PERSISTENT PARADOXICAL EFFECTS ON STRIATAL AND LIMBIC ALPHA-SYNUCLEIN AND TYROSINE HYDROXYLASE FOLLOWING METHAMPHETAMINE WITHDRAWAL

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Introduction

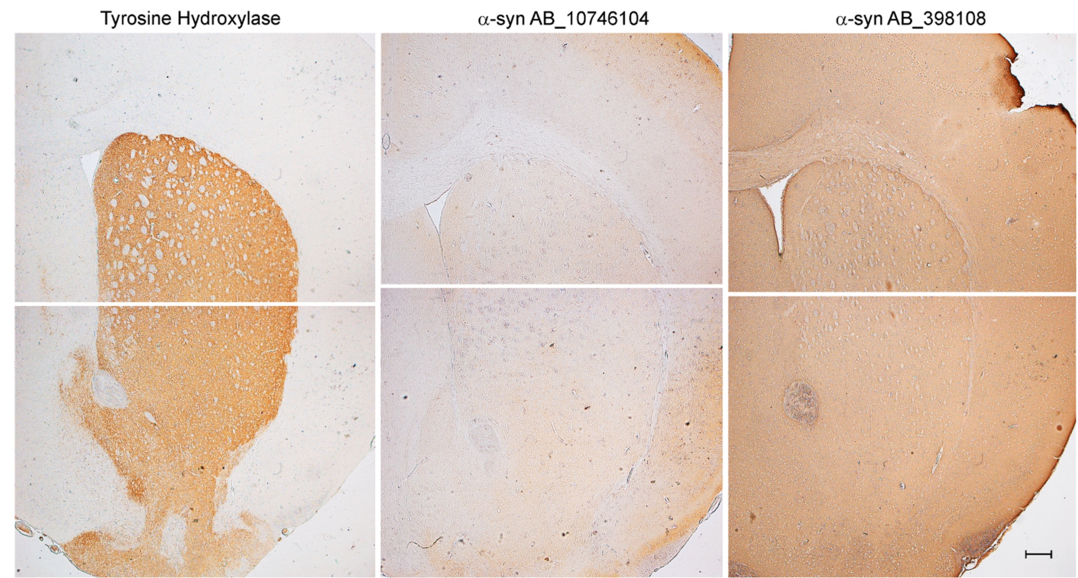
Methamphetamine (Meth) administration leads to experimental parkinsonism in rodents and primates, while in humans long-lasting Meth intake may increase the risk to develop Parkinson's disease (PD). PD is included in a disease group named synucleinopathies. Methamphetamine (METH) produces a variety of epigenetic effects in the brain, which are seminal to establish long-lasting alterations in neuronal activity. A number of studies were carried out aimed at rough assessment of the amount of either histone acetylation and methylation or direct DNA methylation, without a selective analysis of specific genes. In the present study we wish to assess whether METH-induced epigenetic alterations may specifically engage the expression of α-synuclein, which is a key protein in neurodegeneration and synaptic plasticity. In this way, a potential long-term alteration of brain circuitries may produce a variation in the threshold for neurotoxicity, sensitization, addiction and neurodegeneration. Thus, the occurrence of long-term changes in the expression of the protein were analyzed in parallel with persistent changes in a specific marker of integrity of meso-striatal/meso-limbic pathway, which is the expression of tyrosine hydroxylase (TH) both in the mesencephalon and within dorsal striatum. The integrity of dopamine (DA) projection was assessed at the level of the olfactory tubercle, the *nucleus accumbens* and *fundus striati*. Prolonged exposure to small doses of METH, produces nigro-striatal toxicity, when assessed at short time intervals following prolonged exposure. However, at prolonged time intervals a paradoxical increase progressively occurred in TH immunostaining within limbic regions. Such an increase exceeds at large the amount of TH expressed in controls. This occurs concomitantly with an overexpression of the primary transcript as well as the protein alpha synuclein within the same brain regions and dorsal striatum. This increase is persistent at prolonged time interval of METH withdrawal.The increase in the primary a-synuclein transcript is due to hypomethylation of specific CPG islands placed in the SNCA gene promoter which ranged roughly ten-fold of controls, it was steady, and it persisted at least 21 days following METH withdrawal. Thus, such an apparent synucleinopathy induced by METH indeed was associated with increased mesolimbic DA innervation, which equally surpasses several folds the amount which was measured in controls and persists at least for three weeks. The increase in SNCA is not associated with an increase of SNCA copy number. Nonetheless, the amount of the native protein, which is detected by ultrastructural stoichiometry, exceeds the increase reported following genetic SNCA multiplications (ten-fold of controls). These findings are discussed in the light of METH-induced phenotype changes which accompany toxicity, sensitization, addiction and neurodegeneration.

Methods

C57 black 6/J, 8 weeks-old male mice (Charles River Calco, Mi, Italy, N=120) we chose based on previous literature and our previous studies in different mouse strains. Methamphetamine administration and experimental design: A total of 120 mice were dedicated to these experiments. A group of 48 mice was dedicated to electron microscopy, while 72 mice were sacrificed for immunohistochemistry, protein assay, and DNA analysis. These latter 72 mice were divided into different experimental groups (N=24 each) according to three experimental protocols. These protocols consisted in different dosing and timing of Meth or saline administration. Methamphetamine (Meth, Sigma Aldrich Saint Louis, MO, U.S.A., Authorization n° SP/096, 05.15.2016, granted by the Italian Ministry of Health, was dissolved in saline and it was injected i.p. in a volume of 200 µL; saline was administered i.p. at the same volume). In the first subgroup (N=24) mice were sacrificed at different time intervals (1 h, 24 h, 7 d) following the last Meth injection; an additional group of mice (N=6) was administered saline (Controls). Meth was administered at the dose of 5 mg/Kg (×5, 2 h apart). From now on we refer to this protocol as acute time-course protocol since Meth was administered in a single day with a short-time interval between starting Meth and sacrifice. In the second subgroup of mice (N=24) Meth (5 mg/Kg), was injected daily, for 7 d. These mice were sacrificed at different time intervals (24 h, 7 d, 21 d,) after the last administration; an additional group (N=6) was administered saline (Controls). From now on we refer to this protocol as sub-acute time-course protocol (5×7) since Meth was administered for 7 d. The third subgroup of mice (N=24) received Meth (at the dose of 5 mg/Kg) daily, for 21 d and they were sacrificed at different time intervals (24 h, 7 d, 21 d,) after the last injection; an additional group (N=6) was administered saline (Controls). This protocol was named chronic time-course protocol (5×21). This corresponds to the most chronic condition since Meth treatment lasted 21 d. In both sub-acute and chronic protocols, the longest withdrawal between the last Meth injection and sacrifice was 21 d, which allows detecting the persistence of the effects induced by Meth. These mice were sacrificed by deep chloral hydrate anesthesia and their brains were quickly removed and processed according to different procedures. Since the critical data were obtained following acute and chronic protocols, further experiments were aimed at analyzing ultrastructural morphometry according to both acute and chronic time-course protocols. For these additional experiments we used 48 mice, which were sacrificed according to the acute (N=24) or the chronic (N=24) time-course protocol. **Post-sacrifice brain processing:** In the first experimental block (N=72), after sacrifice, the brains were quickly removed from the skull and they were divided into two hemispheres; one hemisphere was constantly used for immune-histochemical studies, while the other hemisphere was used either for immune-blot analysis, or DNA extraction for SNCA mutation detection, and methylation detection assay within SNCA promoter. In the second experimental block (N=48) mice were perfused trans-cardially under deep chloral hydrate anesthesia. The brains were dissected and immersed at 4 °C, overnight, in the perfusing solution. Tissue blocks from striata at the same level of that used for other assays were post-fixed. Immuno-histochemistry: Since Meth administration may lead to nigro-striatal DA denervation, we carried out in all mice immuno-histochemistry and immuno-blotting for Tyrosine-Hydroxylase (TH) to assess indirectly the loss of striatal DA innervation following different protocols of Meth administration, at different time intervals after the last Meth injection. In the same hemisphere, at the same striatal level, we analyzed the effects of Meth administration on TH and α-syn immuno-histochemistry, while in the contralateral hemisphere either striatal TH and α-syn SDS-PAGE immune-blotting, or DNA analysis were carried out. For TH and α-syn immune-histochemistry the hemisphere was removed and placed in Carnoy's solution and 10 µm thick tissue sections were used for immune-histochemical analysis, where TH and α-syn immune-reactivity were counted in serial sections to keep constant and comparable the level of analysis for both antigens. Brain slices were incubated overnight at 4 °C with mouse monoclonal anti-TH antibody (1:100; Sigma Aldrich) rabbit anti- α-syn (1:100; Sigma Aldrich); the sections were incubated with anti-mouse or anti-rabbit biotinylated secondary antibodies (1:200; Vector Laboratories) for 1 h, at 22 °C. Peroxidase activity was revealed by using the 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Sigma Aldrich). The stained sections were dehydrated, cleared and coverslipped with Micromount (Diapath). The specificity and quality of anti- α-syn immune-staining which was detected in brain slices was compared by using antibodies (1:100; Sigma Aldrich) and (1:300; BD). The pilot studies for striatal α-syn immuno-staining compared Sigma Aldrich antibodies with a presently available BD anti-α-syn antibody; both stainings shows a higher specificity for Sigma compared with BD anti-α-syn- antibody. Intensity of striatal TH and α-syn immune-reactivity was semiquantified by measuring relative optical densities. Despite being a semi-quantitative assay, values were consistent with a very low S.E.M. Immuno-blotting: the dorsal striatum was dissected out and placed in an Eppendorf tube containing 150 µL of ice-cold lysis with phosphatase and protease inhibitor to be homogenized. Proteins (20 µg) were separated on SDS-polyacrylamide gels (12%) and transferred on Immuno-PVDF membranes (Biorad) for 1 h. Membranes were blocked for 2 h in Tween-20 Tris-buffered saline (TTBS) containing 5% non-fat dry milk. Membranes were incubated overnight at 4 °C with primary antibodies rabbit anti- α-syn (1:1000; Sigma Aldrich), or mouse anti-TH (1:1000; Millipore). Blots of TH and α-syn were assessed for optical density through (Software, ImageJ) and expressed as the mean ± S.E.M. **DNA extraction**: The mouse striatal DNA following each experimental protocol (Meth- and saline-treated mice) was extracted by NucleoSpin® Tissue (Macherey Nagel GmbH & Co.KG) according to the manufacturer's instructions. The entire SNCA coding sequence (NM_001042451), as well as exon/intron boundaries and flanking intronic regions were analyzed by PCR and direct sequencing. The PCR assay was performed in 25 µL containing 50 ng genomic DNA (GoTaq® Flexi DNA Polymerase, Promega). PCR mix was amplified using the following cycle: 94 °C for 45 s, 58 °C for 30 s, 72 °C for 45 s (35 cycles), followed by a 6 min extension at 72 °C. PCR products were sequenced using ABI BigDye Terminator Sequencing Kit v.3.3 (Applied Biosystems) and ABI 310 Genetic Analyzer (Applied Biosystems). qPCR was performed in a CFX Connect™ Real Time System (Bio-Rad Life Science) using SYBR Green PCR Master (Applied Biosystems). The relative copy number was calculated through a ΔΔCT method, using β-Globin as an internal reference. qPCR was carried out in triplicate for each sample. Ten µL SYBR Green PCR Master (Applied Biosystems), 0.5 µM of each primer and 5 ng of genomic DNA were used as amplification reagents acting in a 15 µL volume. The PCR reaction took place at 95 °C for 10 min, 95 °C for 30 s, 58 °C for 1 min (40 cycles). The samples were homogenized in a lysis buffer and genomic DNA was sonicated using the S220 AFA Ultrasonicator (Covaris Inc.). The sonicated DNA was immune-precipitated with a monoclonal antibody against 5-hydroxymethylcytosine (5-hmC) (Diagenode) and it was incubated for 4 h at 4 °C. The DNA-antibody complex was enriched with Dynabeads Protein G (Life Technologies) and it was digested with Proteinase K. Both DNA fragments in the input and pulled down fractions were purified with phenol-chloroform extraction followed by acid ethanol precipitation. Real-Time PCR was carried out to amplify a region corresponding to CpG Island identified within SNCA promoter. Real-Time PCR was amplified in a CFX Connect™ Real Time System (Bio-Rad Life Science) at 95 °C for 10 min, 95 °C for 2 min, 54 °C for 1 min (40 cycles) using the following primers: SNCA, FW=5'-TCCCTAGGCTTCTGAAGAAC-3' and RW=5'-CGTCCCTCCCTAGGAAG-3'; GAPDH (as an hypomethylated control) and THS2B primers (as an hypermethylated control) (Diagenode) were used as an internal reference. Percentage of methylation for CpG obtained was expressed by averaging the value of all CpGs per assay (0% non-methylated, 100% fully methylated). 5hmC MeDIP Real-Time PCR data were first normalized using the efficacy of each qPCR assay. The ratio IP/input was transformed in percentage. **Immuno-gold-transmission electron microscopy**: Tissue blocks from striata at the same level of that considered for immune-staining and SDS-PAGE immune-blotting were dissected for transmission electron microscopy (TEM). From these blocks, 50 µm thick sections were cut following a coronal plane. All sections were obtained from blocks previously fixed in a solution containing 2.0% paraformaldehyde and 0.1% glutaraldehyde in 0.1M PBS (pH=7.4) for 90 min at 4 °C. After removing the fixing solution, striatal sections were post-fixed in 1% OsO4 for 1 h at 4 °C; they were dehydrated in ethanol and finally embedded in epoxy resin. For ultrastructural morphometry, grids containing non-serial ultrathin sections (40–50 nm thick) were examined at TEM, at a magnification of 8000×. Cells to be counted were carefully selected based on ultrastructural criteria, which were previously described for medium-sized striatal neurons. Plain TEM was implemented by post-embedding immuno-cytochemistry for antibodies against α-syn. At the end of the plain TEM or immuno-cytochemistry, ultrathin sections were stained with uranyl acetate and lead citrate, and they were finally examined using a JEOL JEM-100SX TEM (JEOL). Fixing and post-fixing solutions and the use of epoxy resin were validated in our previous studies for immune-gold-based ultrastructural morphometry. **Statistical analysis**: All data are shown as the mean ± S.E.M. in each group.

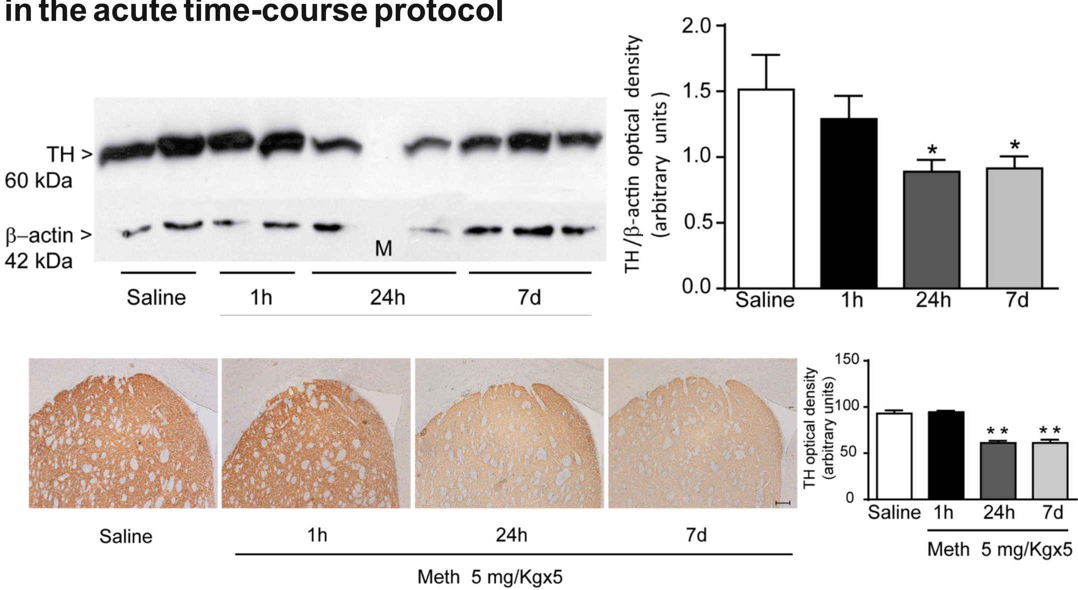
RESULTS

1. Pilot dual alpha-synuclein immuno-staining compared with classic tyrosine-hydroxylase immuno-staining in the whole striatum.



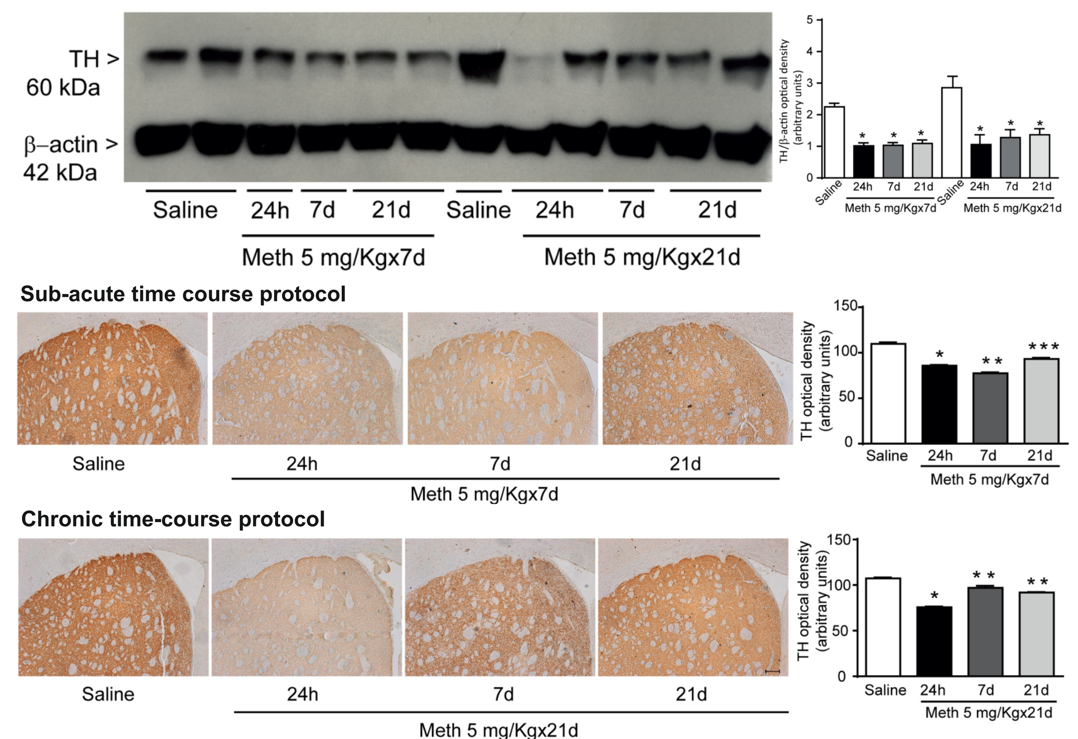
Slices from Controls (salinetreated mice) at striatal level (AP=+1.10 from Bregma) were stained to show representative images of differential α-syn immuno-staining depending on different primary antibody and tissue processing. In this pilot study striatal α-syn immuno-staining with Sigma Aldrich antibody (AB_10746104; middle slice) was compared with a currently available BD anti-α-synantibody (AB_398108; right slice); the left slice reports a classic striatal TH immunostaining (Sigma Aldrich; AB_477560; left slice). Immune-staining with anti-α-syn antibody shows a higher specificity for Sigma compared with BD anti-α-syn-antibody. Scale bar 200 µm.

2. Methamphetamine reduces tyrosine-hydroxylase immuno-reactivity in the acute time-course protocol



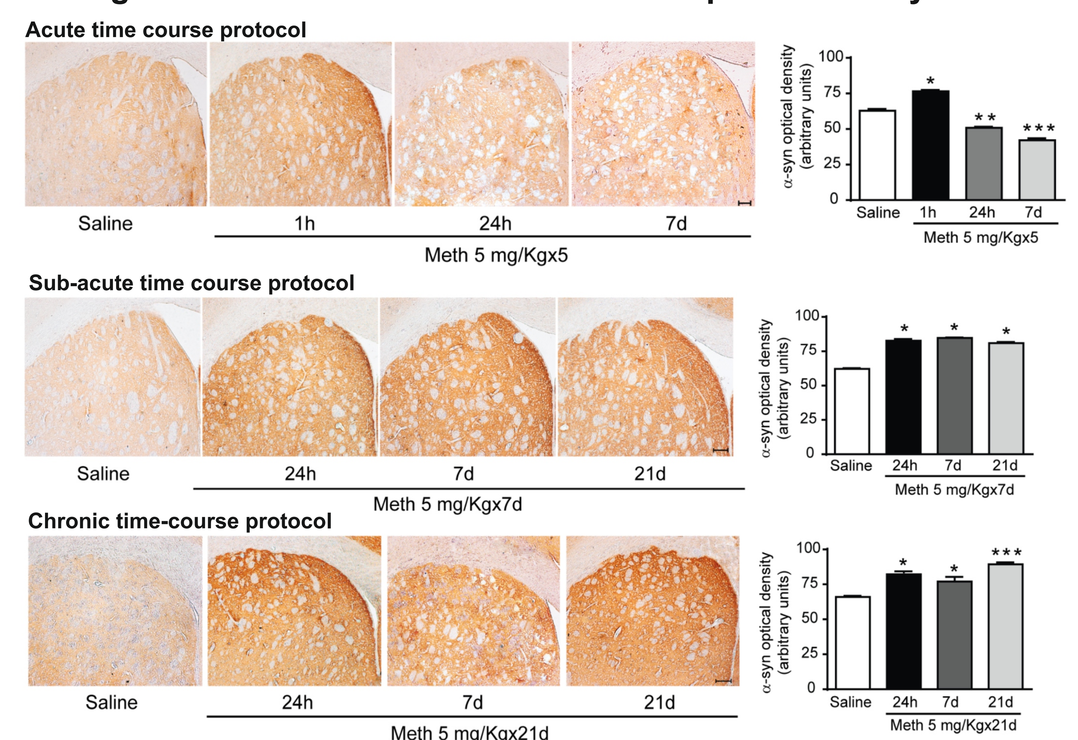
immuno-blot analysis shows a significant reduction in TH protein levels at 24 h and 7d after the last Meth administration according to the acute time-course protocol (5 mg/Kg X 5, 2 h apart). No changes were detected at 1 h (left and right panel) (*p=0.0052 *vs*. saline alone). Immuno-histochemical analysis of TH expression confirm the data obtained from immuno-blot analysis. Densitometric analysis ratifies the data obtained in the striatum of Meth-treated animals compared with Controls (salinetreated mice (**p < 0.0001 *vs.* saline and Meth 1 h). Scale bar 100 µm.

3. Meth reduces tyrosine-hydroxylase immuno-staining following both sub-acute and chronic time-course protocols



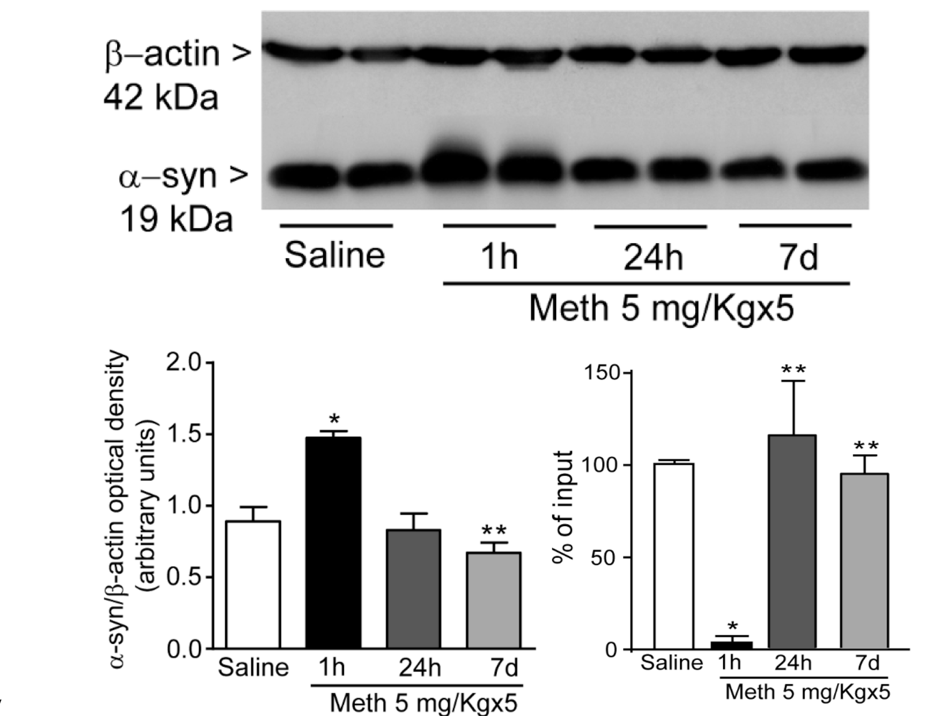
Methamphetamine reduces tyrosine-hydroxylase immuno-reactivity in the sub-acute and chronic time-course protocol. Immuno-blot analysis demonstrates a significant reduction in TH protein levels in the dorsal striatum in both sub-acute and chronic administration protocols (5 mg/KgX7 d or 5 mg/KgX21 d) (*p < 0.0001 *vs.* saline). Immuno-histochemistry shows a significant reduction of TH protein levels at 24 h and 7 d, and a partial recovery at 21 d after the last Meth administration according to the sub-acute time-course protocol (*p < 0.0001 *vs.* saline; **p < 0.0001 *vs.* saline and Meth 24 h; *** p < 0.0001 *vs.* saline, Meth 24 h and Meth 7 d). TH levels were significantly reduced at 24 h after the last Meth administration according to the chronic time course protocol. At 7 d and 21 d of Meth withdrawal, there is a partial recovery of TH, which in any case, remains significantly lower than Controls (saline treated mice; *p < 0.0001 *vs.* saline; **p < 0.0001 *vs.* saline and Meth 24 h). Scale bar 100 µm.

4. Meth persistently increases striatal alpha-synuclein immuno-staining in sub-acute and chronic time-course protocols only



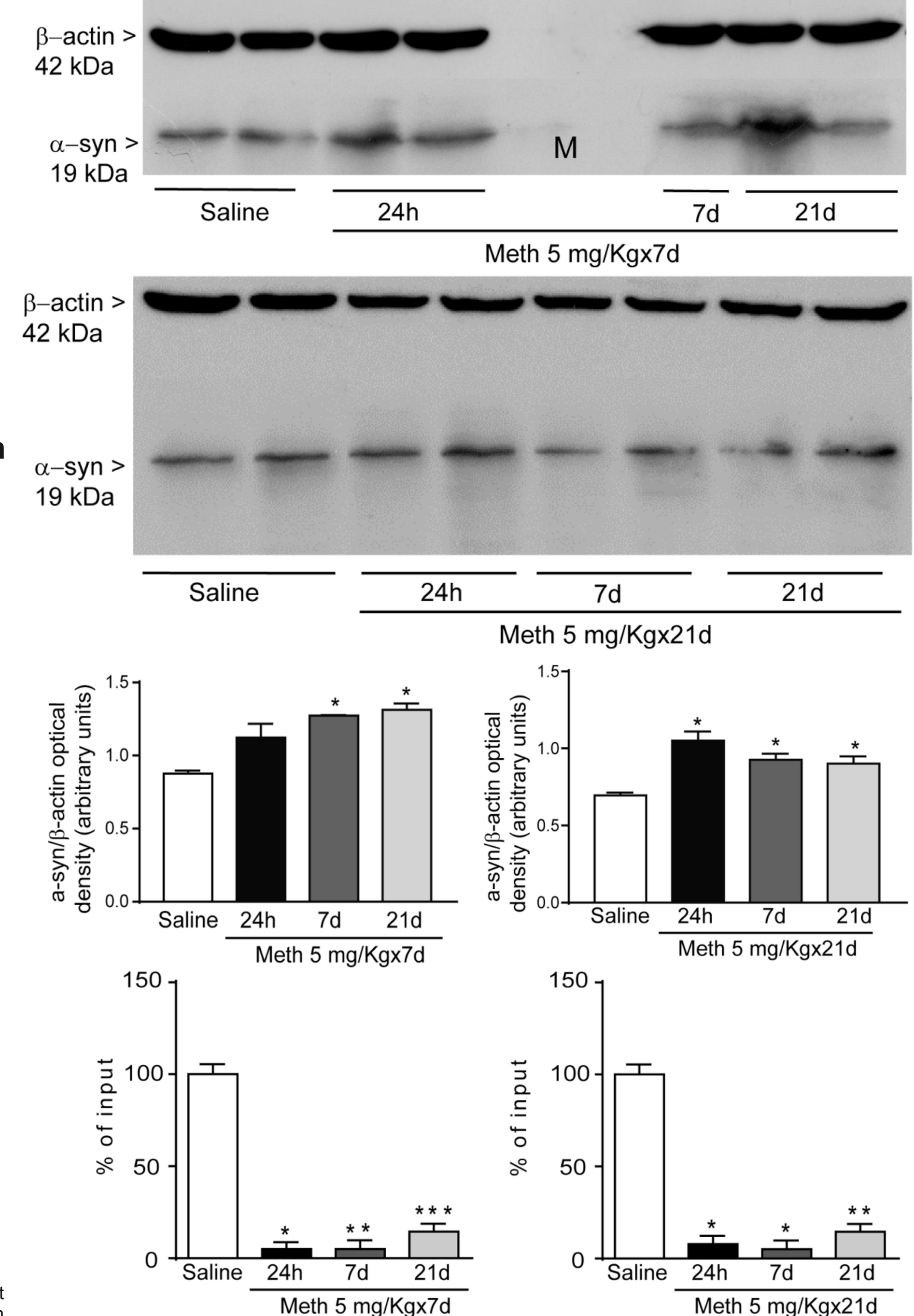
Methamphetamine increases alpha-synuclein immuno-reactivity in the acute, sub-acute and chronic time-course protocols. Immuno-histochemical analysis shows a remarkable increase of α-syn protein in acute time-course protocol at 1 h after the last administration of Meth. This increase is no longer evident at 24 h and 7 d. When administered according to sub-acute and chronic time-course protocols, Meth significantly increases α-syn immune-reactivity at any time of withdrawal analyzed (5 mg/KgX7 and 5 mg/KgX21. Scale bar 100 µm.

5. Meth increases alpha-synuclein protein levels and promotes hypomethylation of *SNCA* promoter in the acute time-course protocol.



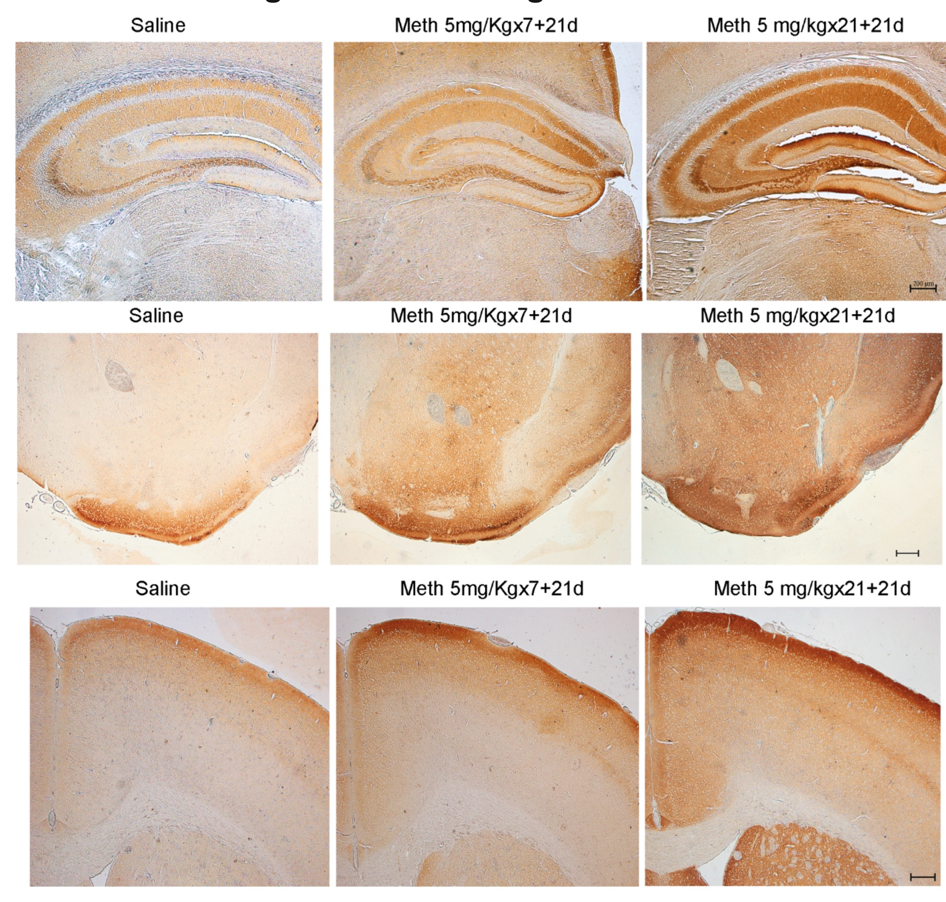
Immuno-blot analysis demonstrates a significant increase in α-syn protein levels at 1 h. Densitometric analysis was obtained as the mean ± S.E.M. The figure shows a representative gel of the mean values quantified in the graph as arbitrary units (*p < 0.0001 *vs.* saline; **p < 0.0001 *vs.* saline and Meth 1 h). Hypomethylation of *SNCA* promoter following acute Meth administration (5 mg/KgX5, 2 h apart). In this experimental condition, significant *SNCA* hypomethylation was observed only at 1 h of Meth withdrawal, while no changes were observed at 24 h and 7 d (*p=0.0036; **p=0.05 *vs.* Meth 1 h).

6. Meth increases alpha-synuclein protein levels and promotes hypomethylation of *SNCA* promoter in the sub-acute and chronic time-course protocol.

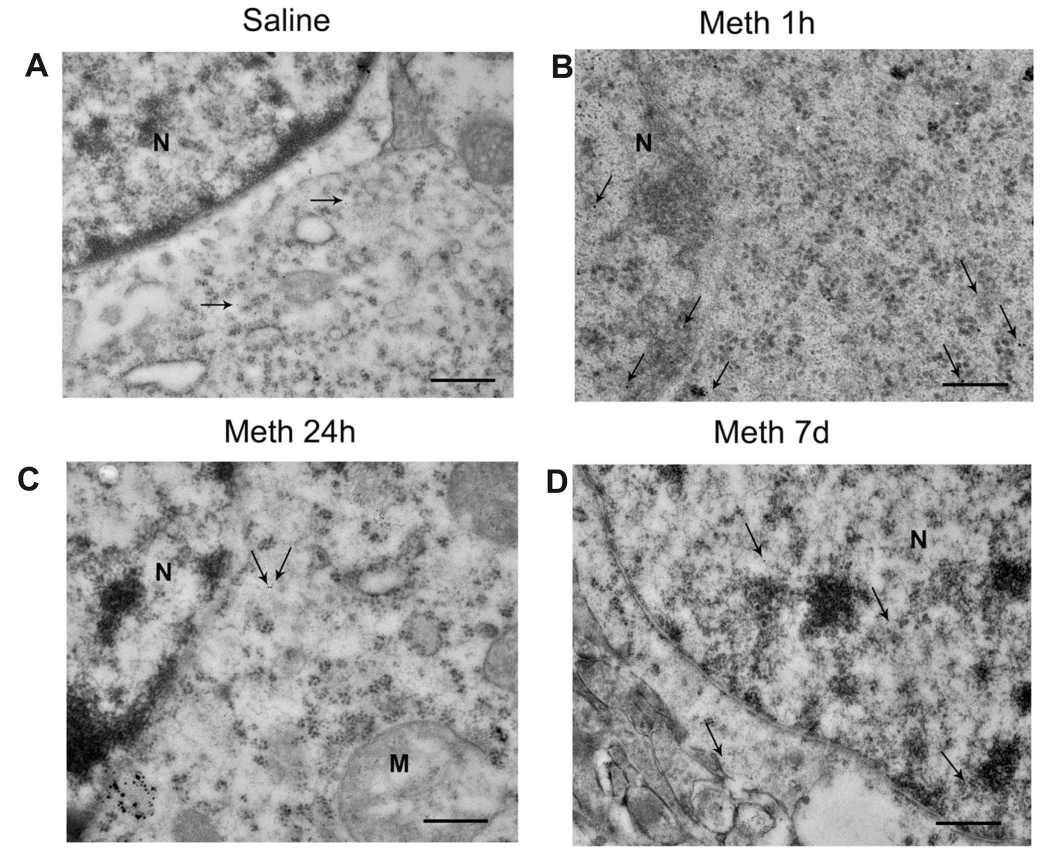


Immuno-blot analysis demonstrates a significant increase in α-syn protein levels at 7 d which persists at 21 d after the last administration of Meth according to the sub-acute (5 mg/KgX7 d) and chronic time-course protocol (*p=0.0016 *vs*. saline alone). A consistent hypomethylation of *SNCA* promoter following a sub-acute and chronic treatment is shown (5 mg/KgX7 d and 5 mg/KgX21 d *p < 0.0001 *vs.* saline alone; **p < 0.0001 *vs*. saline and Meth 24 h; ***p < 0.0001 *vs*. Meth 24 h and Meth 7 d; *p < 0.0001 *vs.* saline alone; **p < 0.0001 *vs*. saline and Meth 7 d).

7. Meth persistently increases striatal alpha-synuclein immuno-staining in other brain regions

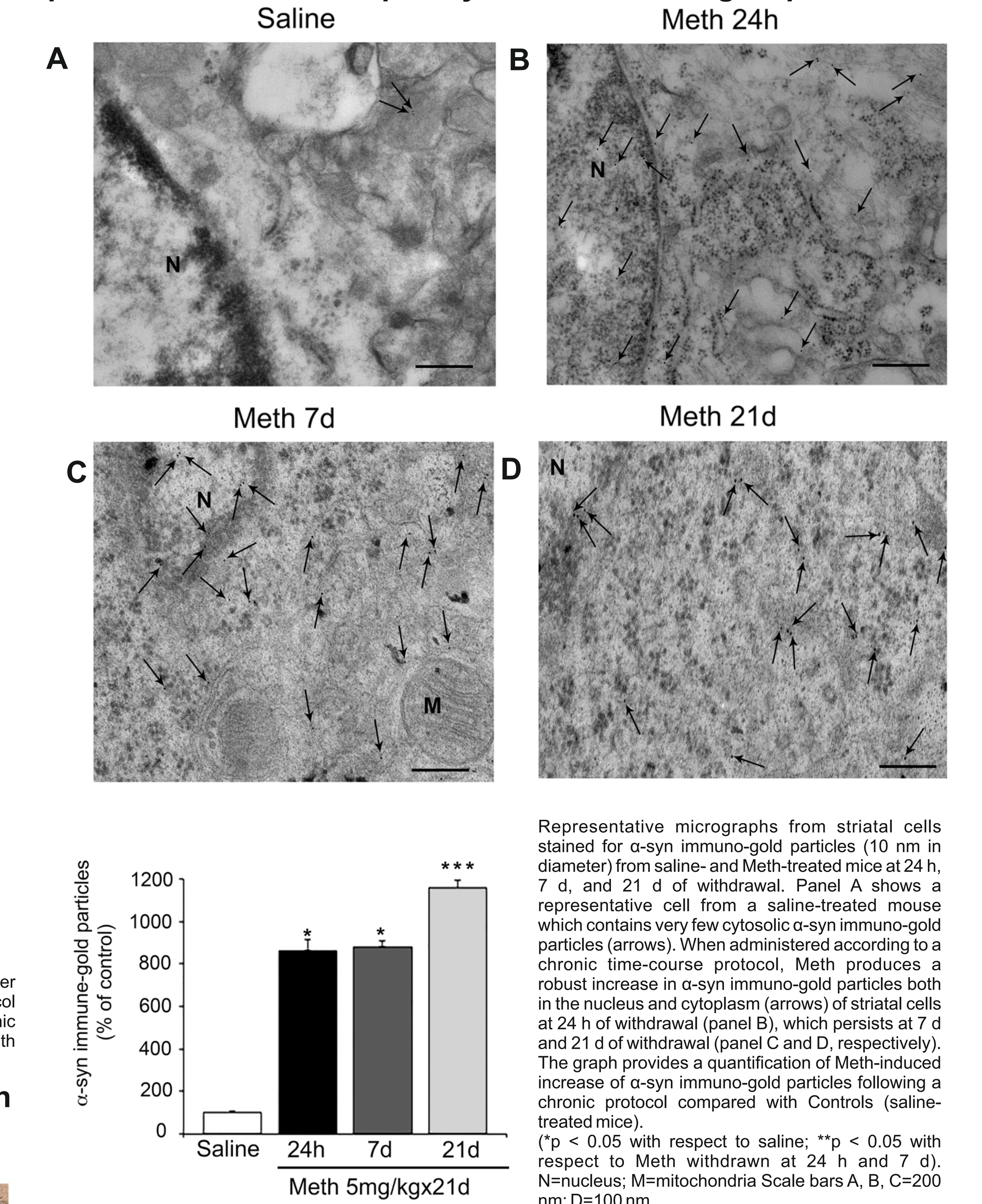


8. A single day of multiple injections transiently alters alpha-synuclein immuno-gold particles within striatal neurons



Representative micrographs from striatal cells stained for α-syn immuno-gold particles (10 nm in diameter) from saline- and Meth-treated mice at 1 h, 24 h, and 7 d of withdrawal. Panel **A** shows a representative cell from a Control (saline treated) mouse which contains very few cytosolic α-syn immuno-gold particles (arrows). At 1 h of withdrawal, acute Meth administration (5 mg/KgX5, 2 h apart) robustly increases α-syn particles both in the nucleus and cytoplasm (arrows), as shown in panel **B**. Instead, at 24 h and 7 d of Meth withdrawal, the amount of α-syn particles (arrows) falls back nearly to control levels (panel **C** and **D**, respectively). The graph reports the stoichiometric count of α-syn immune-gold particles in striatal cells from Meth-treated mice expressed as a percentage of values obtained from Controls (saline-treated mice). The data indicate that, when Meth is administered according to an acute time-course protocol, it significantly increases α-syn only at short-time intervals (1 h) of withdrawal (*p < 0.05 with respect to saline). N=nucleus; M=mitochondria Scale bars A, C, D=400 nm; B=200 nm.

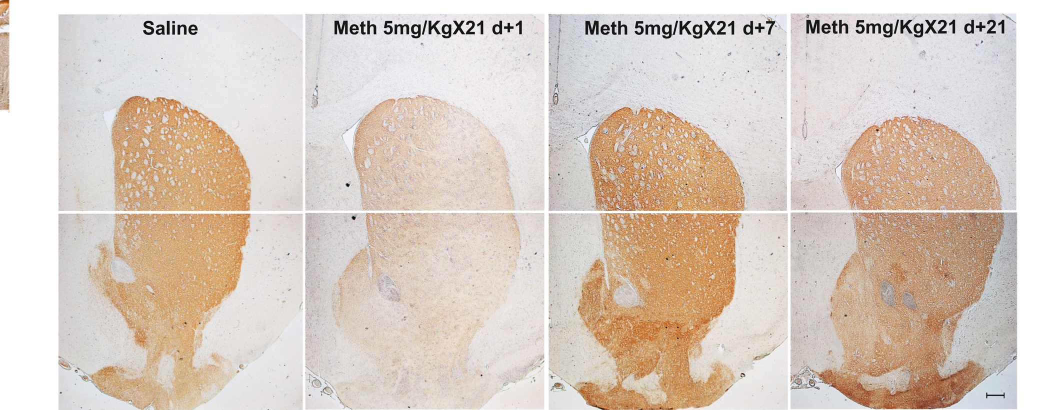
9. Chronic Meth administration produces a dramatic and persistent increase in alpha-synuclein immuno-gold particles



Representative micrographs from striatal cells stained for α-syn immuno-gold particles (10 nm in diameter) from saline- and Meth-treated mice at 24 h, 7 d, and 21 d of withdrawal. Panel A shows a representative cell from a saline-treated mouse which contains very few cytosolic α-syn immuno-gold particles (arrows). When administered according to a chronic time-course protocol, Meth produces a robust increase in α-syn immuno-gold particles both in the nucleus and cytoplasm (arrows) of striatal cells at 24 h of withdrawal (panel B), which persists at 7 d and 21 d of withdrawal (panel C and D, respectively). The graph provides a quantification of Meth-induced increase of α-syn immuno-gold particles following a chronic protocol compared with Controls (saline-treated mice).

(*p < 0.05 with respect to saline; **p < 0.05 with respect to Meth withdrawn at 24 h and 7 d). N=nucleus; M=mitochondria Scale bars A, B, C=200 nm; D=100 nm.

10. Meth produces a paradoxical increases striatal TH immuno-staining that exceeds the amount of TH expressed in controls



CONCLUSION

- Meth produces a nigro-striatal toxicity and a paradoxical icrease of TH immunoreactivity;
- Meth produces a persistently increase of alpha-synuclein immunoreactivity in sub-acute and chronic time-course protcols only;
- Meth increases alpha-synuclein protein levels and promotes hypomethylation of *SNCA* promoter in the sub-acute and chronic time-course protocol.