more faster in the racemate and isomer L group of treated cats compared with the controls and the isomer D treated group.

4. Discussion

4.1. Summary of the results

The findings of the present study can be summarized as follows:

a. Whatever the parameters tested (spontaneous vestibular nystagmus in the light, support surface, global sensorimotor activity and locomotor balance), the functional recovery is strongly and significantly accelerated under pharmacological treatments with N-acetyl-DL-leucine. This finding confirms our previous study on the same animal model (Pascalis, 1990; Lacour, 1995),
b. The N-acetyl-D-leucine isomer has no effect at all on the recovery process. Animals of this group showed the same recovery profile as those receiving no treatment at all or a placebo,
c. The N-acetyl-L-leucine isomer is the active part of the racemate component since it induces a significant acceleration of the vestibular compensation process similar and even better than that observed under treatment with the racemate component only.

4.2. Hypotheses on the sites of action of the drug

Where do N-acetyl-DL-leucine and its isomer N-acetyl-L-leucine act in the brain? A radioautography study in the monkey *Macaca fascicularis* (Benard et al., 2001) showed a wide labeling in CNS structures 2–5 mins after injection of the racemate component marked with ^{14}C in cortical areas overlapping the vestibular cortex (Lopez and Blanke, 2011), in various thalamic nuclei, and in subcortical structures including the VN and associated structures (inferior olive, oculomotor nuclei, cerebellum) known to play a role in vestibular compensation. Measurements of the regional cerebral glucose metabolism (rCGM) by means of μPET, as an index of brain activity, showed in a rat model a significant asymmetry in the VN, vestibulo-cerebellum, thalamus and multisensory vestibular cortex in the acute phase of vestibular imbalance, followed by a rebalanced metabolic activity in the early vestibular compensation stage (1–2 days) (Zwergal et al., 2014). Thereafter (2–7 days), rCGM increased in the ipsilateral spinal trigeminal nucleus, and later on (7–9 days) bilaterally in the vestibulo-cerebellum, hypothalamus, and hippocampus. Using similar μPET imaging methods, Günther et al., (2015) showed that N-acetyl-L-leucine, but not N-acetyl-D-leucine, caused a significant increase of rCGM in the vestibulo-cerebellum on days 3 and 7. In addition, they observed a significant improvement of postural imbalance in the N-acetyl-DL-leucine and N-acetyl-L-leucine groups compared to a sham treatment group, and no effect at all with N-acetyl-D-leucine group, thus confirming our present data in the cat. The authors concluded that N-acetyl-L-leucine improves compensation of postural symptoms after UL, most likely by activating the vestibulo-cerebellum (and also by deactivating the postero-lateral thalamus). This conclusion reflects mainly what happens in the compensatory stage, where relearning processes supporting the sensory and behavioral adaptations involve the cerebellum. But the acute stage implies the VN and associated structures (thalamus, multisensory vestibular cortex), as shown in their study by the early rCGM asymmetry in these structures and the return to symmetrical rCGM over 2–3 days. However, the metabolic changes depicted cannot give access to the underlying recovery mechanisms.

4.3. Hypotheses on the mechanisms of action of the drug

That the static vestibular deficits (nystagmus, posture) are compensated very fast after UVN means that the pharmacological treatment (N-acetyl-DL-leucine or N-acetyl-L-leucine) acts very likely at the level of the VN complexes. There is a consensus today to consider that recovery of the static functions depends mainly on the rebalance of the spontaneous electrical activity of the VN cells on both sides (Lacour et al., 1989; smith and Curthoys, 1989; Darlington and Smith, 2000, Lacour, 2006, Vidal et al., 2015 for reviews). This was demonstrated in both the rat (Ris et al., 1995) and cat (Zennou-Azogui et al., 1993) models, in which the drastically reduced activity on the ipsilesional side was progressively restored over time with time constants corresponding to those for the postural compensation (1 week in rat, 6 weeks in cat).

Synaptic plasticity mechanisms (Gacek et al., 1998), axonal sproutings (Dieringer, 1995), astroglial reactions (De Waele et al., 1996; Dutheil et al., 2009, 2013), microglial reactions (Campos-Torres et al., 1999), Gabaergic, cholinergic and histaminergic changes (Tighilet and Lacour, 1998, Tighilet and Lacour, 2001, Tighilet et al., 2006; Dutheil et al., 2013) neurogenesis and astrogenesis in the VN (Tighilet et al., 2007a; Dutheil et al., 2009, 2013), and changes in the membrane properties of the VN neurons (Vidal et al., 2015), are potential mechanisms that could explain such a restoration of electrical activity in the VN cells on the lesion side.

Several studies have attempted to elucidate the mechanisms by which N-acetyl-DL-leucine could facilitate vestibular compensation. Lacour (1995) were the first to report the effects of the drug on the activity of lateral VN neurons during static (whole body tilt) and dynamic (sinusoidal whole body oscillations in roll) conditions in totally awake cats. Unitary extracellular recordings of these vestibulo-spinal neurons showed that N-acetyl-DL-leucine significantly inhibited the recovery of the resting firing rate of the lateral VN neurons, but increased strongly their sensitivity to dynamic stimuli as well as the proportion of responsive neurons to head roll tilts. A possible explanation was provided later on by

Vibert and Vidal (2001) from in vitro whole brain investigations of labyrinthectomized guinea pig. N-acetyl-DL-leucine reduced the prominent asymmetry characterizing the VN-related networks by decreasing the activity of the abnormally depolarized neurons on the hyperactive side. They suggested that N-acetyl-DL-leucine might act mainly on abnormally hyperpolarized and/or depolarized MVN neurons, by bringing back their membrane potential towards a mean value of −65 to −60 mV. The effect may be mediated by modulation of the ion channel activity. Supplementary investigations of these authors performed with the isomers L and D suggested that isomer D only would be able to reproduce the effects obtained with the racemate, and particularly the depolarization of the abnormally hyperpolarized cells (Eugène et al., 2005). Our behavioral data in the awake cat as well as the metabolic investigations reported above do not support this finding. Such a discrepancy could be attributed to species differences or, most likely, to the in vivo versus in vitro preparations.

Among the other possible mechanisms is the binding of N-acetyl-DL-leucine and N-acetyl-L-leucine to stereospecific sites of receptors located in brain structures involved in vestibular compensation. Indeed, evaluation of the affinity of N-acetyl-DL-leucine for glycinergic and glutamatergic receptors specific neurotransmitters, widely distributed in the VN neurons, suggested that N-acetyl-DL-leucine could be a partial agonist of these two types of receptors (Neuzil et al., 2002). Their coactivation on the same neuron might explain why the effects of N-acetyl-DL-leucine are function of the membrane potential of the VN neurons, and would be greater on cells abnormally depolarized or hyperpolarized.

4.4. Why a better efficacy of the isomer L compared to the isomer D?

A highly interesting hypothesis is based on the relationship between the activity level of the enzyme «D-amino-acid oxidase » and the D-serine amino acid. The enzymatic activity of « D-amino-acid oxidase» is localized strictly in the astrocytes of adult brain stem (VN, inferior olive) and CNS (cerebellum, oculomotor nuclei) structures implicated in vestibular compensation (Moreno et al., 1999; Horiike et al., 1994; Puyal et al., 2006). The astroglial reaction described in the VN on the lesioned side both in the rodent (De Waele et al., 1996; Campos-Torres et al., 2005) and the cat (Dutheil et al., 2009, 2011, 2013) could induce an increase in the expression of «D-amino-acid oxidase». This increased expression would accentuate the degradation of the dextrogyre amino acids, a hypothesis that would explain the total inefficacy of the isomer D in vestibular compensation. Another hypothese, besides this one on enzymatic deactivation, is based on the differences in isomer transport over the blood brain barrier (Oldendorf, 1973) or incompatibility to stereospecific binding site.

N-acetyl-DL-leucine is a drug used in clinical practice for the treatment of acute vertigo (Ferber-Viart et al., 2009). **T**he neurovegetative signs are attenuated early after intravenous N-acetyl-DL-leucine administration, an observation that could be due to reduction of the asymmetrical activity in the VN complexes on both sides. But there are today no controlled trials on the acute effects of N-acetyl-DL-leucine administration on symptoms of patients with acute vestibular syndromes. In the study of Ferber-Viart et al. (2009), the first follow-up examination was done on day 8 after a unilateral vestibular lesion, that is, in a subacute stage of compensation.

Moreover, vestibular compensation is not slowed down, but speed up following N-acetyl-DL-leucine administration, suggesting that the drug has no sedative effects. This is confirmed by the sensorimotor activity level recorded in the cats treated with the racemate component or the isomer L. Indeed, these animals looked strongly motivated, very active, and alert compared to the cats treated with the isomer D. Freyss et al., (1990) noted that the early prescription of N-acetyl-DL-leucine allows more easily the active mobilization of patients, considered as a key recommendation in vestibular rehabilitation therapy (Lacour and Bernard-Demanze , 2015).

5. Conclusions

We confirm here the facilitatory effects of N-acetyl-DL-leucine on vestibular compensation in the cat, as described many years ago by our group. These effects are seen on the recovery of both the static (posture, nystagmus) and dynamic (sensorimotor activity, locomotor balance) functions, and lead to a 50% reduction of the time constant of the recovery process. And we provide on the same animal model, which vestibular neurophysiology is well documented and which vestibular compensation have been fully investigated with multidisciplinary approaches, that these effects are due to the isomer L (the isomer D being totally inefficient), thus confirming data recently recorded in the rat (Günther et al., 2015). Further investigations have however to be done to determine the mechanisms of action of the drug and to know why isomer D has no beneficial effect. Dose-dependent effects should be tested to determine the optimal dosage.

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