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Methylated tin toxicity a reappraisal using rodents models

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ABSTRACT

Trimethyltin-induced intoxication has a great impact on human health due to the widespread occurrence of methyltin compounds. Acute TMT intoxication in humans leads to a variety of neurological symptoms which involve primarily the limbic system. In the present review we summarized the neuromorphological correlates of this neurological syndrome extending the analysis to various extra-limbic regions and detailing the fine ultrastructure of TMT-induced neuronal alterations. In order to comprehend the pathophysiology of TMT-induced neuronal damage we analysed the various experimental models of TMT-induced neurotoxicity. When comparing various animal species, it seems that the variety of neuropathological correlates are not related to species difference in the sensitivity to TMT toxicity but to a different susceptibility to secondary effects produced by TMT. In fact, apart from a primary neurotoxic damage induced by TMT at neuronal level, this compound promotes the onset of limbic and generalized seizures, which in turn add a secondary damage to that induced immediately by TMT. Thus, the different neuropathology observed in different animal species is produced mainly by a different sensitivity to epilepsy-induced brain damage.

Key words
Tin • Trimethyltin • Hippocampus • Seizures

Introduction: the wide occurrence of TMT and its relevance to environmental health

The great impact of Trimethyltin (TMT) on human health is primarily related to workers in plastic manufacturing who are potentially exposed to TMT by skin contact or inhalation (Ross et al., 1981; Rey et al., 1984; Besser et al., 1987; Yanofsky et al., 1991; Kreyberg et al., 1992; Saary and House, 2002). This is due to the fact that tin, which is quite ubiquitous, easily undergoes biomethylation thus producing methyltin compounds, which in turn, are released in the environment (Fent, 1996).

Research on neuropathology produced by this compound as well as its mechanism of action and cell biology represent the standpoint to develop an in depth perspective to prevent and/or curing TMT toxicity in humans. This is mandatory for health-involved agencies since the widespread contamination from this compound leads to intoxications which still remain without a remedy. TMT represents a methylated metal (organotin), which is often conjugated with chloride to form trimethyltin chloride. In the process of plastic production it is used to inhibit dissociation of hydrochloric acid from polyvinylchloride (PVC); thus, TMT can be found in several domestic products (Gomez et al.,

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2007) and supplies causing the presence of TMT in drinking water (Fent, 1996; Hoch, 2001; Mundy and Freudenrich, 2006). TMT is also found in marine environment and it is spread among aquatic specimens (Shawky and Emons, 1998). This might justify why TMT is also found in urine samples from humans who apparently were never exposed to common tin sources (Braman and Tompkins, 1979). Organotins are also used as pesticides and they occur as intermediates in the synthesis of other chemicals (Kimbrough, 1976; Rüdel, 2003; Appel, 2004). TMT exposure occurs directly from TMT containing products or via the endogenous methylation of DMT (dimethyltin). In fact, there are reports of workers who were exposed to DMT and suffered severe hypokalemia and neurological disturbances such as ataxia, and memory loss similarly to what occurs following TMT exposure. It is likely that neurotoxicity in these subjects depends on the endogenous conversion of DMT into TMT (Piver, 1973; Mushak et al., 1982; Yoo et al., 2007). Confirming such an hypothesis, urine and blood samples from these workers contain both TMT and DMT (Jiang et al., 2000; Yoo et al., 2007). This is also confirmed by experiments in rodents, where i.p. injection of DMT chloride produces detectable amount of TMT in blood and urine (Furuhashi et al., 2008). In humans, the greater toxicity of TMT compared with DMT led to limit the use of organotins to DMT only. However, when DMT is synthesized from inorganic tin and methylchloride under a pressure of 4 atm, the reaction product is a mixture of DMT (88%), TMT (8%) and MMT (4%) (Besser et al., 1987). Thus, occurrence of TMT contamination remains a critical issue in environmental health.

Toxic effects produced by TMT became manifest for the first time following a severe intoxication reported in 1958, when 219 people were exposed to organic tin salts (Alajouanine et al., 1958); further reports were published later in the 70s (Fortemps et al., 1978). These patients suffered from a variety of symptoms including: cognitive deficit, hyperactivity, aggressiveness, partial complex seizures, altered sexual behavior, ataxia, and leg paresthesias. These symptoms remained in search for a pathological correlate until the seminal study of Besser et al. (1987), who published the first anatomo-clinical correlation for TMT intoxication in human beings. They found that acute TMT exposure was responsible for the onset of a specific clinical syndrome named "limbic-cerebellar syndrome", which is unique since it does not reproduce any neuropsychiatric disorder described so far. Such a specificity was confirmed by neuropathology showing that brain damage occurred in selective brain areas which were uniquely clustered. The nosographical effort produced by Besser et al. (1987) led to extend the previous concept of selective limbic toxicity as reported by the experimental studies of Dyer et al. (1982a) to a prevalent limbic-cerebellar damage (Besser et al., 1987). In keeping with this, updated literature on TMT intoxication confirms the occurrence of extra-limbic toxicity involving the cerebellum and extending to other brain regions (see later).

From what reported above it is evident why TMT research spreads over a variety of matters:

- Environmental toxicity.
- Occupational diseases.
- Research on cognitive deficit.
- Research on epilepsy.
- Common neurobiological substrates for neuronal susceptibility within the cerebellum and limbic system.

When a human neurotoxin is identified, in order to detail its mechanism of action and develop effective remedies, the first approach consists of setting up an experimental model which is predictable of acute toxicity and neuropathology observed in humans. In this way, by profiting of this animal model, specific mechanisms of action and accurate neuropathology can be easily investigated and then translated into humans with a reasonable chance of success.

Thus, in the present review we focus at first on the behavioral syndrome produced by TMT in humans, followed by the analysis of the available neuropathology and the potential correlation between anatomy and behavior in human patients. In order to extend the paucity of informations on human neuropathology, we will analyze which animal model is best suitable to predict the human syndrome. Since we are mainly basic neuroscientists this section of the review, comparing different animal species and specific strains, covers a large part of the manuscript. We will discuss to which extent these basic findings are translatable to patients and the great opportunity to have experimental models which allow to draw cause-effects relationship in TMT neurotoxicity. From a morphological standpoint we wish to define the concept of TMT intoxication as site-specific and

we wish to highlight underestimated target areas. Within affected brain regions we wish to understand the temporal pattern of cell death in different subfields. In this way a distinction needs to be done between primary neurotoxicity triggered by TMT and secondary neurotoxic events which follow TMT administration (seizures, ischemia ecc.).

The TMT in humans and the limbic cerebellar syndrome

In humans acute TMT intoxication represents a dramatic syndrome, which as previously mentioned was comprehensively described by Besser et al. (1987). The most severe phase of acute TMT intoxication is observed when organotin levels in the urine reach the peak. At this point symptoms engage several organs. On the other hand, few days after exposure neurological effects become prevalent and persist mainly as cerebellar and limbic dysfunction. In fact, the TMT syndrome is also known as "limbic cerebellar syndrome" since it features symptoms which may be caused by limbic as well as cerebellar impairment. In keeping with the former, intoxicated patients show confabulation and disorientation up to psychotic behaviour, retrograde and anterograde amnesia occurs and loss of impulse control is manifest as hyperphagia, aggressiveness, and altered sexual behavior. Limbic toxicity is also manifest as partial complex seizures and delta rhythm. On the other hand, cerebellar signs are evident as ataxia and mild gaze-evoked nystagmus. Although both limbic and cerebellar signs are predominant, the TMT syndrome in humans also features peripheral neuropathy with parehestesia and slowed sensory nerve conduction. The involvement of central vestibulo-cochlear pathways is marked and it contributes to the extended definition of the TMT syndrome beyond the limbic-cerebellar domain. In fact, we are now aware that specific brainstem nuclei related with the vestibulo-cochlear pathways are markedly and specifically engaged during TMT exposure. Such a feature is probably underestimated since it might cover a variety of symptoms beyond the mere hearing impairment such as the onset of tonic clonic seizures (which in rodents are triggered by the inferior colliculus). Similarly, there is a clear evidence indicating that a vestibular dysfunction greatly contributes to nistagmus originally attributed to a mere cerebellar damage. Seizure activity represents a typical feature, being limited to limbic structures in most cases (partial complex seizures), while it spreads over non-limbic cortical areas when the syndrome is more severe and less defined as it occurs during the acute phase. The relationship between TMT and epilepsy is key to understand species differences and the role of primary and secondary neuronal toxicity following TMT exposure (see later). When acute intoxication is severe, breathing difficulties and death may occur in human patients early after exposure, otherwise the TMT-induced syndrome persists as a neurological disorder within 1 year after exposure.

In search for the best animal model to study TMT toxicity

A variety of animal species ranging from fishes to non-human primates can be compared. When looking at the bulk of the available literature, despite a wide range of species were exposed to TMT, most of the data comes from experimental studies carried out in rodents. Typing trimethyltin and rodents on a PubMed search sorts 419 references at the beginning of August 2009; most of them (328) refer to rats. In fact, acute TMT neurotoxicity in rodents is comparable with what observed in humans both for behavior and neuropathology. The multi-faceted behavioral syndrome recorded following rodents exposure was named originally as "The trimethyltin syndrome" (Dyer et al., 1982a). However, talking about rodents in general is rather simplistic and may hide important details which depend on which rodent species is under analysis. Although both mice and rats are sensitive to TMT-induced toxicity, there are marked discrepancies between these species, being the rat the most studied. In keeping with limbic toxicity, the pattern of neurodegeneration in rats and mice appears to be totally opposite at "first glance". While in the mouse the main target of TMT appears to be the granule cell of the dentate gyrus (DG) of the hippocampus (Chang et al., 1982a; Wenger et al., 1984a; Bruccoleri et al., 1998; Bruccoleri and Harry, 2000; Fiedorowicz et al., 2001; Geloso et al., 2002; Ogita et al., 2004) (Fig. 1), in the rat the preferred hippocampal target of TMT corresponds to the pyramidal cell of Cornu Ammonis (CA) subfields (Chang et al., 1983a; Chang and Dyer, 1983a,

1985; Perretta et al., 1993) (Fig. 1). This apparent discrepancy becomes slighter when a careful analysis of the available data is carried out. In fact, the large prevalence of rat studies compared with a few studies in mice, led to discrepancies concerning the age of the rodent, the dose, the time interval, and the time window elapsing between the last TMT dose and the sacrifice. Thus, the great amount of rat studies is not matched by the few mice studies. Nonetheless, in keeping with rat studies we can draw a comparison with mice by selecting those few experimental conditions which were replicated in both rodent species.

Behavioral alterations in rodents

As mentioned above, when translating the human disease into an experimental model it is critical to analyze to which extent behavioral deficits observed in humans can be reproduced in animals. Following a single intraperitoneal injection of TMT, mice show persistent tremor with tail biting, hindlimb paralysis, hyperactivity, tonic clonic and partial seizures, aggression, and body weight loss (Wenger et al., 1984b; Ogita et al., 2004; Shintani et al., 2007). Rats show behavioural alterations such as hyperactivity, tremors, seizures, learning impairment, mutilation of the tail, weight loss and aggressiveness that appeared a few days after TMT (Bouldin et al., 1981; Ruppert et al., 1982; Dyer et al., 1982a; Noland et al., 1982; Ishida et al., 1997). Impairment in learning and memory represents a critical issue in humans and it is replicated in rodents (Kim et al., 2007), in fact a single intraperitoneal injection of TMT (2,5 mg/kg), leads to a memory loss coupled with reduced ChAT activity in the cerebral cortex (Kim et al., 2007). In keeping with memory loss, Tsutsumi et al. (2002) described the impairment of spatial learning (as measured by the swimming time in the Morris water maze test) in rats 16 weeks following a single oral dose of TMT.

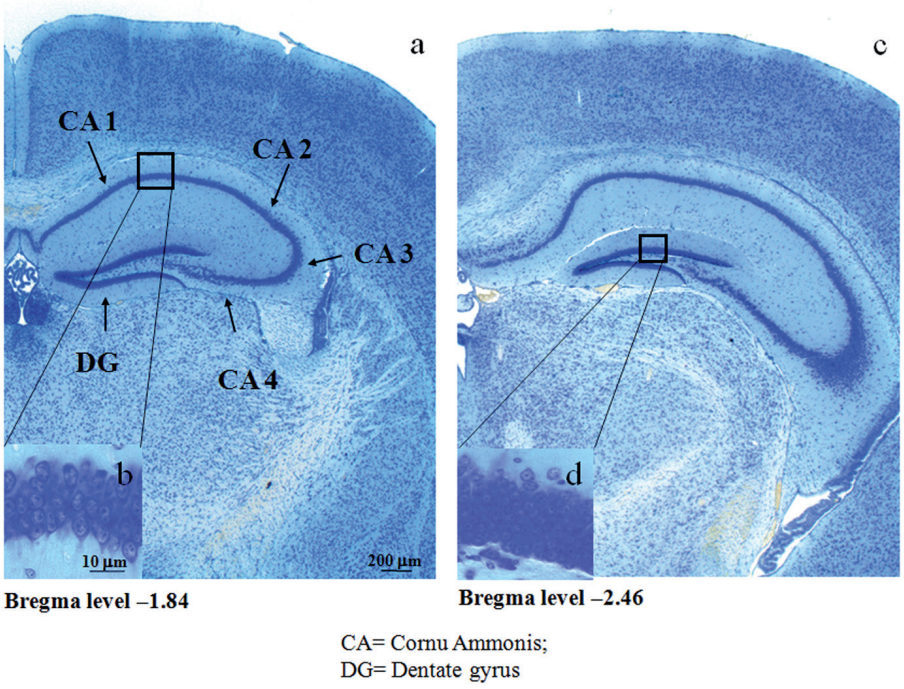

Fig. 1. - Hippocampal subfields as target of TMT toxicity. In (a) a low magnification shows the hippocampus where the various subfields of the Cornu Ammonis are reported. These areas represent the main target of TMT toxicity at prolonged time intervals and they are commonly described as the main target of TMT-induced degeneration in rats. Among various subfields of the Cornu Ammonis, the CA1 and CA3 are preferentially lesioned in rats while the dentate gyrus DG is lesioned in mice. (b) Shows a higher magnification of the CA1 detailing the pyramidal cells which correspond to the neuronal phenotype which is targeted by TMT. In (c) a similar view of the hippocampus detailing the dentate gyrus at a more caudal level is reported. The dentate gyrus corresponds to the hippocampal area which is damaged early following acute TMT intoxication and represents the main target described in TMT-induced degeneration in mice. At higher magnification (d) it is visible the classic cytology of the dentate gyrus with the giant granular cells which are targeted by TMT.

TMT-induced seizures appear after a few days following exposure. This is unexpected since common chemoconvulsants are known to trigger seizure activity from a few min up to 1 h after their administration (depending on the specific compound: bicuculline, kainate, or pilocarpine). This delay was originally noted by Sloviter et al. (1986), who described the onset of seizure activity in TMT-treated rats (Sloviter et al., 1986). In this study, authors related TMT-induced hippocampal damage to such a delayed ictal events. In particular, they found a correlation between the severity of seizures and the subsequent hippocampal damage. Rats which did not exhibit seizures exhibited minimal hippocampal damage (Sloviter et al., 1986). Lending substance to this analysis, the delayed hippocampal damage involves the specific subfields of the CA while it spares the DG, which is exactly the zonal pattern of epilepsy-induced cell death (Sloviter et al., 1986). On the other hand, early hippocampal damage, which occurs before and independently from seizures, targets the DG while it spares the CA. In this way we might introduce the term of "direct" (primary) and "indirect" (secondary, here epileptic) TMT-induced neuronal cell death.

Neuropathology following TMT intoxication in humans

Apart from the original definition, the damage induced by TMT spans all over different regions as originally noted by Besser et al. (1987). In fact, despite defining the TMT syndrome as limbic and cerebellar the authors detail, these authors found neuronal damage also in the pons, and basal ganglia. In these areas as well as in the cerebellum and limbic system they documented cell loss, which was mostly severe at the level of the amygdala and the Purkinje cells of cerebellar cortex. In a further study, Kreyberg et al. (1992), who examined a variety of brain areas following TMT exposure, documented necrosis both in the granule cells of the DG and pyramidal neurons of the CA within the hippocampus. Thus, if one considers the few reports in humans limbic structures appear to be damaged at the level of hippocampal cortex in a way which overlaps quite well with what reported in rodents (see later). The limbic damage extends to the amygdaloid nucleus but there is no documentation concerning other limbic regions such as the olfactory bulb, the piriform cortex, the entorhinal cortex and related nuclei. This point is critical since the recruitment of limbic structures appears to be widespread in rodents and probably it is likely to be the same in humans. Probably a great part of this damage is not directly induced by TMT but it may be due to secondary neuronal toxicity induced by ischemia or epilepsy. In this way, the hippocampal damage seems to be more an effect of TMT toxicity in the DG while it could be an indirect consequence of brain ischemia/seizures when it involves the CA (as noted above). This point will be discussed in detail in the following part of the review. However, it is worth to mention here that rats undergo easily to limbic seizures (which spare the DG and damage the CA) while mice are much more resistant to limbic seizures. When mice and rats are challenged with TMT early toxicity involves the DG in both species. However, only rats undergoing complex partial seizures develop a damage to the CA resembling mesial temporal sclerosis which occurs in patients affected by limbic seizures. Thus, the issue of secondary epileptogenesis may represent a bias when interpreting direct TMT toxicity in humans and the key to understand differential TMT sensitivity between species. In any case, given the scarce amount of pathological findings in humans, the use of animal models is key to understand the specific (primary, direct) toxicity induced by TMT ruling out the neuronal damage which is produced by a variety of concomitant systemic alterations.

At the same time, more extensive neuropathological analysis in human patients is needed to substantiate this concept. Apart from the limbic system, additional areas of neuronal damage were scattered along various brain regions such as the pons, basal ganglia, and temporal lobes (Besser et al., 1987); or isocortex, and spinal cord including dorsal root ganglia (Kreyberg et al., 1992).

Experimental pathology following TMT intoxication

The hippocampal formation

When making a comparative analysis between mice and rats, the extent of similarities with humans in each rodent species is critical in order to establish which model better mimics the human pathology.

From a general standpoint, TMT administration damages the hippocampal formation in both species (Bouldin et al., 1981; Chang et al., 1982b, 1983a; Balaban et al., 1988; Fiedorowicz et al., 2001; Ogita et al., 2004). Nonetheless, as mentioned above, there are critical differences between rats and mice concerning various hippocampal subfields. In fact, whereas mice undergo a marked toxicity in the DG (Ogita et al., 2004), rats are proner to cell loss in the CA (Dyer et al., 1982b; Chang et al., 1983a). However, when looking in depth such a difference, this is also related to the dosing of TMT. In other words, there is not such a rigid separation between rats and mice. The few studies carried on in mice were based on acute administration of TMT, whereas most rat studies are carried out using chronic TMT injections. When chronic dosing is used, the amount of TMT is diluted and each dose is lower compared with that used in acute studies. As reported by Balaban et al. (1988), there are early and late sites of lesion following TMT administration. Therefore, in considering degeneration it is mandatory to take into account the time window between the end of TMT administration and the sacrifice of the animal. If the dosing is protracted over several days (just like it occurs in a chronic treatment), the outcome is expected to produce a pattern of degeneration which overlaps more with later than earlier effects induced by TMT. This happens even when the rodent is sacrificed at short time intervals after the last TMT injection.

In keeping with this, the time window and the dosing more than the animal species and the strain are supposed to generate the regional pattern of hippocampal degeneration. Thus, if one considers the available data on acute administration of TMT in rats a clear vulnerability of the DG occurs at earlier time intervals. For instance, Brown et al. (1979) in a very articulated experimental design in rats combining different genders, strains, dosing and time intervals found that female Wistar rats administered TMT at 10 mg/kg developed a slight DG damage at two days with a few effects within CA subfields. Again, at 4 days, there was a serious damage of the DG compared with CA regions. Conversely, when they administered a chronic protocol (up to 4 mg/kg weekly): chronic low doses produced only a slight damage in the CA while DG was intact. Following acute administration in rats, when a single high dose of TMT was administered and the rats were sacrificed after a short time interval, a frank damage to the DG was observed. In the seminal study of Bouldin et al. (1981), authors administered 1-5 daily doses of TMT (each dose, 5 mg/kg, via gastric intubation) to Long Evans rats. They sacrificed rats at 24 h after the last dose. Using this paradigm they found the earliest occurrence of hippocampal damage at 24 h following 3 daily doses. At this time interval, the hippocampal damage consists of a selective neuronal loss in the DG, while the CA was spared (Bouldin et al., 1981). Such a preferential toxicity for the rat DG compared with the CA was confirmed at 24 h following 4 doses of TMT; although the amount of cell loss was increased (Bouldin et al., 1981). A similar study was performed in male Long Evans rats receiving 8 mg/kg of TMT i.p. by Balaban and co-workers (1988). They followed an experimental design which slightly differs from that used by Bouldin et al. (1981). In fact, they administered a lower cumulative dose (8 mg/kg) compared with 15 mg/kg (5X3) used by Bouldin et al. (1981). On the other hand, Haga et al. (2002) found that toxicity following a low dose of TMT to Sprague-Dawley rats produced a selective damage to the CA hippocampal fields at 4 days following TMT administration, while a high dose of TMT anticipated at 2 days the cell death making it more pronounced in the DG compared with CA.

In summary, when analyzing the results obtained in different strain of rats, despite a variability due to the susceptibility of each strain, there is a consistent effect which occurs in condensed time interval: the preferential damage to the DG with relative preservation of CA. On the other hand, when low doses are administered for longer time intervals (chronic treatment) CA is preferentially involved. When high doses are administered and rodents are sacrificed at prolonged (at least four days) time intervals, a combined damage involving to a similar extent the CA and DG is observed (Haga et al., 2002).

Therefore, high TMT doses acting for a small time window preferentially target the granular cells of the DG, whereas small repeated doses of TMT tend to spare such a class of neurons and damage preferentially the pyramidal cells of the CA. This analysis turns to be seminal when observing the hippocampus of people intoxicated by TMT, where the hippocampal damage involves both the DG and CA depending on the dose and time window (Kreyberg et al., 1992).

Most of the papers on TMT in mice demonstrate that this neurotoxin produces severe neuronal damage in the granule cells of the DG (Fiedorowicz et al., 2001; Ogita et al., 2004, 2005; Fiedorowicz et al., 2008; Yoneyama et al., 2008). However, this evidence (as outlined previously) is grounded on a very limited pattern of toxin administrations where chronic delivery of small doses was never carried out. In this way, the range of analysis following TMT administration in mice is limited to 72 h, which corresponds to the time window where in the rat fairly selective damage to DG in the absence of significant CA damage occurs.

Thus, it is not surprising that mice sacrificed at 42 and 72 h after TMT administration undergo neuronal degeneration in the DG (Bruccoleri and Harry, 2000). Again, in mice a single injection of TMT (2,8 mg/kg) produces cell loss in the DG but not in the CA at 2 days (Ogita et al., 2004). These data were confirmed by Nagashima et al. (2008) following a single i.p. injection of TMT (2,8 mg/kg), which led to DG damage in the absence of CA cell loss at 2 days. Despite lower doses and higher vulnerability to neuronal damage for the DG in mice compared with rats, at present we do not possess evidence allowing a comparative analysis at prolonged time intervals. This is critical to establish whether hippocampal CA toxicity occurs in mice as it happens in rats following lower doses for protracted time intervals. In summary, a higher susceptibility exists for the mouse hippocampus compared with the rat keeping constant the time window between toxin exposure and brain analysis. This is based on the fact that much lower doses (5 fold less) are necessary to induce hippocampal damage in mice compared with rats. When this occurs, the acute hippocampal effects recruit the DG. It remains unclear why the DG is preferentially involved by acute TMT toxicity (all the mice studies and those rat studies which analysed acutely the hippocampal formation), whereas the CA subfields undergo severe cell loss only at prolonged time interval following multiple doses (chronic rat studies but never tested in the mice). One might argue that a species difference underlies these effects. However, for what we just reported there is no data to draw such a conclusion. An alternative hypothesis is that most of acute studies are carried out by using higher TMT doses compared with chronic studies (in which small amount of TMT need to be administered daily in order to avoid systemic lethal effects). Though, it is odd that a high TMT dose produces a damage to DG while sparing CA neurons and a low TMT dose spares the DG and kills CA neurons. Therefore, one might consider the cumulative dose of small TMT shots, which in chronic studies may lead to a higher amount compared with single big shot used in acute studies. This might explain the progressive recruitment of CA neurons in the chronic toxic effects but it would leave unexplained the sparing of the DG under the same experimental conditions. Finally, such puzzling results might be faced considering that the damage to these hippocampal fields owns a different nature. Namely, if one assumes that direct toxicity of TMT is directed towards granule cells of the DG, then it becomes natural to observe a selective damage of this region occurring immediately after the exposure to a toxic dose of TMT. On the other hand, the loss of CA pyramidal cells might be an indirect damage which results as a consequence of the epileptogenesis induced by TMT. Within this context it is worth mentioning that during protracted limbic seizures a selective damage to the CA area occurs in the absence of DG cell loss (Sloviter et al., 1986).

It is well known that partial complex (limbic) seizures occur in humans after TMT exposure. In particular, depending on the severity of the syndrome seizures can be limited to limbic episodes or becoming generalized as tonic clonic phenomena. In rodents, exposure to TMT produces comparable effects (see later paragraph on experimental behavioural effect of TMT). It is likely that protracted TMT administration at low doses albeit being not directly neurotoxic produces repeated limbic seizures, which in turn, are responsible for the delayed (and indirect) damage to the CA regions. This hypothesis might explain the apparent contradiction in finding an intact DG concomitantly with the occurrence of cell loss in the CA area for low doses at prolonged time intervals following TMT. Similarly, this would explain the occurrence of an intact CA in the presence of lesioned DG following high doses at shorter time intervals following TMT. Again, despite being more resistant to neurotoxicity induced by TMT (the threshold dose required to induce cell loss is much lower in mice than rats) rats are much more prone than mice to limbic seizures (as can be witnessed by the effects of various chemoconvulsant or the effects induced by electrical stimulation, kindling).

Moreover, the occurrence of brain damage to extra-hippocampal limbic brain regions described in the rats following the chronic pattern of TMT administration offers a remarkable similarity with the site-specificity produced in all animal species during brain damage induced by protracted limbic seizures. In fact, following a variety of limbic seizure models, apart from the hippocampal damage (restricted to CA1-4 subfields) the piriform, periamygdaloid and entorhinal cortex are affected in addition to the olfactory bulb and the amygdala.

It is very likely the subtle, persistent limbic seizures of small intensity were not carefully recorded during behavioural observations following TMT. Despite a few reports which described the occurrence of limbic seizure (Dyer et al., 1982c; Sloviter et al., 1986; Ishida et al., 1997), no study carried out EEG following TMT administration. This experiment needs to be done in order to confirm or rebut such an hypothesis. When going back to humans it should be emphasized that EEG abnormalities accompanying partial complex seizures were continuously recorded and persisted for more than a week, which implies the occurrence of status epilepticus. This condition is defined by the persistence of seizures for more than 30 min without noticeable seizure free intervals and it is well established as a primary cause for seizure-induced brain damage to CA (Fornai et al., 2005). Limbic status epilepticus can be induced easily in rats but it is difficult to reproduce in mice. This explains variation between rodent species to TMT indirect toxicity. In fact, status epilepticus leads to a TMT-like delayed cell death as observed in rat TMT chronic studies. This inference we just made follows up the few comments of Kreyberg et al. (1992) who suggested that the primary toxicity of TMT was directed towards the DG whereas the damage of the CA regions was likely to be the effects of secondary phenomena (seizures, as we suppose here) induced by TMT. In line with this, the specific damage affecting the CA region may be further classified depending on the various subfields. In Sprague-Dawley rats it is known that while the CA1 region undergoes a corticosterone-dependent neuronal loss, in the CA3 subfield cell damage is corticosterone-independent (Shirakawa et al., 2007). These novel findings, while fostering new avenues in interpreting the finer pathways involved in TMT-induced hippocampal damage, confirm the variety of indirect mechanisms involved in producing TMT-induced neuronal loss. As usual, "nothing new is under the sun", since in a pioneer study, Sloviter et al. (1986), found that morphological changes induced by TMT in the CA subfields reproduce those occurring following limbic seizures. These consist of neuronal necrosis and dendritic swelling mainly in the hilar cells projecting to the inner third of the granule cell dendrites and the projection from CA3 pyramidal cells to the statum oriens and radiatum of CA3, CA2 and CA1 (Giorgi et al., 2003, 2005, 2006a, 2006b).

Extra-limbic regions

Apart from an apparent wider toxicity spectrum in the hippocampal cortices, in rats TMT affects a variety of additional brain areas including both limbic and extra-limbic regions such as the pyriform cortex, amygdaloid nuclei (Earley et al., 1992; Nilsberth et al., 2002). Even in mice extra-hippocampal toxicity has been described but, again, due to the lack of extended temporal analysis and the scarcity of mice experiments, it is likely that extra-hippocampal toxicity of TMT remains underestimated in mice. In describing all the areas affected by TMT in rats, Bouldin et al. (1981), demonstrated a damage in the isocortex, the allocortical piriform area, basal ganglia, brainstem, spinal cord, dorsal root ganglia. Again, Balaban et al. (1988) using silver staining techniques provided evidence for other areas involved in TMT-induced neurodegeneration. These discrete brain regions might help to clarify the bulk of behavioral changes. According to the study by Balaban et al. (1988):

At 1 day degeneration starts in the intermediate and ventral division of the lateral septal nucleus. At 2-4 days, degeneration occurs in: septohippocampal nucleus, septohypothalamic nucleus, anterior olfactory nucleus, bed nucleus of the stria terminalis, endopiriform nucleus, parafascicular nucleus, superior colliculus, intersitial nucleus of the posterior commissure, inferior colliculus, pontine nuclei, raphe nuclei, pars caudalis of the spinal trigeminal nucleus, the caudal aspect of nucleus tractus solitarius, dorsal vagal motor nucleus, dorsal cochlear nucleus and reticular nucleus, granule cells in the DG, pyramidal cells in CA fields of hippocampus. At 5-7 days: ventral posterolateral and ventral posteromedial thalamic nuclei, amygdaloid nuclei, periaqueductal gray (Balaban et al., 1988). In mice, a

detailed study was done in the brain stem and spinal cord by Chang and co-workers (Chang et al., 1983b, 1984). In the brain stem degeneration is marked in the mesencephalic trigeminal nucleus, while in the spinal cord neurodegeneration was seen mainly in the medial and lateral motor nuclei of the anterior horns (Chang et al., 1983b, 1984). In another study a damage in the olfactory structures was demonstrated by Chang and Dyer, (1983b). In particular, this occurs in the granule cell layer, the olfactory bulb (OB) and anterior olfactory nucleus (AON) of the mouse brain (Kawada et al., 2008). Recently, following a single injection of TMT, after 2 days, using immunostaining for ssDNA, Shuto et al. (2009) have demonstrated neuronal degeneration in the cerebral frontal cortex in addition to the anterior olfactory nucleus, olfactory bulb and DG (Shuto et al., 2009).

Fine Morphology in TMT toxicity in humans

In keeping with TMT and the human brain, damaged neurons in the amygdala, isocortex and brainstem undergo neuronal necrosis and neuronophagia and possess swollen perikaria and/or pyknotic nuclei and cytoplasmic inclusions within a slight eosinophilic cytoplasm. Electron microscopy shows loss of Nissl substance and enlargement of the cytoplasmatic membranes forming big vacuoles (Chang et al., 1983b) as Besser et al. (1987) and Bouldin et al. (1981) reported in human and rats, respectively. Electron microscopy shows also increased number of lysosomes (Chang et al., 1984). These latter features, joined with the abundance of cytosolic big vacuoles are suggestive of enhanced autophagy following TMT intoxication.

In its original paper, Besser et al. (1987) described ultrastructural changes which are suggestive of altered autophagy. In describing the fine structure of TMT-altered human neurons Besser and co-workers relate to rodents studies reporting a specific involvement of multilamellar bodies which again are reminiscent of altered autophagy. In keeping with pathological findings obtained from the human brain it is evident that TMT produces intracellular bodies which are made up of concentric membranes which now could be considered as witnesses for non-efficient autophagic vacuoles. Interestingly, differing from common stagnant autophagic vacuoles, those induced by TMT in human amygdala contain alternate electron dense bars, which lead to vacuoles named “zebra bodies”. We may hypothesize that the autophagy pathway which is now considered to be the main clearing system of the cells is engulfed by organotins which cannot be metabolized by eukariotic cells. Such a substrates accumulation would lead to an autophagy impairment which might be responsible for organotin-induced cell death. Confirming such an hypothesis only prokariotic organisms (such as Pseudomonas) are able to degrade organotin compounds under certain conditions (Barnes et al., 1973). If this is the case we would expect that specific compounds acting as powerful autophagy inducers might rescue neuronal cells from TMT-induced neurotoxicity. This working hypothesis is leading at present our group to try lithium as a powerful autophagy inducer to protect from TMT toxicity.

These findings in humans call for more in depth analysis of the finer ultrastructural changes in order to prompt further evidence bridging autophagy with TMT-induced toxicity and planning effective therapy based on this altered cellular pathway which remains a key since at present there is no effective therapy for TMT-induced toxicity.

TMT-induced ultrastructural changes: From Humans to Rodents and back again

As reported above in reference with human studies, at ultrastructural level TMT produces a variety of neuronal alterations. Among these, it is intriguing the accumulation of lysosome-like bodies and the derangement of the endoplasmic reticulum (Besser et al., 1987). In fact, this suggests a derangement of the autophagy pathway. In fact, preserved cells possess many whorls (i.e. multi-membrane bodies). A number of these bodies possess alternating electron-dense strip forming the so-called zebra-bodies prominent within amygdala neurons. The availability of reliable rodent models confirmed the involvement of an altered autophagy pathway in the neurons affected by TMT. When analyzing the fine structure of these pathological changes in rodents Bouldin et al. (1981) provided evidence that neuronal necrosis

in the hippocampal formation and pyriform cortex in rats is anticipated by subcellular pathologic changes consisted with altered autophagy. In the rat as in the human, the perikaryon cytoplasm and the proximal dendrite of these neurons were found to be filled with dense-cored vesicles and tubules containing acid phospatase activity and deriving from the Golgi-associated endoplasmic reticulum (GERL) system (Novikoff et al., 1971). This first alteration is followed by a sudden cytoplasmic accumulation of polymorphic dense bodies and autophagic vacuoles in many of the neurons in the DG and CA in which the number of granulated neurons was more conspicuous. These ultrastructural changes were found both acutely and chronically following TMT in rats.

How much neuropathology and behavior are related?

Interestingly, in humans intoxicated by TMT partial complex seizures (limbic seizures) are observed before and more often than tonic clonic seizures (Besser et al., 1987). In rodents, the bulk of the available data only refers to tonic clonic seizures (Ishida et al., 1997), which is quite unusual since this type of convulsions are triggered by hindbrain alterations, while the hipppocampal dysfunction is manifested through limbic seizures. As we reported before, it is likely that these latter attacks are underestimated in rodents' studies since they do not lead to the death of the animals and consist of slight changes of normal behavioral patterns (see for instance Racine, 1972). Thus, it is very likely that the occurrence of hippocampal and brainstem pathology is accompanied by limbic and tonic clonic seizures, respectively as reported in humans following TMT intoxication. On the other hand a damage/dysfunction of the superior colliculus in humans is unlikely to lead to epileptic activity but it is tightly related with acoustic dysfunction (Besser et al., 1987). In all these cases, since seizures occur transiently (a few days following acute TMT administration) it is likely that trigger lesions are responsible to fire the epileptic circuitry. For limbic seizures, this seems to occur specifically for the DG (Brown et al., 1979). In fact, acute TMT administration produces a serious damage to the DG which is otherwise minimally affected following chronic TMT administration (Bouldin et al., 1981).

In keeping with the extended limbic damage both in humans and rodents (this extends beyond the hippocampus to recruit the amygdaloid complex, septal nuclei and pyriform cortex), behavioral alterations are consistent with such findings. In humans this is associated with aggressive behaviour (rage-fear response, Ruch, 1965), impaired learning and memory loss (Halladay et al., 2006). The same effects are observed in rodents where marked self mutilations and aggressive behaviour when housing a few animals per cage is observed (Fiedorowicz et al., 2001; Lefebvre and Harry, 2005; Harry et al., 2008). Moreover, additional symptoms are observed in humans like disorientation, confabulation, retrograde and retrograde amnesia, dysregulation of feeding and sexual behavior. In agreement with the original definition given by Besser et al. (1987), the TMT intoxication syndrome in humans has been defined as a limbic cerebellar dysfunction. In fact, in addition to these symptoms, specific movement disorders due to cerebellar dysfunction are reported ranging from mild gaze-induced nystagmus to severe ataxia. According to these latter symptoms the pathology of the cerebellum was evident including a severe loss of Purkinje cells, (Balaban et al., 1988), the deafness and tinnitus found in humans may reflect the damage in the dorsal cochlear nucleus, inferior colliculus and medial geniculate body, while the somatosensory dysfunction and self-mutilation from thalamic nuclei (Balaban et al., 1988).

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