PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
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Tesis Doctoral

Individual differences in amphetamine sensitization: Role of dorsolateral striatum

Por

Rafael Ignacio Gatica Hernández,

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Individual differences in amphetamine sensitization: Role of dorsolateral striatum

Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Neurociencias

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Dedicatoria

It was all this reimagined

It was a different kind of speech

It was repetition

It was what you want it to be

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LIST OF ABBREVIATIONS

AMPH: Amphetamine

ANOVA: Analysis of Variance

cp: cerebral peduncle

DA: Dopamine

DLS: Dorsolateral Striatum

DREADD: Designer Receptor Exclusively Activated by Designer Drug

DS: Dorsal Striatum

eEPSC: evoked Excitatory Post Synaptic Currents

EP: Entopeduncular Nucleus

FR/L: Firing rate vs Locomotor activity

Geph: Gephyrin

GP: Globus Pallidus

GPCR: G Protein Coupled Receptor

HR: High Responders

ic: Internal capsule

ISI: Interspike interval

LR: Low Responders

LTD: Long Term Depression

LTP: Long Term Potentiation

LV/L: Lv index vs Locomotor activity

Lv: Local variation

MDMA: 3,4-methylenedioxy-methamphetamine

mPFC: medial Prefrontal Cortex

MSN: Medium Spiny Neuron

NAc: Nucleus Accumbens

PAR: Peak to Amplitude Ratio

PBS: Phosphate Buffer Solution

PFA: Paraformaldehyde

pMSN: putative Medium Spiny Neuron

SNc: Substantia Nigra pars compacta

SNr: Substantia Nigra pars reticulata

VTA: Ventral Tegmental Area

RESUMEN

Evidencia clínica ha mostrado que el desarrollo de adicción luego de consumo repetido de drogas de abuso no se observa en todos los sujetos. Estas diferencias individuales en las consecuencias de la exposición a drogas también han sido observadas en modelos animales. Cerca de la mitad de los animales muestra sensibilización locomotora luego de la exposición repetida a psicoestimulantes. Adaptaciones neuroquímicas y neuroplásticas diferenciales han sido encontradas en áreas involucradas en la búsqueda de drogas y la sensibilización a psicoestimulantes, incluyendo el núcleo accumbens y la corteza prefrontal medial. Estudios recientes han mostrado que el estriado dorsolateral (DLS), una región de los ganglios basales, también contribuye a la adicción a drogas. Sin embargo, no hay suficiente evidencia respecto al rol del DLS en las diferencias individuales observadas luego de la administración repetida de psicoestimulantes. El objetivo principal de esta tesis doctoral fue el estudio de las diferencias individuales en la actividad neuronal del DLS, así como también de las proyecciones axonales de áreas de salida estriatales, en ratas que fueron expuestas repetidamente a anfetamina (AMPH).

Ratas fueron tratadas repetidamente con AMPH 1.0 mg/kg ip y clasificadas como no-sensibilizadas y sensibilizadas. Registros unitarios de neuronas putativas espinosas medianas (pMSNs) del DLS fueron realizados en ratas anestesiadas 24 horas después de la última inyección de AMPH. Además, registros unitarios de pMSNs del DLS fueron realizados en ratas conductuando durante la administración repetida de AMPH. Luego de los registros en animal conductuando, la densidad de gefrina (Geph) fue medida usando estereología para cuantificar las sinapsis GABAérgicas de los núcleos de salida del estriado dorsal, véase, de la vía directa, el núcleo entopeduncular (EP) y la sustancia negra reticular (SNr), y de la vía indirecta, el globo pálido (GP).

En ratas anestesiadas, la expresión de sensibilización locomotora a AMPH se acompaña con un incremento de la frecuencia de disparo basal de pMSNs del DLS. En ratas conductuando, una inicialmente alta variación local (Lv) del DLS, un índice de patrón de disparo, fue observada en ratas vulnerables a la sensibilización. Durante la primera inyección de AMPH, ambos grupos mostraron un alto porcentaje de neuronas que cambian su frecuencia de disparo, así como una correlación directa entre la excitación producida por AMPH vs la frecuencia de disparo basal, y una disminución en el índice Lv. Por otra parte, durante la última inyección de AMPH, los efectos observados durante la primera inyección de AMPH se siguieron observando en ratas sensibilizadas, pero fueron revertidos en ratas no sensibilizadas. En otras palabras, una disminución del porcentaje de neuronas que cambian su frecuencia de disparo, una ausencia de correlación entre la excitación producida por AMPH vs la frecuencia de disparo basal, y la ausencia de cambios en el índice Lv se observaron en ratas no sensibilizadas en la última inyección de AMPH. En la misma línea de estos resultados, mientras que modificaciones en la

densidad de Geph no fueron observadas en el EP, SNr ni GP de ratas sensibilizadas, un incremento en la densidad de Geph en el GP fue observada en las ratas no sensibilizadas.

En resumen, la persistencia de los cambios producidos por AMPH en la frecuencia de disparo y el índice Lv del DLS, sin cambios en las sinapsis estriatales sobre la vía directa o indirecta, acompañan la expresión de sensibilización locomotora a AMPH. Por otra parte, una reversión de las modificaciones producidas por AMPH en la frecuencia de disparo y el índice Lv del DLS, así como un incremento en las sinapsis estriatales de la vía indirecta, acompañan la ausencia de expresión de sensibilización locomotora a AMPH. Juntos, estos resultados sugieren que diferencias individuales en las neuroadaptaciones del DLS son observadas entre ratas no sensibilizadas y sensibilizadas. En este sentido, una alta irregularidad de disparo puede subyacer a la vulnerabilidad a expresar sensibilización locomotora. Además, nuestros datos sugieren que la potenciación de la vía indirecta del estriado dorsal se podría considerar como un mecanismo protector a expresar sensibilización locomotora.

ABSTRACT

Clinical evidence have shown that the development of addiction after repeated consumption of drugs of abuse is not observed in all subjects. These individual differences in the consequences of drug exposure have been also observed in animal models. Nearly a half of animals show locomotor sensitization after the repeated exposure to psychostimulant. Differential neurochemical and neuroplastic adaptations have been found in areas involved in drug seeking and psychostimulant induced sensitization, including the nucleus accumbens and medial prefrontal cortex. Recent studies have shown that the dorsolateral striatum (DLS), a basal ganglia region, also contributes to drug addiction. However, evidence is lacking regarding the involvement of the DLS in the individual differences observed after repeated psychostimulant administration. Then, the main objective of this doctoral thesis was to study the individual differences in activity of DLS neurons, as well as their axonal projection on striatal output areas, in rats that has been repeatedly exposed to amphetamine (AMPH).

Rats were repeatedly treated with amphetamine 1.0 mg/kg ip and classified as non-sensitized and sensitized rats. Single unit recording of DLS putative medium spiny neurons (pMSNs) in anesthetized rats were performed 24 hours after the last AMPH injection. Also, single unit recording of DLS pMSNs were performed in freely moving animals during the repeated AMPH administration. After freely moving recording, gephyrin (Geph) density was measured using stereology to quantify to GABAergic synapses in the output nuclei of dorsal striatum, namely the entopeduncular nucleus (EP) and substantia nigra part reticulata (SNr) from the basal ganglia direct pathways, and the globus pallidus (GP) from the indirect pathway.

In anesthetized rats, the expression of AMPH locomotor sensitization was accompanied by an increase in DLS pMSNs baseline firing rate. In freely moving rats, an initially higher DLS local variation (Lv), an index of firing pattern, was observed in sensitization-vulnerable rats. During the first AMPH injection, both groups showed a high percentage of neurons showing change in their firing rate, as well as a direct correlation between AMPH excitation vs baseline firing rate, and a decrease in Lv index. On the other hand, during the last AMPH injection, the effects observed during the first AMPH injection were still observed in sensitized rats, but they were reverted in non-sensitized rats. In other words, a decrease in the percentage of neurons showing change in their firing rate, a lack of correlation between AMPH excitation vs baseline firing rate, and no changes in Lv index were observed in non-sensitized rats during the last AMPH injection. In line with these results, while no modifications in Geph density were observed on EP, SNr and GP of sensitized rats, an increase in Geph density on GP was observed in non-sensitized rats.

In summary, a persistence of AMPH changes on DLS firing rate and Lv index on DLS, without changes in striatal synapses on direct or indirect pathways, accompanies the expression of AMPH locomotor sensitization. On the other hand, a reversion of AMPH modifications on DLS firing rate and Lv index, as well as an increase in striatal synapses of the indirect pathway (GP)

accompanies the lack of expression of AMPH locomotor sensitization. Together, these results suggest that individual differences in DLS neuroadaptations are observed between non-sensitized and sensitized rats. In this sense, an increased firing irregularity could underlie the vulnerability to express locomotor sensitization. Moreover, our data suggest that the potentiation of dorsal striatum indirect pathway could be considered a protective mechanism to express locomotor sensitization.

1. INTRODUCTION

1.1. Drug Addiction

Drug addiction is a disease that has multiple impacts in people's life. It is defined as a cluster of cognitive, behavioral and physiological symptoms associated to the compulsive use of drugs, despite the adverse consequences following dependence (Frey, 2018). Chronic drug use is associated with cardiovascular pathology, psychotic disorders, increased mortality, and others diseases (Degenhardt and Hall, 2012). In the world, problematic consume of 'unsanctioned psychoactive drugs", such as cocaine and amphetamine (AMPH) has a prevalence of 1% (Gowing et al., 2015). In Chile this is not different, where the problematic use of cocaine, coca paste and AMPH have a prevalence of 1-2 % (Senda, 2014). Drug addiction has a high relapse rate (40-60%), that shows how complex is to achieve adherence to the treatment (McLellan et al., 2000). Currently, there are few options to treat this disorder (Christopher Pierce et al., 2012).

Interestingly, not all the subjects exposed to drugs of abuse progress to a chronic use (Anthony et al., 1994), a result also observed in animal models of the disease (Piazza et al., 1989). Initial differences and neuronal adaptations are observed after repeated drug administration. Knowing how the brain changes specifically in addicted subjects can help to develop more effective therapies.

1.2. Behavioral sensitization to psychostimulants

The term sensitization is used to refer to an increase of a drug effect when it is repeatedly administered. Robinson and Berridge stated that one of the most important psychological changes in drug addiction is a "sensitization or hypersensitivity to the incentive motivational effects of drugs and drug associated stimuli" (Robinson and Berridge, 1993). This process is accompanied by changes in structural plasticity in nucleus accumbens (NAc), a brain area related to incentive salience and motivation to stimuli, and the medial prefrontal cortex (mPFC), related to the inhibitory control of behaviors (Robinson and Kolb, 2004). This sensitization is not related to the euphoric effects of drugs (referred as drug "liking"); instead, it is sensitized the drug incentive salience (or "wanting") that is responsible of drug seeking and taking (Robinson and Berridge, 2001).

In drug addiction research, behavioral sensitization is one valid preclinical model that allows the study of hypersensitivity to the incentive motivational effects of drugs (Steketee and Kalivas, 2011). This increased salience by the drug can be indirectly measured by an augmented locomotor activity, a change that reflects the engagement of the brain incentive systems (Robinson and Berridge, 2008). Repeated use of psychostimulant drugs like AMPH or cocaine is associated with behavioral sensitization and increased locomotion in animals. These behavioral changes can be examined in two distinctive phases: induction and expression. Induction is referred as the transient neuroadaptations related to the psychostimulant administration responsible of the augmented locomotion. In turn, expression is the stage when these transient changes pass to be permanent after a period of abstinence (Pierce and Kalivas, 1997). AMPH sensitization is also observed in humans (Boileau et al., 2006). These changes

can persist for months or even years after drug discontinuation (Castner and Goldman-Rakic, 1999; Paulson et al., 1991).

One of the most studied anatomical pathways in behavioral sensitization is the motivation circuit. This circuit includes the mesocortical dopamine (DA) system, originated from dopaminergic neurons of the ventral tegmental area (VTA), that send axonal projections to the NAc and mPFC (Fallon and Moore, 1978). A bulk of evidence shows that behavioral sensitization to psychostimulants it is a process associated to neurochemical and neuroplastic changes in this circuit (for review, Steketee and Kalivas, 2011).

An increase in the somatodendritic DA release on VTA during the induction but not the expression of cocaine sensitization was found (Kalivas and Duffy, 1993), that was related to a decreased sensitivity of D2 autoreceptors (White and Wang, 1984). Furthermore, intra-VTA infusions of D1 antagonist blocked the development of AMPH sensitization (Stewart and Vezina, 1989). Also, an increase in VTA extracellular glutamate levels was observed after AMPH locomotor sensitization (Xue et al., 1996). In this sense, intra-VTA injection of NMDA antagonist blocked the development of AMPH locomotor sensitization (Kalivas and Alesdatter, 1993). Other neuroplastic changes involves an AMPA mediated long-term potentiation (LTP) in VTA dopaminergic neurons after acute and repeated cocaine exposure (Borgland et al., 2004; Ungless et al., 2001). Overall, these evidence supports that VTA function is relevant during the induction phase of psychostimulants sensitization.

In the mPFC, an increase in stimulated DA release was observed after the expression of AMPH locomotor sensitization (Casanova et al., 2013). In this same line, repeated administration of a D2 receptor antagonist into mPFC generates cross-sensitization to an acute cocaine injection

(Steketee and Walsh, 2005). mPFC extracellular glutamate levels were also increased after cocaine locomotor sensitization (Williams and Steketee, 2004). Relevantly, induction of LTP was increased in the mPFC after repeated cocaine administration (Huang et al., 2007).

In the NAc, increased DA levels were associated with the expression of behavioral sensitization to AMPH (Robinson et al., 1988) and cocaine (Kalivas and Duffy, 1990). Furthermore, repeated cocaine administration increased D1 receptor sensitivity in the NAc (Henry and White, 1991). Also, D1 agonist injection into NAc potentiate the development of cocaine sensitization (De Vries et al., 1998). In contrast to VTA and mPFC, a decrease in basal extracellular glutamate levels in NAc was observed after cocaine sensitization (Pierce et al., 1996). Moreover, intra-NAc pretreatment using a AMPAR antagonist prevented the development of cocaine locomotor sensitization (Ghasemzadeh et al., 2003). Interestingly, withdrawal from repeated cocaine administration was accompanied with an increase in AMPAR/NMDAR ratio, showing a synaptic potentiation, that was reverted after cocaine challenge (Kourrich et al., 2007), showing a change from a synaptic potentiation to a synaptic depression in NAc.

In summary, specific neuroplastic changes, both transient and persistent, are observed in the motivation circuit, that accompanies the maintenance of the behavioral changes observed in behavioral sensitization.

1.3 Individual differences to develop drug addiction

Not all the subjects exposed to addictive drugs develop drug addiction. In humans, between a 15 to 17 % of those who use addictive drugs pass to a chronic use (Anthony et al., 1994). At least, part of this individual vulnerability is associated to the subjective effects after the acute consumption of the drug of abuse. In a clinical study, subjects blindly received both AMPH and

placebo. Then, they were asked for their preference. The group that preferred AMPH reported stimulant and euphoric effects after use the drug, whereas the group that did not choose AMPH reported less of these stimulant and euphoric effects and more depressive effects (de Wit et al., 1986). These data show that drugs of abuse do not produce the same rewarding effects in all individuals. The understanding of the neurobiological basis of these individual differences in drug addiction are relevant, because it could allow to develop preventive therapies for addiction-susceptible subjects and to generate more effective pharmacotherapies to revert the neuroplastic changes associated to chronic drug use. Considering that preclinical models reproduce different phases of drug addiction disease (Sanchis-Segura and Spanagel, 2006), the use of pre-clinical models appears as an indispensable tool for studying the neurobiological bases underlying the individual differences in drug of abuse vulnerability.

Individual differences are also observed in animal models of drug addiction. In cocaine self-administration experiments, around a 17% of the animals show the characteristic traits of addictive behavior: persistence of drug seeking, resistance to punishment and motivation for the drug (Deroche-Gamonet et al., 2004). In behavioral sensitization, about a 50% of the animal exposed to repeated AMPH develop and express AMPH sensitization (Casanova et al., 2013; Scholl et al., 2009). One individual trait that relates to this differential response to psychostimulant is locomotor response to a novel environment. Rats classified as “low responders” (LR) to novelty develop AMPH sensitization, while rats classified as “high responders” (HR) to novelty do not show an increase in locomotor activity after repeated exposure to AMPH (Piazza et al., 1989). This differential reactivity to novelty is accompanied with a higher baseline firing rate and burst frequency of substantia nigra pars compacta (SNc) and VTA dopaminergic neurons in HR rats compared to LR rats (Marinelli and White, 2000).

In addition, specific individual responses such as differential locomotor response to mild stress (Herrera et al., 2013) or low cocaine dose (Yamamoto et al., 2013) can also predict behavioral sensitization.

Moreover, specific neuroplastic modifications induced by the repeated exposure to psychostimulants contribute to the individual differences the expression of locomotor sensitization For example, AMPH sensitized rats show higher DA concentrations in NAc and lower DA concentrations in VTA compared to non-sensitized rats (Scholl et al., 2009). Results from our laboratory have shown that specific neurochemical modifications in mPFC cortex accompany the reinforcing properties of AMPH. Thus, an increase in potassium-stimulated DA release in mPFC is observed in rats that express locomotor sensitization, in relation to non-sensitized rats after AMPH repetitive administration (Casanova et al., 2013). Interestingly, Kasanetz and collaborators have shown that the persistence of neuroplastic changes observed in the early phase of drug use is critical to develop drug addiction. Specifically, those animals showing controlled drug consumption reverse these initial neuroplastic changes. This deficit in the ability to reverse neuroplastic changes caused by repeated drug use has been called "anaplasticity" (Kasanetz et al., 2010). Taking together, the evidence suggests that the maintenance of the reinforcing properties of psychostimulants depend on persistent specific neuroplastic changes.

Overall, these data show that specific innate neural differences and drug-induced neuroplastic modifications in motivation circuits areas are important for the progression of addictive-like behaviors. However, other brain areas could be relevant to stablish these individual differences after repeated drug administration.

1.4 Role of dorsolateral striatum on drug addiction

The striatum is part of the basal ganglia system. It is divided in a ventral area (NAc) related to motivation and reinforcement (as described above), an associative dorsomedial area and a sensorimotor dorsolateral area, the dorsolateral striatum (DLS, Haber, 2003). In particular, the DLS has been related to habit formation (Graybiel, 2008; Yin and Knowlton, 2006), motor responses and instrumental learning (Lovinger, 2010).

Everitt and Robbins defined drug addiction has "the endpoint of a series of transitions from initial drug use, when a drug is voluntary taken because it has reinforcing effects, through loss of control over this behavior as it emerges as a stimulus-response habit, ultimately to become compulsive and not easily relinquished" (Everitt and Robbins, 2013). These authors state that there is a shift in brains areas that control behavior; specifically, a dopaminergic transition from ventral to dorsal striatum and this change is related to habitual drug taking . At early states of cocaine self-administration, NAc core (but not shell) is necessary for acquisition of cocaine seeking, as showed with lesion experiments (Ito et al., 2004). But, when cocaine seeking behavior becomes habitual, DA receptor antagonism in DLS, and not NAc core, decreases cocaine seeking (Vanderschuren et al., 2005). This change from ventral to dorsal striatum control on habitual cocaine seeking it has been associated with an increase of DA in DLS (Ito et al., 2002).

This ventral-to-dorsal striatum shift could be mediated by the spiral DA loop that links these areas (Haber et al., 2000; Ikemoto, 2007). By disconnecting ventral from dorsal striatum with unilateral lesion of NAc core and using DA receptor antagonist in DLS, it has been shown a decrease in drug-seeking behavior (Belin and Everitt, 2008). Moreover, modifications in DLS

DA release has been observed after repetitive psychostimulant administration. Using voltametric measurement of DA release, Willuhn and colaborators showed a change in DA release over the course of drug taking behavior: a decrease in DA release was observed in ventral striatum, but at the same time DA release in DLS is increased. Furthermore, this increase in DA release on DLS was prevented in animals with a lesion on the ipsilateral ventral striatum (Willuhn et al., 2012). Together, these data show the relevance of DLS in the progression to chronic drug taking, which depends on its connections with the NAc via spiral DA loop (Haber et al., 2000; Ikemoto, 2007).

1.5 Role of dorsolateral striatum on behavioral sensitization

The dorsal striatum receives dopaminergic afferents principally from substantia nigra pars compacta, through the so called nigrostriatal pathway. This pathway has a critical role in movement. In turn, two distinctive GABAergic output pathways emerge from the dorsal striatum: the striato-nigral and striato-pallidal pathway, usually called direct and indirect pathway, respectively (Dudman and Gerfen, 2015). Optogenetic activation of the direct pathway is related to initiation and expression of voluntary movement, whereas optogenetic activation of indirect pathway is related to suppression of movements (Kravitz et al., 2010). Given that behavioral sensitization has a strong locomotive component, various research approaches have been carried out to unveil the role of the dorsal striatum in behavioral sensitization. For instance, a loss of nigrostriatal DA neurons using mutant mice lacking of Pitx3 resulted in animals that did not show development of behavioral sensitization (Beeler et al., 2009). Also, to study the relative contribution of DLS efferent pathway in AMPH sensitization, Ferguson and collaborators used a viral mediated strategy in dorsal striatum to express a DREADD (Designer Receptor Exclusively Activated by Designer Drug) Gi/o G protein coupled receptor (GPCR)

specifically expressed in direct or indirect pathway neurons. This data shows that direct pathway inactivation disrupt expression but not the induction and development of AMPH sensitization. On the other hand, indirect pathway inactivation facilitated both the development and expression of AMPH sensitization (Ferguson et al., 2011). Finally, activation with a DREADD Gs GPCR expressed in indirect pathway neurons prevented development and expression of AMPH sensitization (Farrell et al., 2013). With this evidence, it is tempting to suggest that modifications of the indirect pathway are enough to the development of behavioral sensitization. On the other hand, both pathways are necessary for the expression of behavioral sensitization.

The important role of DLS in behavioral sensitization is highlighted by psychostimulant induced neuroplastic changes. As showed by Willuhn and collaborators with cocaine self-administration (Willuhn et al., 2012), an increase in DLS basal DA levels was observed after the expression of AMPH locomotor sensitization (Azocar et al., 2019). Furthermore, an intra-dorsal striatum infusion of D1 antagonist blocked the expression of AMPH locomotor sensitization (Wang et al., 2013). Cocaine sensitization increases depolarization-evoked glutamate release in DLS, with AMPAR antagonism on DLS blocking the development of sensitization (Parikh et al., 2014). Also, an increase in DLS dendritic spine density was observed after withdrawal from methamphetamine sensitization (Jedynak et al., 2007). Interestingly, Wang and collaborators showed specific functional adaptations in medium spiny neurons (MSNs) from DLS, induced by presynaptic plasticity after repeated administration of AMPH (Wang et al., 2013). Specifically, while an in vitro AMPH challenge decreased eEPSC (evoked excitatory post synaptic currents) amplitude in saline group, an increase in eEPSC amplitude after in vitro AMPH challenge was observed in repeated AMPH administration group. This effect, that was defined as an “paradoxical” presynaptic potentiation, was associated particularly to D1 MSNs.

On the other hand, a heterogeneous response was observed in D2 MSNs: while a group of D2 MSNs showed this "paradoxical" presynaptic potentiation, other D2 MSNs showed chronic presynaptic depression (Wang et al., 2013). These data indicate that repeated psychostimulant administration result in differential changes in direct pathway compared to the indirect pathway.

1.6 Role of dorsolateral striatum on individual differences on behavioral sensitization: anatomo-functional changes

Given the relevance of DLS in locomotor sensitization to psychostimulants, could this area be differentially changed after repeated drug administration? Evidence have shown a decrease in total DA content only in non-sensitized rats after repeated AMPH administration (Scholl et al., 2009). Moreover, DLS baseline firing rate was decreased after repeated 3,4-methylenedioxy-methamphetamine (MDMA or 'ecstasy') administration only in sensitized rats (Ball et al., 2010). These data support the hypothesis that differential functional neuronal adaptations underlie the expression of locomotor sensitization to psychostimulants. Yet, data is still lacking regarding if individual differences after repeated psychostimulants are observed in firing pattern and the degree of correlation between locomotor activity and firing rate in DLS. Also, it has been not studied if individual differences in DLS neuronal activity can predict the expression of locomotor sensitization. Importantly, it has been observed a high baseline firing rate and burst frequency in SNc and VTA of HR responders compared to LR rats (Marinelli and White, 2000). Together with the data showing that LR rats are vulnerable to express AMPH locomotor sensitization (Piazza et al., 1989), these results suggest that a decreased SNc and VTA neuronal activity is required to develop and express psychostimulant behavioral sensitization after repeated drug administration.

The first main objective of this doctoral thesis was to study the individual differences on DLS neuronal activity during repeated AMPH administration. To assess these questions, two approaches were used. Firstly, single unit recordings in anesthetized rats were carried out to determine the effect of repeated AMPH administration on DLS putative MSNs (pMSNs) neuronal activity measures (firing rate, firing pattern and the effect of an acute AMPH injection). Firing pattern of pMSNs was studied using the local variation index (Lv, Shinomoto et al., 2009). Secondly, single unit recordings in freely moving rats were performed. DLS pMSNs neuronal activity measures were studied before the beginning of AMPH locomotor sensitization, to determine if the individual differences in DLS neuronal measures can be used as a predictor of the expression of AMPH locomotor sensitization. Then, DLS firing measures and locomotor activity/neuronal measures correlation were studied to determine individual differences during repeated AMPH administration. Considering that dopaminergic (Azocar et al., 2019) and presynaptic (Wang et al., 2013) adaptations accompanies the expression of AMPH locomotor sensitization, an increase in DLS pMSNs firing rate and firing irregularity is expected only in sensitized rats, given the role of dopamine and glutamate modulating striatal neurons activity (Kiyatkin and Rebec, 1996)

Considering that differential DLS functional adaptions are expected after repeated AMPH administration, what DLS structural adaptations could underlie the expression of AMPH locomotor sensitization? Also, which striatal neuronal population is mainly modified: direct or indirect pathway neurons? As previously shown, basal ganglia direct and indirect pathway have an opposite role during the development and expression of psychostimulant locomotor sensitization (Farrell et al., 2013; Ferguson et al., 2011). One limitation of single unit recordings in striatum is that is not possible to differentiate between direct or indirect pathway MSNs. Also,

the lack of a specific marker for MSNs can be a problem to study morphological changes in dorsal striatum. But, alternative methods have been proposed. One of these is to study the changes in GABAergic synapses of the output regions of dorsal striatum: for the direct pathway, the substantia nigra pars reticulate (SNr) and the entopeduncular nucleus (EP), and for the indirect pathway, the globus pallidus (GP). Given that these output regions mainly receive projections from dorsal striatum (Dudman and Gerfen, 2015), this method can give us reliable information of the contribution of dorsal striatum synapses in the expression of AMPH locomotor sensitization. It has been demonstrated that gephyrin (Geph) colocalize in post-synaptic clusters of GABAergic synapses (Essrich et al., 1998). For this, it has been largely used to determine the number of GABAergic synapses in neuroanatomical studies (Groeneweg et al., 2018). Also, Geph estimation has been used to quantify changes GABAergic synapses in Parkinson's disease (Fan et al., 2012), Alzheimer's disease (Agarwal et al., 2008), seizure (Kumar and Buckmaster, 2006) and Huntington's disease models (Thompson-Vest et al., 2003). In relation to drug of abuse studies, a two-week withdrawal after the expression of cocaine conditioned place preference was accompanied by a decrease the protein expression levels of Geph in mPFC (Yang et al., 2017). Taking together, Geph measurement could be relevant to quantify changes in GABAergic synapses from dorsal striatum outputs after repeated AMPH administration.

The second main objective of this doctoral thesis it is to quantify GABAergic synapses in SNr, EP and GP of AMPH sensitized and non-sensitized rats. With this neuroanatomical technique, we want to determine if differential changes in direct and indirect pathway are related to the individual susceptibility to express AMPH sensitization after repeated drug administration. Given that changes in the indirect pathway are important for both the development and

expression of behavioral sensitization (Farrell et al., 2013; Ferguson et al., 2011), we propose that an increase in GABAergic synapses to GP is a protective mechanism observed in non-sensitized rats. On the other hand, an increase GABAergic synapses to direct pathway (SNr and EP) and a decrease in GABAergic terminals in GP could be found in rats that express AMPH locomotor sensitization.

1.7 Hypothesis

The individual differences observed in behavioral sensitization after repeated amphetamine administration depend on functional changes on dorsolateral striatum and structural changes on striatal output pathways.

1.7.1 Specific hypothesis

1. The expression of locomotor sensitization is accompanied by an increase in baseline firing rate and firing irregularity in dorsolateral striatum after repeated amphetamine administration.

2. The expression of locomotor sensitization increase GABAergic synapses in substantia nigra pars reticulata and entopeduncular nucleus, and decrease GABAergic synapses in globus pallidus after repeated amphetamine administration.

3. The lack of expression of locomotor sensitization increase GABAergic synapses in globus pallidus after repeated amphetamine administration.

1.8 Objectives

1.8.1 General Objectives

To study dorsolateral striatum neuronal activity and density of inhibitory post-synaptic markers on striatum output areas in rats repeatedly treated with amphetamine.

1.8.2 Specific Objectives

1. To determine the firing rate and firing pattern of dorsolateral striatum neurons after amphetamine repeated administration in anesthetized rats.

2. To determine neuronal activity and locomotion correlation of dorsolateral striatum neurons during amphetamine repeated administration in freely moving rats.

3. To quantify the density of gephyrin puncta in the entopeduncular nucleus, substantia nigra pars reticulata and globus pallidus after amphetamine repeated administration.

2. MATERIALS

2.1. Animals

Adult male Sprague-Dawley rats (300-340 g) were grown in the Animal Care Facility of the Biological Sciences, Pontificia Universidad Católica de Chile, under the supervision of a veterinarian. During drug treatment, rats were maintained in the Animal Care Facility of the Department of Pharmacy, Pontificia Universidad Católica de Chile, following the instruction of a protocol approved by the veterinarian. Rats were housed in a colony room in groups of two-three per cage (15x47x26 cm) and were kept at room temperature between 20 to 24°C on a 12 h light/dark cycle (lights on at 7 AM, Eastern Standard Time) with access to food and water *ad libitum*. All procedures were in strict accordance with the guidelines published in the "NIH Guide for the Care and Use of Laboratory Animals" (8º Edition) and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. Also, the protocols were approved by the local bioethics committees (Project # 151005003, Pontificia Universidad Católica de Chile). Rats were handled for one week before starting the experiments. All procedures were performed by the main experimenter (Rafael Gatica).

2.2. Locomotor sensitization

-For anesthetized rat experiments, horizontal locomotor activity was measured in a test cage of 15x47x26 cm, equipped with two pairs of infrared lights located lengthwise, separated by 25 cm and 5 cm above the floor. Data was acquired in a Windows 98 PC with an Excel sheet programed with a macros code.

-For freely moving rat experiments, horizontal locomotor activity was measured in a test box of 40x54x36 cm and video recorded (Facecam 1000x, Genius). Locomotor activity was measured analyzing videos with manual tracking using mTrackJ plugin in ImageJ 1.51s (Meijering et al., 2012).

-D-amphetamine (Laboratorio Chile) and saline (NaCl 0.9%, Winkler) were injected using Terumo 1 mL syringes with a 25G needle.

-Locomotor activity boxes were cleaned using alcohol 70% (Winkler).

2.3. Surgery

-Rats were anesthetized using urethane 1.5 g/kg ip (Sigma-Aldrich) for anesthetized single unit recordings, and a mixture of ketamine 100 mg/kg ip (Troy Laboratories) and xylazine 10 mg/kg ip (Centrovet Ltda) for freely moving experiments.

-Rats were placed in a stereotaxic apparatus (Stoelting Co. and RWD Life Sciences Co.) and a heating pad was used to regulate temperature (Sensomed).

-For freely moving experiments, rats received ophthalmic drops (Systane, Alcon) to lubricate the eyes during surgery. After surgery rats received ketoprofen 1 mg/kg ip (Biosano), tramadol 25 mg/kg po (Opko) and enrofloxacin 5 mg/kg ip (Chemie).

2.4. Electrophysiology

-Eight custom-made nichrome tetrodes were used, constructed by twisting four 12 µm wires together (Sandvik Tetrode Wire). Tetrodes impedance was modified with a gold plate solution (Neuralynx), using an impedance tester (Bak electronics Inc.) and stimulator (Grass Instruments Co.). A custom manipulator was used for the tetrode array (manufactured by Marcelo Aguilar-Rivera, University of California San Diego). In freely moving experiments, the tube array of the micromanipulator was positioned at the cortical surface and glued using a low viscosity silicone (Kwik-Sil, World Precision Instruments). Micromanipulator was anchored to the animal's head with four stainless steel screws (Small Parts) and dental acrylic. A 76 µm stainless steel coated wire (A-M systems) welded to a stainless-steel screw (Small Parts) was anchored to the animal head and used as ground.

-During electrophysiological recording, signals from tetrodes were preamplifed using a 32 channels micro preamplifer (µPA 32, Multichannel Systems). Signals were then amplifed with a 32 channel amplifer (PGA 32, Multichannel Systems). This data was sent to an analog-to digital converter (NI USB-6363, National Instruments), connected to a Windows 10 laptop running a custom made LabWindows program (National Instruments), allowing the visualization and storage of the digital information. Spikes were analyzed using a custom made LabWindows program (National Instruments). These data were further analyzed using Matlab R2018a (Mathworks).

2.5. Histology

-An electrode impedance tester (Bak electronics Inc.) and stimulator (Grass Instruments Co.) were used for electrolytic lesions.

-A peristaltic pump (LongerPump) was used for intracardial perfusion of phosphate buffer solution (PBS, Winkler) and paraformaldehyde (PFA) 4% (Merck). Brains were cryoprotected with sucrose 30% (Winkler).

-Brain sections were obtained using a microtome equipped with a freezing stage.

-Sections were incubated with Cresyl Violet (Sigma-Aldrich) and latter mounted to verify the tetrodes placement.

2.6. Inmunofluorescence

-Table 1 resume the primary antibodies used in this study

Antigen	Host Species	Source	Cat. #	Inmunogen
Tyrosine Hydroxylase	Rabbit	Santa Cruz	sc-14007	TH (H-196) is a rabbit polyclonal antibody raised against amino acids 1-196 of TH of human origin.
Gephyrin	Mouse	Synaptic System	147 011	Monoclonal antibody. Purified rat gephyrin. Specific for brain specific 93 kDa splice variant.

TABLE 1 List of primary antibodies used.

-The following secondary antibodies were used:

-Cy3-conjugated AffiniPure Goat Anti-mouse IgG (115-165-003, Jackson InmunoResearch).

-Cy3-conjugated AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (711-166-152, Jackson InmunoResearch).

-Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Rabbit IgG (711-545-152, Jackson InmunoResearch).

-Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Mouse IgG (715-545-150, Jackson InmunoResearch).

-Sections were mounted using Fluoromount (Sigma-Aldrich-Aldrich).

2.7. Stereology

-Imaging was performed using an epifluorescence microscope (Nikon Ecliple Ci) equipped with a camera (Microfire, Optronics), coupled with a motorized x-y-z stage, transmitted light and filters suitable to the two fluorescent markers.

-A Windows 7 PC with Stereo Investigator software (MBF Bioscience) was used for gephyrin profiles estimation and volume estimation.

3. METHODS

3.1. To determine the firing rate and firing pattern of dorsolateral striatum neurons after amphetamine repeated administration in anesthetized rats.

3.1.1 Sample size

Sample size was calculated using G*Power 3.1.9.4 (Faul et al., 2007). The change in baseline firing rate between groups was selected to estimate the sample size. Using a one-way ANOVA test (α error=0.05, power=0.95, number of groups=3) and considering a determined 'effect size f' of 0.47, the total sample size was 75 (25 neurons/group). Considering that around 7 neurons could be recorded in each rat, a total of 5 rats will be required for each group.

3.1.2. Amphetamine locomotor sensitization

The process of AMPH locomotor sensitization was developed according to a schedule as described previously (Azocar et al., 2019). Horizontal locomotor activity was measured in a test cage. Once in the test cage, rats had an habituation period of 30 minutes. Horizontal locomotor activity was measured for 50 minutes post treatment. Locomotor sensitization protocol is showed in Figure 1A. Rats were injected with AMPH 1.0 mg/kg ip for five consecutive days (days 1-5). After four days of withdrawal (days 6-9), Saline (NaCl 0.9%) ip was injected on day 10. A challenge dose of AMPH 1.0 mg/kg ip was injected on day 11. We consider locomotor

sensitization when the rat had at least doubled the crossover/50min on day 11 compared to day 1.

3.1.3. Electrophysiological recordings

Eight custom-made nichrome tetrodes were used, constructed by twisting four 12 µm wires together. Tetrodes impedance were set at 200-300 kΩ with gold platting procedure. A custom manipulator was used for the tetrode array. Twenty-four hours after the last AMPH injection, rats were anesthetized with urethane 1.5 g/kg i.p and placed in a stereotaxic apparatus. The skull of the rat was exposed, and a hole is drilled targeting the DLS: +1.2 mm anterior to bregma, +3.6 mm posterior to bregma. Tetrodes array was positioned in the DLS coordinates and lowered 2.8-4.0 mm under dura. During electrophysiological recording, signals from tetrodes were preamplifed (10x) using a 32 channels micro preamplifer. Signals were then amplifed (500x) with a 32 channel amplifer and band pass filtered (1-5000 Hz). This data was sent to an analog-to digital converter, connected to a Windows 10 laptop running a custom made LabWindows program, allowing the visualization and storage of the digital information. Data was sampled at 30 kHz. Extracellular waveforms that exceeded a voltage threshold above two standard deviations from the average noise were recorded. Neurons with a percentage of ISI < 2 ms greater than 1% are considered multiunits and excluded of further analysis. Recording protocol was as follow: 20 minutes after an acute Saline ip injection (baseline activity), 40 minutes after an acute AMPH 1.0 mg/kg ip injection (post-AMPH activity).

3.1.4. Data analysis

Spikes were analyzed using a custom made LabWindows program. Waveforms were sorted using two dimensional plots of peak to peak and principal component of spike waveforms.

Isolated clusters were used to obtain average waveforms and time stamp of neurons. These data were further analyzed using Matlab R2018a. Firing rate was calculated dividing the total number of spikes in a period (baseline or post-AMPH) to the total time during that period. Lv index was used to measure the firing pattern of pMSNs. Lv index allows to analyze the instantaneous variability of interspike intervals (ISIs), with the advantage that Lv index is less sensitive to changes in firing rate compared to other firing pattern measures, like coefficient of variation. Lv index was calculated using the following equation (Shinomoto et al., 2009):

$$Lv = \frac{3}{n-1}\sum_{i=1}^{n-1}\left(\frac{I_i - I_{i+1}}{I_i + I_{i+1}}\right)^2$$

were I_i and I_{i+1} correspond to the *i*-th and $i+1^{st}$ ISIs and *n* is the number of ISIs. Lv values larger than 1 correspond to bursty neurons, whereas Lv values lower than 1 correspond to regular firing neurons.

Striatal neurons were classified according to their waveform and firing properties (Schmitzer-Torbert and Redish, 2008; Yarom and Cohen, 2011). First, each waveform was normalized with their peak value. As showed in figure 2A, the waveform width is one of the main differences between these two populations of neurons. Two waveform measures were used to classify striatal neurons (Figure 2B): repolarization at 200 µs after the peak (Vinck et al., 2016) and the peak to amplitude ratio (PAR, Yarom and Cohen, 2011). Using matlab k-means clustering algorithm, two clusters were defined. Short waveform neurons (black cluster, Figure 2B) were excluded from further analysis. Regarding to firing properties, large waveform striatal neurons (gray cluster, Figure 2B) were classified according to proportion of time spent in long interspike-intervals (Prop ISIs > 2 seg), an index used to identify putative medium spiny neurons (pMSNs,

Schmitzer-Torbert and Redish, 2008; Yarom and Cohen, 2011). Large waveform neurons showing a Prop ISIs>2 sec greater than 0.4 were classified as pMSNs (Figure 2C). Large neurons showing a Prop ISIs>2 sec lower than 0.4 were excluded of further analysis.

To analyze changes in firing rate induced by AMPH, a non-parametric permutation test was used (Aguilar-Rivera et al., 2015; Maris and Oostenveld, 2007). First, baseline and post-AMPH firing rate of an individual neuron were divided in 1 minute bins. The mean firing rate of baseline and post-AMPH bins were calculated and then obtained the differences between them (original mean). After this, baseline and post-AMPH bins were randomly mixed across the entire time and a new mean was calculated between the surrogate baseline and post-AMPH bins. This permutation process was repeated 1000 times and a distribution of all the new mean firing rate differences was obtained. Finally, from this distribution, the value representing the 5% limit of the distribution was used as the significant limit for the original mean firing rate of each neuron. pMSNs that showed a significant increase in firing rate after AMPH were classified as 'Excited', whereas those who showed a significant decrease their firing rate were classified as 'Inhibited'. pMSNs not showing change in firing rate after AMPH were classified as 'Not changed'.

Statistical analyses were performed on GraphPad Prism 5.0 and Matlab Matlab R2018a. Data were analyzed using one way ANOVA, Kruskall-Wallis test, Wilconxon sign rank test, Wilcoxon rank sum test and Fisher's Exact test. Bonferroni's post-test and Dunn's multiple comparisons post-test were used when required.

3.1.5. Histology

Electrolytic lesions were performed at the end of the recording session by applying an anodic current of 25 μA for 10 s through 2 wires of each tetrode. Then, rat brains were removed and

post-fixed for at least 1 day in paraformaldehyde 4% and cut in coronal series of 50 µm. Brain sections were stained with Cresyl Violet and examined by light microscopy to determine tetrode placement. Figure 1C shows representative placement of tetrodes tips.

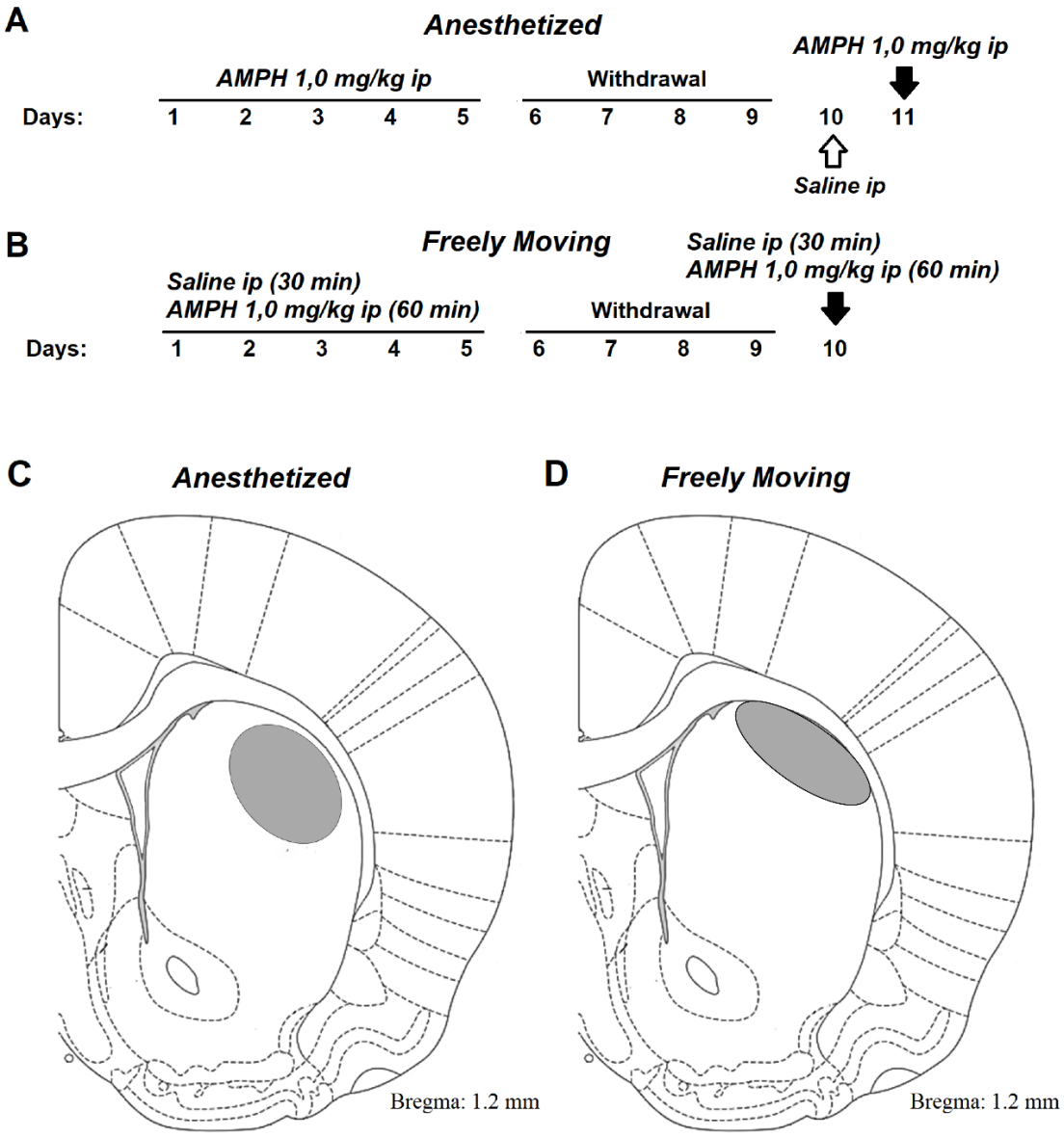

FIGURE 1 Experimental protocols and tetrodes placement.

A) Repeated AMPH administration protocol for anesthetized single unit recordings. Rats were daily treated with AMPH 1.0 mg/kg ip for 5 consecutive days. After 4 days of abstinence, Saline ip was injected on day 10. A challenge dose of AMPH 1.0 mg/kg ip was injected on day 11. Twenty-four hours after the last AMPH injection, anesthetized single unit recordings were performed. B) Repeated AMPH administration protocol for freely moving single unit recordings. Rats were daily treated with Saline ip and AMPH 1.0 mg/kg ip for 5 consecutive days. After 4 days of abstinence, a Saline ip injection and a challenge dose of AMPH 1.0 mg/kg ip were injected on day 10. Single unit recordings were performed during baseline (after saline ip, 30 min) and post-AMPH (60 minutes) on days 1 and 10. C-D) Representative placement of tetrodes for anesthetized (C) and freely moving (D) experiments. Gray shading represents the area were tetrodes tips are identified. Diagrams were adapted from Paxinos and Watson, 2009.

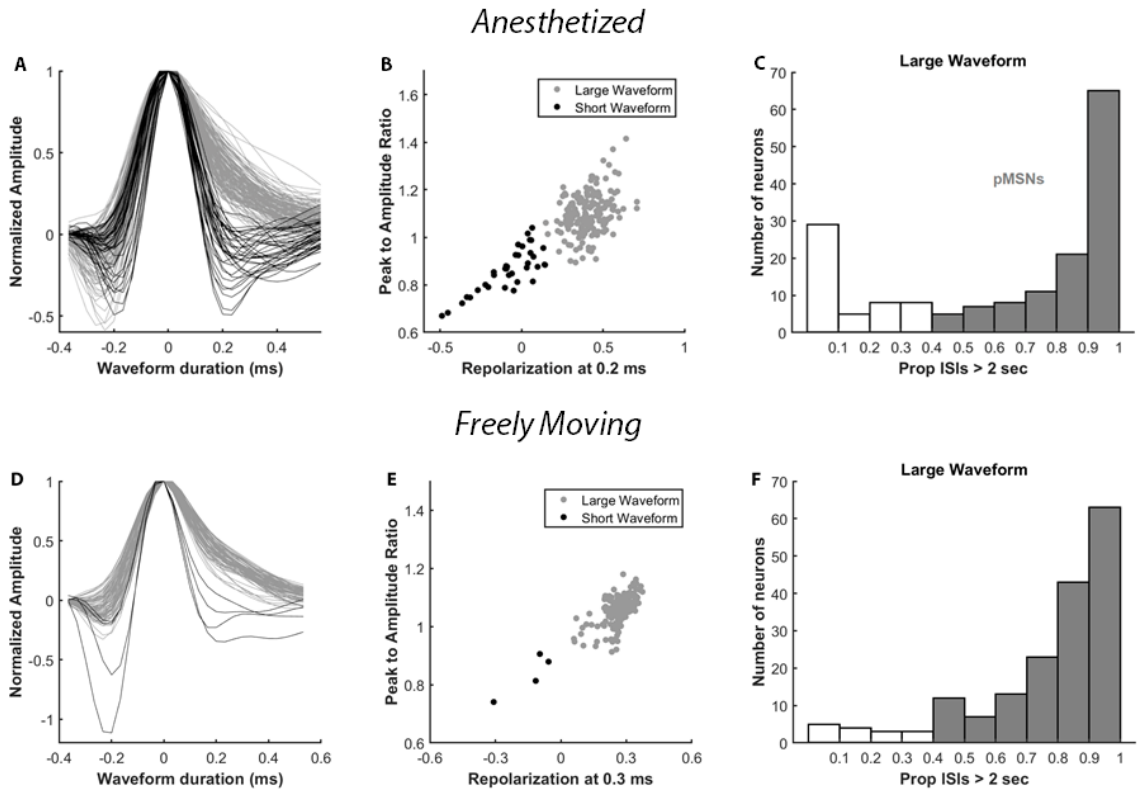

FIGURE 2 Classification of DLS neurons in anesthetized and freely moving rats.
A,D) Normalized waveform aligned at peak for all the units, from anesthetized (A) and freely moving (D) experiments. Gray lines: Large waveform; Black lines: Short waveforms. B,E) Scatterplot for peak to amplitude ratio vs repolarization at 0,2 ms (B, anesthetized rats) and 0,3 ms (E, freely moving rats). Using k-means clustering, two populations of neurons were defined: large waveform and short waveform. C,F) Histogram of Prop ISIs > 2 seg of large waveforms neurons, for anesthetized (C) and freely moving (F) rats. Neurons with Prop ISIs > 2 seg greater than 0.4 were classified has putative pMSNs. Neurons with Prop ISIs > 2 seg lower than 0.4 were excluded.

3.2 To determine neuronal activity and locomotion correlation of dorsolateral striatum neurons during amphetamine repeated administration in freely moving rats.

3.2.1 Sample size

Sample size was calculated using G*Power 3.1.9.4 (Faul et al., 2007). The change in baseline firing rate between groups on day 10 was selected to estimate the sample size. Using a Wilcoxon-Mann Whitney test (α error=0.05, power=0.95, two tails) and considering a determined 'effect size d' of 1, the total sample size was 58 (29 neurons/group). Considering that around 7 neurons could be recorded in each rat, a total of 5 rats will be required for each group.

3.2.2. Surgery

Rats were anesthetized with ketamine 100 mg/kg i.p and xylazine 10 mg/kg i.p. For single unit recording, eight custom-made nichrome tetrodes were used, constructed by twisting four 12 µm wires together. Tetrodes impedance were set at 200-300 kΩ with gold platting procedure. A custom micromanipulator was used to allow all tetrodes movement at the same time. A craniotomy was performed in the following coordinates according to bregma: 1.2 mm anterior to bregma and 3.0-4.0 mm lateral to bregma . The tube array of the micromanipulator was positioned at the cortical surface and glued using a low viscosity silicone. Micromanipulator was anchored to the animal's head with four stainless steel screws and dental acrylic. Additionally, ground electrode was fastened with a stainless-steel screw in the contralateral posterior part of the head. Immediately after surgery, the tetrodes was lowered ~315 µm every 1 minute until reach 2.8-3.0 mm under dura. After surgery, the rats received ketoprofen (1 mg/kg ip), tramadol (25 mg/kg oral) for three days, and enrofloxacin (5 mg/kg ip) for 5 days in total. In the days following surgery, the tetrodes were moved in 100 µm steps until detect stable

unitary activity in at least 4 tetrodes. Locomotor sensitization protocol starts when stable unitary activity last at least 1 day (normally, within 1-3 weeks after surgery).

3.2.3. Amphetamine locomotor sensitization

The process of AMPH locomotor sensitization was developed according to a schedule as described previously (Azocar et al., 2019). Horizontal locomotor activity was measured in a test box and video recorded. Locomotor activity was obtained analyzing videos with manual tracking using mTrackJ plugin in ImageJ 1.51s (Meijering et al., 2012). Once in the test box, rats had an habituation period of 30 minutes. After 30 min of habituation, horizontal locomotor activity was measured for 30 minutes after an acute injection of saline ip and 60 minutes after AMPH 1.0 mg/kg ip injection (Figure 1B). Rats were injected with Saline ip and AMPH 1.0 mg/kg ip for five consecutive days (days 1-5). After four days of withdrawal (days 6-9), a Saline ip injection and a challenge dose of AMPH 1.0 mg/kg ip were injected on day 10. We consider locomotor sensitization when the rat had at least doubled the locomotor activity on day 10 compared to day 1.

3.2.4. Electrophysiological recordings

Single unit recordings are performed as showed in Section 3.1.2 (except for anesthesia and surgery). Electrophysiological recordings are performed every day of the AMPH locomotor sensitization protocol (3.2.2) as follows: 30 minutes after Saline ip injection (baseline activity), 60 minutes after AMPH 1.0 mg/kg ip injection (post-AMPH activity).

3.2.5. Data analysis

Data analysis for firing rate, firing rate change after AMPH injection, Lv index and pMSNs classification were performed as described in 3.1.3. Figure 2D-F show the results of pMSNs classification in freely moving rats.

For each excited pMSNs, magnitude of AMPH excitation is equal to the difference between mean post-AMPH firing rate and the mean baseline firing rate. Then, the correlation between magnitude of AMPH excitation and baseline firing rate was assessed on days 1 and 10 of each group using a Kendall correlation.

To analyze the covariance of pMSNs neuronal activity measures (firing rate and Lv index) with locomotor activity, each neuron firing rate and Lv index, and the rat locomotor activity during baseline and post-AMPH were grouped using a 1 minute bin. Then, for each neuron a Kendall correlation between firing rate and locomotor activity was performed. Also, a Kendall correlation between Lv index and locomotor activity was performed. Finally, a pMSN with a Kendall correlation $p<0.05$ was classified as a correlated pMSN. Neurons with a $tau>0$ were directly correlated pMSNs and those with a $tay<0$ were inversely correlated pMSNs.

Statistical analyses were performed on GraphPad Prism 5.0 and Matlab R2018a. Data were analyzed using two way ANOVA, Wilconxon sign rank test, Wilcoxon rank sum test and Fisher's Exact test. Bonferroni's post-test was used when required.

3.2.6. Histology

Rats were anesthetized 24 hours after the last AMPH injection with ketamine 100 mg/kg ip and xylazine 10 mg/kg ip . Electrolytic lesion was performed by applying an anodic current of 25 µA for 10 s through 2 wires of each tetrode. Forty-eight hours after electrolytic lesion, rats were

anesthetized (ketamine 100 mg/kg i.p, xylazine 10 mg/kg i.p) and intracardially perfused with PFA 4% (Merck). Rat brains were removed and post-fixed for at least 1 day in PFA 4%, cryoprotected with sucrose 30% for 2-3 days and cut in coronal sections of 50 µm for electrode probe placement and in coronal sections 25 µm for immunofluorescence (next section). Brain sections were stained with Cresyl Violet and examined by light microscopy to determine electrode probe placement. Figure 1D shows representative placement of tetrodes tips.

3.3 To quantify the expression of gephyrin in the entopeduncular nucleus substantia nigra pars reticulata and globus pallidus after amphetamine repeated administration.

3.3.1 Sample size

Sample size was calculated using G*Power 3.1.9.4 (Faul et al., 2007). The change in Geph density between groups was selected to estimate the sample size. Using a one-way ANOVA test (α error=0.05, power=0.95, number of groups=3) and considering a determined 'effect size f' of 2.35, the estimated sample size was 9 (3 rats/ group).

3.3.2 Gephyrin immunofluorescence

As previously stated (3.2.4), rats from freely moving experiments were anesthetized (ketamine 100 mg/kg i.p, xylazine 10 mg/kg i.p) and intracardially perfused with PFA 4%. Extracted brains were post-fixed for 1 night with PFA 4%, cryoprotected with sucrose 30% for 2-3 days and 25 µm sections were cut using a microtome. EP, SNr and GP serial sections were obtained. Eight serial sections, with a section interval of 8 for GP and SNr (EP sections are part of the GP serial sections) were obtained. Geph and tyrosine hydroxylase (TH) immunofluorescence were performed. TH immunofluorescence was performed to delimitate each GP and SNr. Sections were washed with PBS and later incubated with citrate 10 mM for 30 minutes at 80 °C. Then, sections were washed with PBS and incubated with 3% horse serum in PBS with Triton-100

0.3% for 2 hours at room temperature (blocking solution). Sections were incubated with mouse Geph primary antibody (1:30.000) and rabbit TH primary antibody (1:15.000) for 1 night at room temperature. Next, sections were washed with PBS and latter incubated using a fluorescent secondary antibody for 2 hours at room temperature. Two combinations were tested: 1) anti-mouse AlexaFlour488 and anti-rabbit Cy3 secondary antibody; 2) anti-rabbit AlexaFlour488 and anti-mouse Cy3 secondary antibody. Finally, sections were washed with PBS and mounted.

3.3.3. Stereological measurement of gephyrin profiles

Serial sections were examined using a stereology microscope equipped with an x-y-z motor stage and camera. EP, SNr and GP were contoured using Stereo Investigator software. Estimation of Geph was performed using the optical fractionator method in Stereo Investigator software (West et al., 1991). Experimenter was blind to what sample was measuring. Software place systematically random disector boxes in the areas of interest. Stereological counting has the advantage that avoid counting bias for profiles size, shape, orientation or distribution. The optical fractionator probe was used with the following settings: Section Evaluation Interval: 8; Counting Frame Size: 3 x 3 µm; Grid Size: 120 x 120 µm (EP), 320 x 320 µm (SNr), 420 x 420 µm (GP); Dissector Height: 3 µm; Top Guard Zone: 1 µm. These settings allowed to have a coefficient of error (Gundersen m=1) lower than 0.1. Volume estimation for EP, SNr and GP were obtained using the planimetry function in the optical fractionator probe on Stereo Investigator software. Results were presented as Geph density (total number of estimated Geph profiles/volume estimation of the nucleus).

4. RESULTS

4.1. To determine the firing rate and firing pattern of dorsolateral striatum neurons after amphetamine repeated administration in anesthetized rats.

4.1.1. Individual differences in locomotor activity after repeated AMPH administration

To determine individual differences in locomotor activity during a first AMPH administration, post-AMPH locomotor activity of non-sensitized (n=5) and sensitized (n=6) rats on day 1 was analyzed (Figure 3A). Locomotor activity after AMPH 1.0 mg/kg ip was measured in locomotor activity boxes (see methods 3.1.2.). A trend of increased locomotor activity in non-sensitized rats compared to sensitized rats was observed on day 1 (Figure 3A; p=0.3022, unpaired t-test), suggesting that rats that will express locomotor sensitization show a lower hyperlocomotion after a first AMPH administration.

Figure 3B-C show temporal course of locomotor activity of non-sensitized (Figure 3B) and sensitized (Figure 3C) rats during repeated AMPH administration protocol. AMPH 1.0 mg/kg ip was administered for 5 consecutive days (days 1-5). On day 10, saline ip was injected as a control. A challenge dose of AMPH 1.0 mg/kg ip was injected on day 11.

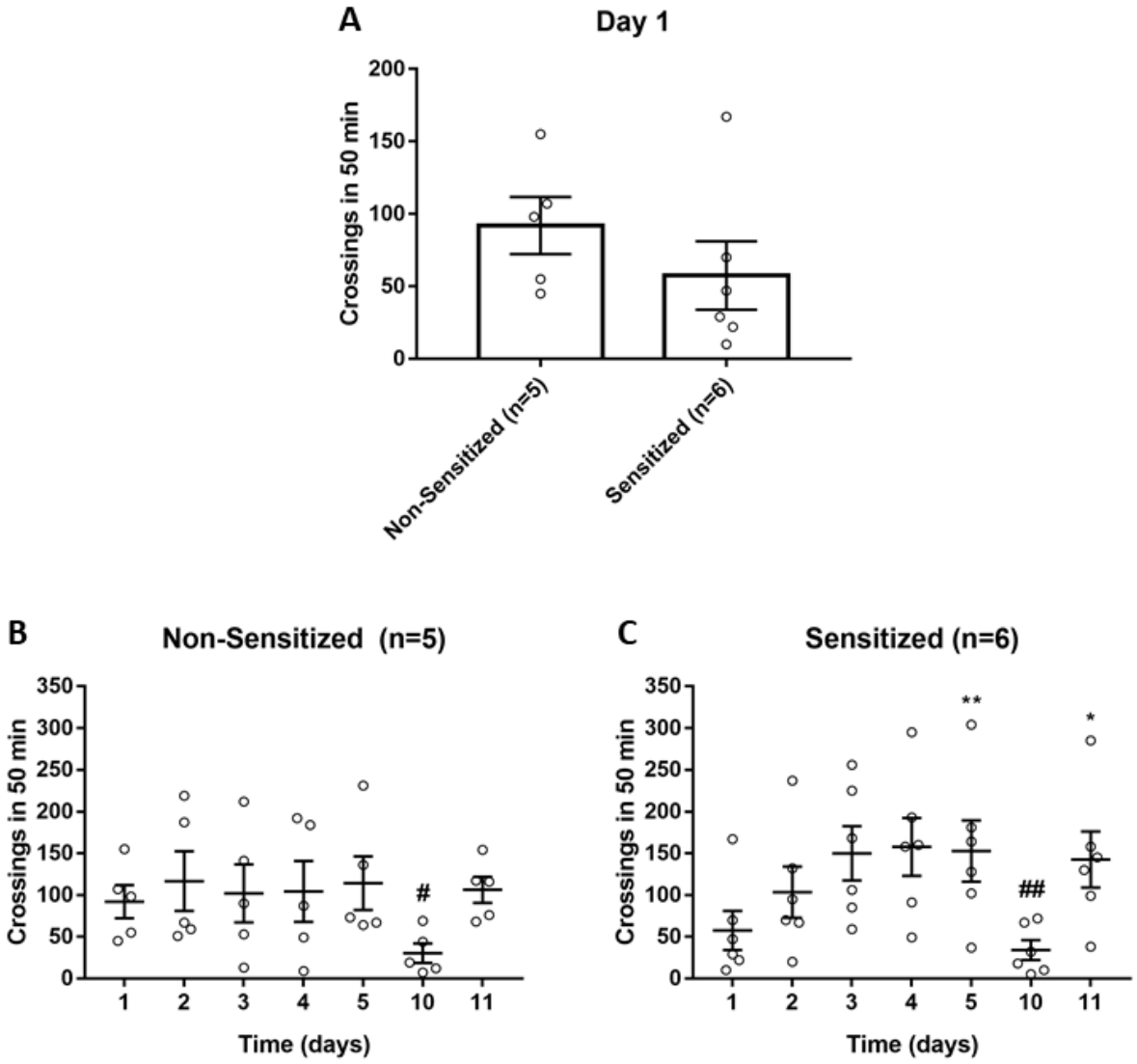

FIGURE 3 Individual differences in locomotor activity after repeated AMPH administration.

Locomotor activity after AMPH 1.0 mg/kg ip injection was measured for 50 minutes in locomotor activity boxes. Saline ip was injected on day 10. A) Effect of AMPH 1.0 mg/kg ip on locomotor activity of non-sensitized and sensitized rats on day 1. A trend of increased locomotor activity is observed in non-sensitized rats after AMPH injection ($p=0.3022$, unpaired t-test). B-C) Effect of repeated AMPH administration on locomotor activity in non-sensitized (Figure 3B) and sensitized (Figure 3C) rats. Development and expression of locomotor sensitization was only observed in sensitized rats. * $p<0.05$, ** $p<0.01$ vs day 1 of sensitized rats, using Bonferroni post-hoc test; ## $p<0.01$ v/s day 5 and 11 of the respective groups, using Bonferroni post-hoc test. Each line represents mean $\pm$ SEM, points represent individual data.

Using a repeated measure ANOVA, a significant effect between days in non-sensitized ($F_{(6, 30)}$= 6.24; $p<0.05$) and sensitized ($F_{(6, 30)}$= 9.58; $p<0.001$) rats was observed. Non-sensitized rats did not show an increase in their locomotor activity after repeated AMPH administration and thus, no devolvement nor expression of locomotor sensitization was observed (Figure 3B, $p>0.99$ crossings in 50 min, Bonferroni's post-test). In contrast, in sensitized rats, an increase in locomotor activity on days 5 and 11 compared to day 1 was found (Figure 3C, $p<0.05$, Bonferroni's post-test), showing the development and expression of locomotor sensitization in these rats. On the other hand, on day 10 (saline injection day), locomotor activity of sensitized and non-sensitized rats was significantly lower compared to their respective days 5 and 11 ($p<0.01$,Bonferroni's post-test), showing that the induced hyperlocomotion is in fact due to AMPH, and not to stress related to repeated injections.

4.1.2. Individual differences in DLS baseline firing rate after repeated AMPH administration in anaesthetized rats

Twenty-four hours after the last AMPH injection rats were anesthetized and electrophysiological experiments were carried out (see methods 3.1.3.). A control group (n=4) without previous treatments was also used for electrophysiological recordings. A total of 117 neurons were obtained from pMSNs neuron classification (see methods 3.1.4. and Figure 2A-C; Control=29 neurons, 7$\pm$2 neurons/rat; Non-sensitized=40 neurons, 8$\pm$1 neurons/rat; Sensitized=48 neurons, 8$\pm$1 neurons/rat). pMSNs baseline firing rate was compared between groups to study individual differences in DLS baseline neuronal activity after repeated AMPH administration. Baseline firing rate was measured for 20 minutes after an acute saline ip injection (Figure 4). A significant effect between groups was found ($p<0.001$, Kruskall-Wallis test). Sensitized rats showed a higher baseline firing rate compared to control and non-sensitized

rats ($p<0.01$, Dunn's multiple comparisons test). No significant differences were found between control and non-sensitized rats ($p>0.99$, Dunn's multiple comparisons test).

Together, these results indicate that the expression of AMPH locomotor sensitization is accompanied with an increase in DLS baseline neuronal activity.

4.1.3. Repeated AMPH administration increase population response to an acute AMPH injection in anaesthetized rats

To determine the effect of an acute AMPH injection in individual pMSNs firing rate, a non-parametric permutation test was carried out (see methods 3.1.4.). Figure 5 shows examples of the three types of responses observed after acute AMPH injection: excitation (Figure 5A), inhibition (Figure 5B) or no change (Figure 5C) in firing rate. Then, the percentage of pMSNs changing (excited and inhibited) their firing rate after acute AMPH injection was analyzed between groups to study individual differences in the DLS population response to an acute AMPH injection after repeated AMPH administration (Figure 5D).

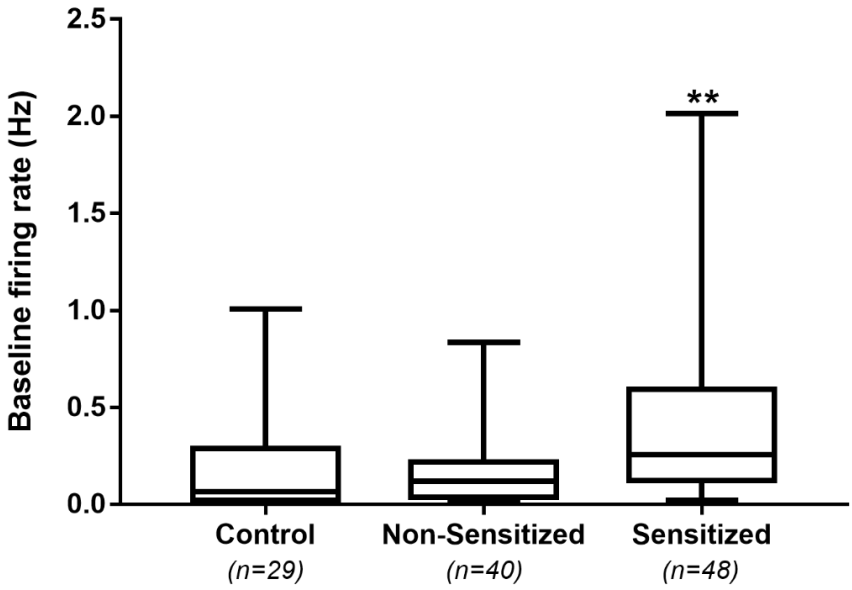

FIGURE 4 Individual differences in baseline firing rate of pMSNs after repeated AMPH administration in anesthetized rats.
DLS pMSNs were recorded for 20 min after saline ip injection (baseline). The expression of AMPH locomotor sensitization is accompanied by an increase in baseline firing rate (** $p<0.01$ vs Control and Non-sensitized rats, Dunn's multiple comparisons test). Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers).

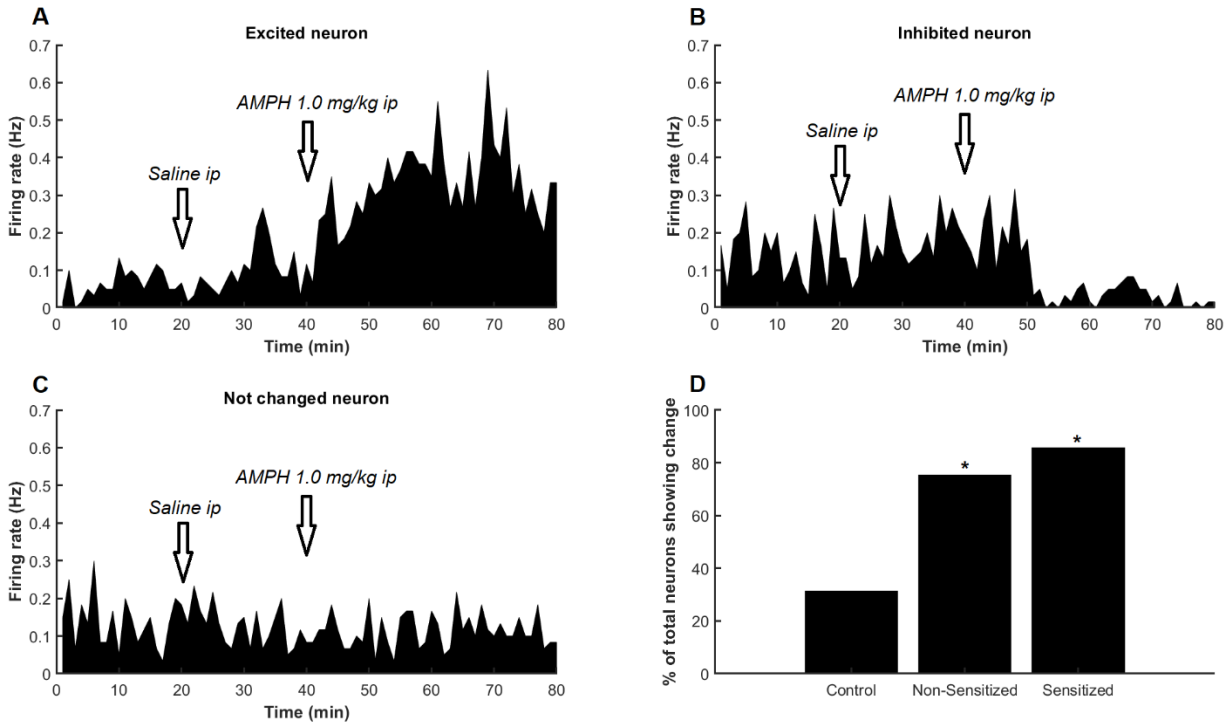

FIGURE 5 Repeated AMPH administration increases neuronal population response to an acute AMPH injection in anesthetized rats.
A-C) Peristimulus time histogram of excited A), inhibited (B) or not changed (C) pMSNs after an acute AMPH 1.0 mg/kg ip injection. D) Bar chart showing the percentage of neurons with a significant change in their firing rate ('excited' neurons + 'inhibited' neurons) after an acute AMPH 1.0 mg/kg ip injection. Both sensitized and non-sensitized rats have a higher percentage of neurons that change their firing after AMPH compared to control (* $p<0.05$ vs control rats, Fisher exact test).

In control rats, 31% of the neurons changed their firing rate after acute AMPH administration. On the other hand, sensitized and non-sensitized rats showed a higher percentage of neurons that changed their firing rate after AMPH compared to control rats (Figure 5D, $p<0.05$ Fisher exact test). Furthermore, no significant differences were found in the percentage of excited or inhibited pMSNs between non-sensitized and sensitized rats (Annexes Figure S1).

These results indicate that repeated AMPH administration is associated with an increase in the DLS population response to an acute AMPH injection. In this sense, no individual differences after repeated AMPH administration were observed in this parameter.

4.1.4. Individual differences in DLS firing pattern after repeated AMPH administration in anaesthetized rats.

To determine the effect of repeated AMPH administration on firing pattern of DLS pMSNs, Lv index, a measure of firing pattern (Shinomoto et al., 2009; see methods 3.1.4.), was analyzed during baseline and post-AMPH periods (Figure 6).

Firstly, pMSNs baseline Lv index was analyzed between groups to assess individual differences in DLS baseline firing pattern after repeated AMPH administration (Figure 6, white boxes). A Kruskall-Wallis test showed a significant effect between groups on Lv index during baseline period ($p<0.01$, Kruskall-Wallis test). A Dunn's multiple comparisons test showed a significant decrease in Lv index during baseline period in sensitized rats compared to controls ($p<0.05$), indicating that the baseline firing pattern of sensitized rats pMSNs was less irregular compared to control rats.

Secondly, pMSNs post-AMPH Lv index was compared between groups to study individual differences in DLS firing pattern after an acute AMPH injection (Figure 6, gray boxes). A

significant effect between group was observed on Lv index after an acute AMPH injection ($p<0.05$, Kruskall-Wallis test). An acute AMPH injection was accompanied by a significant decrease in Lv index in sensitized rats compared to controls ($p<0.05$, Dunn's multiple comparisons test), showing that the post-AMPH firing pattern of sensitized rats pMSNs was less irregular compared to control rats pMSNs. A within group analysis shows a significant decrease in Lv index after an acute AMPH injection in control and sensitized rats compared to their corresponding baseline period ($p<0.01$, Wilcoxon sign rank test). Interestingly, no effect on Lv index was observed after an acute AMPH injection in non-sensitized rats compared to baseline ($p=0.2479$, Wilcoxon sign rank test). Then, these results show that the persistence of a decrease in firing irregularity after an acute AMPH injection accompanies the expression of AMPH locomotor sensitization.

All together, these results indicate that the expression of AMPH locomotor sensitization is accompanied with specific modifications in DLS pMSNs activity. Specifically, an increased pMSNs baseline firing rate and a decreased baseline Lv index were observed in sensitized rats. Relevantly, a decrease in firing irregularity after an acute AMPH injection was observed in sensitized but not in non-sensitized rats.

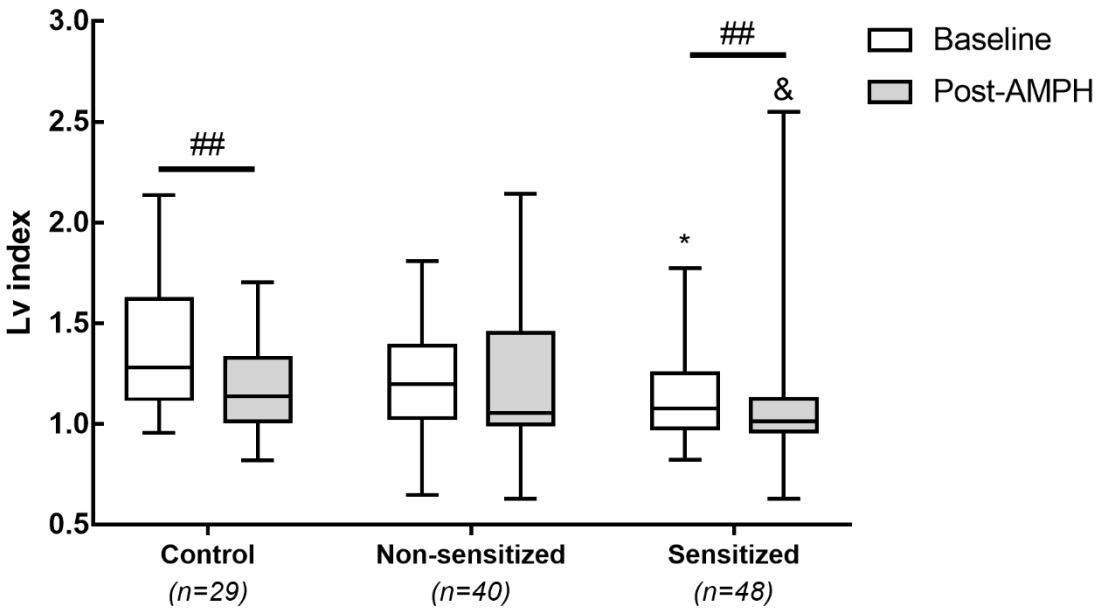

FIGURE 6 Individual differences in firing pattern of pMSNs after repeated AMPH administration in anesthetized rats.

Lv index was analyzed for 20 minutes after saline ip (Baseline) and for 40 minutes after an acute AMPH 1.0 mg/kg ip injection (post-AMPH). AMPH locomotor sensitization is accompanied by a significant decrease in baseline Lv index compared to controls (* $p<0.05$ compared to baseline in control rats, Dunn's multiple comparisons test). Also, a decrease in post-AMPH Lv index is observed in sensitized rats compared to control rats (& $p<0.05$ compared to AMPH in control rats, Dunn's multiple comparisons test). An acute AMPH injection decreased Lv index in both control and sensitized rats, compared to their corresponding saline period (## $p<0.01$, Wilcoxon sign rank test). Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers).

4.2 To determine neuronal activity and locomotion correlation of dorsolateral striatum neurons during amphetamine repeated administration in freely moving rats.

4.2.1. Individual differences in locomotor activity after repeated AMPH administration in freely moving rats

Figure 7 shows the locomotor activity of non-sensitized (n=4) and sensitized (n=4) rats during repeated AMPH administration protocol in freely moving rats. Firstly, baseline locomotor activity was measured for 30 minutes on day 1 to study individual differences in locomotion induced by novelty before any injection has been performed (Figure 7A). Locomotor activity was video recorded and analyzed (see methods 3.2.3.). A trend of increased locomotor activity in those that will become non-sensitized rats compared to those that will become sensitized rats was observed ($p=0.1474$, unpaired t-test). This data suggests that a lower locomotion induced by novelty is observed in rats that will express AMPH locomotor sensitization.

Secondly, post-AMPH locomotor activity on day 1 was measured to study individual differences in locomotor activity during a first AMPH injection (Figure 7B). Locomotor activity was measured for 60 minutes after AMPH injection. A trend of increased locomotor activity in those that will become non-sensitized rats compared to those that will become sensitized rats was observed ($p=0.1505$, unpaired t-test). Then, this data suggest that a lower locomotion induced by AMPH injection is observed in rats that will express AMPH locomotor sensitization.

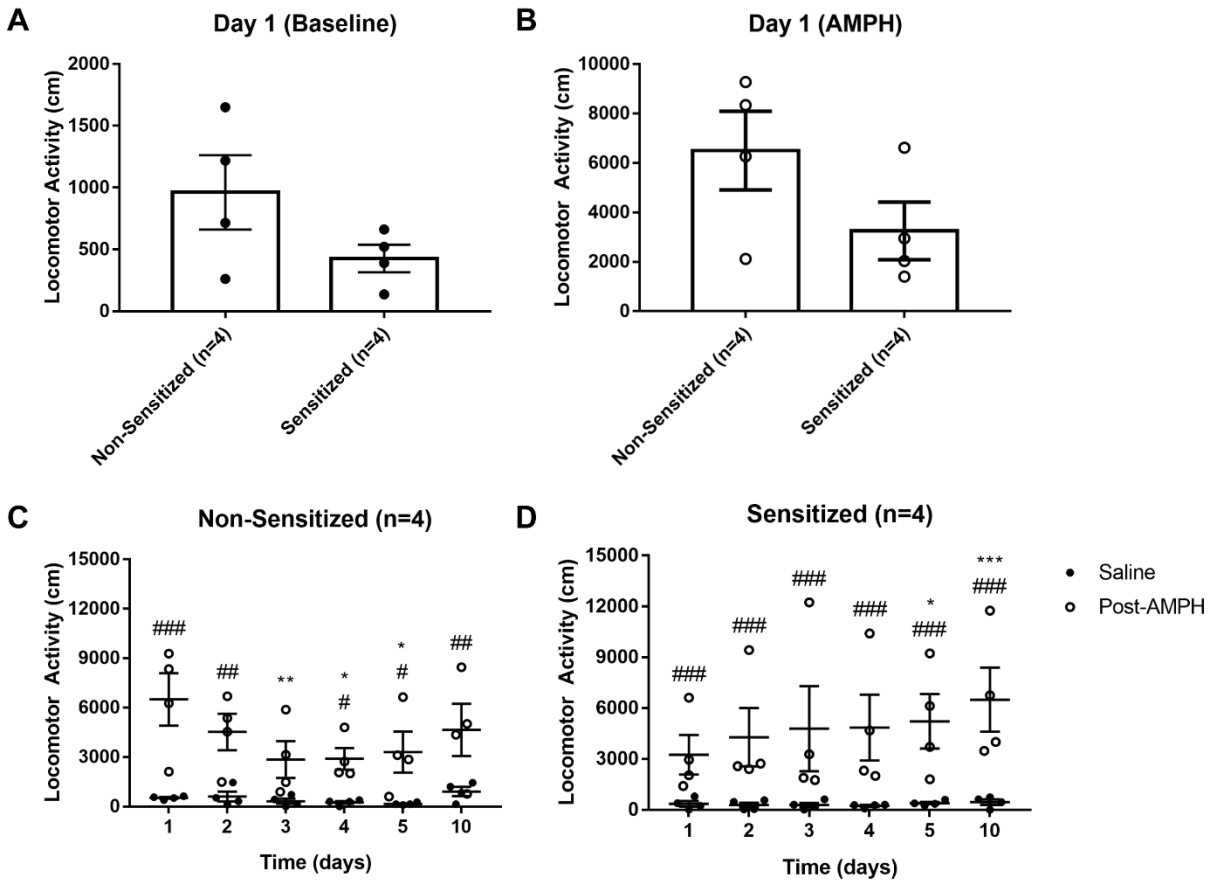

FIGURE 7 Individual differences in locomotor activity after repeated AMPH administration in freely moving rats.

A) Baseline locomotor activity of non-sensitized and sensitized rats on day 1. Locomotor activity was measured for 30 minutes in a locomotor activity box, to study the effect of novelty in locomotion. A trend of increased locomotor activity is observed in non-sensitized rats after AMPH injection ($p=0.1474$, unpaired t-test). B) Effect of AMPH 1.0 mg/kg ip on locomotor activity of non-sensitized and sensitized rats on day 1. Locomotor activity after AMPH 1.0 mg/kg ip injection was measured for 60 minutes in a locomotor activity box. A trend of increased locomotor activity is observed in non-sensitized rats after AMPH injection ($p=0.1505$, unpaired t-test). C-D) Effect of repeated AMPH administration on locomotor activity in non-sensitized (Figure 7C) and sensitized (Figure 7D) rats. Development and expression of locomotor sensitization was only observed in sensitized rats. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs day 1, Bonferroni post-hoc test; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs Saline on the same day, Bonferroni post-hoc test Each line represents mean $\pm$ SEM. Circles represent individual data.

Lastly, figure 7C-D show temporal course of locomotor activity after saline ip and AMPH ip injections of non-sensitized (Figure 7C) and sensitized (Figure 7D) rats between day 1 to 5 and day 10 of the repeated AMPH administration protocol. As indicated previously (see methods 3.2.3), locomotion was measured for 30 minutes after saline ip injection, followed by 60 minutes after AMPH 1.0 mg/kg ip injection. Saline ip and AMPH 1.0 mg/kg ip was administered for 5 consecutive days (days 1-5). Then, after 4 days of abstinence saline ip, followed by a challenge dose of AMPH 1.0 mg/kg ip, was injected on day 10.

Using a repeated measure two-way ANOVA, a significant effect of AMPH treatment ($F_{(1,3)}$= 16.58, $p<0.05$) and days ($F_{(5,15)}$= 3.263, $p<0.05$) but not interaction ($F_{(5, 15)}$= 2.224, $p=0.1057$) was found in non-sensitized rats (Figure 7C). Multiple comparisons analysis shows that locomotor activity after AMPH injection was significantly higher compared to saline injection in all days except day 3 (Day 1: $p<0.001$; Day 2: $p<0.01$; Day 3: $p=0.0557$; Day 4: $p<0.05$; Day 5: $p<0.05$; Day 10: $p<0.01$ Bonferroni's post-test), indicating that hyperlocomotion is related to AMPH and not due to stress related to repeated injections. As expected, non-sensitized rats did not show an increase in their locomotor activity after repeated AMPH and thus, no development nor expression of locomotor sensitization was observed ($p>0.05$, Bonferroni's post-test). Furthermore, locomotor activity in this group after AMPH injection on day 1 was significantly higher compared to days 3, 4 and 5 ($p<0.05$ Bonferroni's post-test), showing a tolerance to AMPH effect on these days.

On the other hand, repeated measure two-way ANOVA showed no significant effect in AMPH-treatment ($F_{(1,3)}$= 6.662 , $p=0.0817$) in sensitized rats (Figure 7D). Relevantly, a significant effect of days ($F_{(5, 15)}$= 2.932 , $p<0.05$) and interaction ($F_{(5, 15)}$= 3.822, $p<0.05$) was observed in

sensitized rats. Multiple comparisons analysis shows that locomotor activity after AMPH injection was significantly higher compared to saline injection in all days (Day 1: $p<0.001$; Day 2: $p<0.001$; Day 3: $p<0.001$; Day 4: $p<0.001$; Day 5: $p<0.001$; Day 10: $p<0.001$ Bonferroni's post-test), indicating that also in sensitized rats hyperlocomotion is related to AMPH and not due to stress related to repeated injections. An increase in locomotor activity on days 5 and 10 compared to day 1 was found ($p<0.001$, Bonferroni's post-test), showing the development and expression of locomotor sensitization in these rats.

4.2.2. Individual differences in DLS baseline firing rate during repeated AMPH administration in freely moving rats

Freely moving single unit recordings were performed on day 1 and day 10 of repeated AMPH administration procedure. A total of 161 neurons were classified as pMSNs (see methods 3.2.5. and Figure 2D-F; Non-sensitized: Day 1=29 neurons, 7±2 neurons/rat; Day 10=35 neurons, 9±4 neurons/rat; Sensitized: Day 1=56 neurons, 14±3 neurons/rat; Day 10=41 neurons, 10±4 neurons/rat).

Baseline firing rate was measured for 30 minutes after saline ip injection (Figure 8). pMSNs baseline firing rate was analyzed on days 1 and 10 to study individual differences in DLS firing rate before a first AMPH injection and after withdrawal, respectively (Figure 8). No significant difference was observed in baseline firing rate between groups on day 1 ($p=0.5112$, Wilcoxon rank sum test). Also, there was no significant difference in baseline firing rate between groups on day 10 ($p=0.09$, Wilcoxon rank sum test). Furthermore, no significant differences were observed in baseline firing rate between day 1 and day 10 within sensitized rats nor within non-sensitized rats (Non-sensitized: $p=0.1622$; Sensitized: $p=0.3413$, Wilcoxon sign rank test).

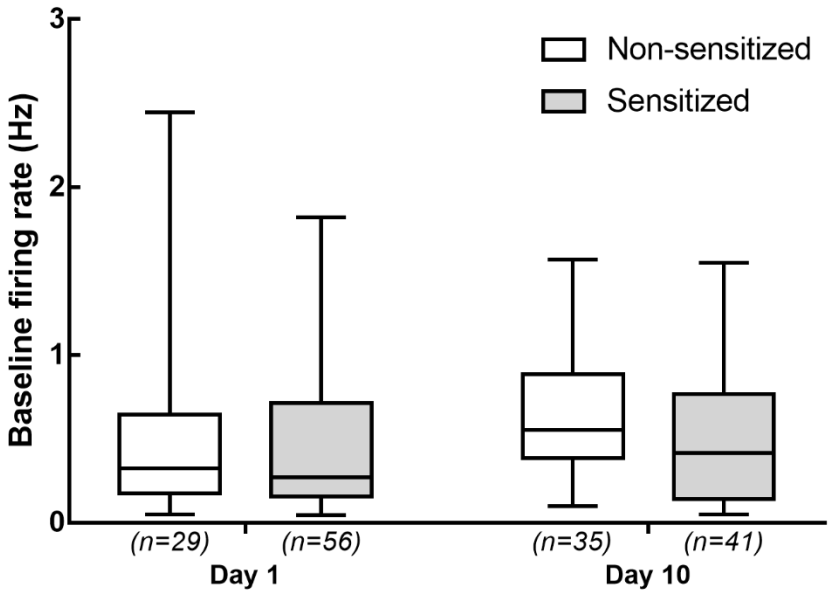

FIGURE 8 Individual differences were not observed in DLS pMSNs baseline firing rate during repeated AMPH administration in freely moving rats.
DLS pMSNs firing rate were recorded for 30 minutes after Saline ip (baseline). No significant differences between groups were observed in baseline firing rate ($p>0.05$, Wilcoxon rank sum test). Also, no significant differences between day 1 and day 10 were observed within groups comparing day 1 and day 10 ($p>0.05$, Wilcoxon rank sum test). Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers).

Together, these data indicate that individual differences in DLS baseline firing rate were not observed before AMPH exposure. Also, the repeated exposure to AMPH did not modify DLS baseline firing rate.

4.2.3. Individual differences in population response to AMPH during repeated AMPH administration in freely moving rats

To determine the specific effect of AMPH on each pMSNs firing rate, a non-parametric permutation test was carried out (see methods 3.2.4.). Post-AMPH firing rate was measured for 60 minutes. Figure 9 shows representative examples of three types of pMSNs neuronal responses observed after an AMPH injection: excitation (Figure 9A), inhibition (Figure 9B) or no change (Figure 9C).

The percentage of pMSNs showing change after an AMPH injection was analyzed to assess individual differences in DLS population response to AMPH during a first AMPH injection (day 1) and during a last AMPH injection (day 10, Figure 9D). No significant differences were observed between groups on day 1 ($p>0.05$, Fisher's exact test). Interestingly, a higher percentage of neurons showing change was observed in sensitized rats compared to non-sensitized rats on day 10 ($p<0.05$, Fisher's exact test). Also, non-sensitized rats showed a significant decrease in the percentage of neurons showing change after AMPH on day 10 compared to their day 1 ($p<0.05$ Fisher's exact test). On the other hand, no changes in the percentage of neurons showing change after AMPH were observed in sensitized rats within days ($p=0.835$, Fisher's exact test).

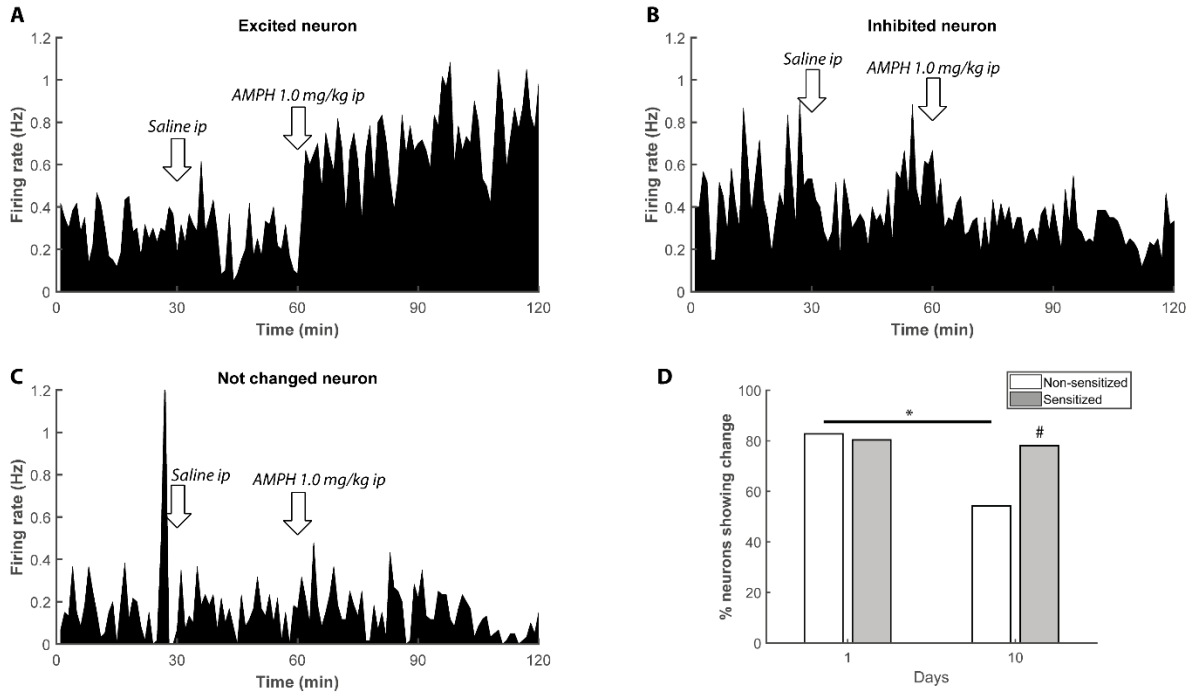

FIGURE 9 Individual differences in DLS neuronal population response to AMPH in freely moving rats.

A-C) Peristimulus time histogram of excited A), inhibited (B) or not changed (C) pMSNs after an acute AMPH 1.0 mg/kg ip injection in freely moving rats. D) Bart charts show the percentage of pMSNs showing change (increase neurons + decrease neurons) after an AMPH 1.0 mg/kg ip injection in days 1 and 10 of the repeated AMPH administration protocol. No significant differences were observed between groups on day 1 ($p>0.05$, Fisher's exact test). Non-sensitized rats percentage of neurons showing change was significantly lower compared to sensitized rats on day 10 (# $p<0.05$ vs non-sensitized rats day 10, Fisher's Exact test). Also, a decrease in the percentage of neurons showing change was observed on non-sensitized rats comparing day 10 with day 1 of the same group (* $p<0.05$, Fisher's exact test). No changes were observed within days in sensitized rats ($p=0.835$).

It is worth to note that no significant differences in the percentage of excited or inhibited pMSNs between groups were observed, with excitation being the main response in both non-sensitized and sensitized rats (Annexes Figure S2).

Together, these results show that DLS pMSNs population response to AMPH is preserved in sensitized rats after repeated AMPH administration. On the other hand, a decrease in DLS pMSNs population response to AMPH was observed in non-sensitized rats after repeated AMPH administration.

4.2.4. Individual differences in AMPH excitability modulation during repeated AMPH administration in freely moving rats

To determine the relationship between baseline firing rate and the magnitude of change in firing rate of excited pMSNs during repeated AMPH administration, Kendall correlations were performed on days 1 and 10 of non-sensitized and sensitized rats (Figure 10, method described in section 3.2.5.). Inhibited neurons were not considered due to the low sample size in each group (Non-sensitized= 5 to 7 inhibited neurons; Sensitized= 13 to 7 inhibited neurons).

The correlation between pMSNs magnitude of excitation and baseline firing rate on days 1 and 10 was analyzed to assess individual differences in AMPH excitability modulation during a first AMPH injection and a last AMPH injection of repeated AMPH administration (Figure 10). AMPH magnitude of excitation was directly correlated with baseline firing rate in both groups on day 1 (Day 1: Non-sensitized: tau = 0.43791, $p<0.05$; Sensitized: tau = 0.64516, $p<0.001$ Kendall Correlation). This means that a greater baseline firing rate is accompanied with a greater magnitude of excitation. On day 10, AMPH magnitude of excitation was directly correlated with baseline firing rate in sensitized rats too (tau = 0.62, $p<0.001$ Kendall Correlation).

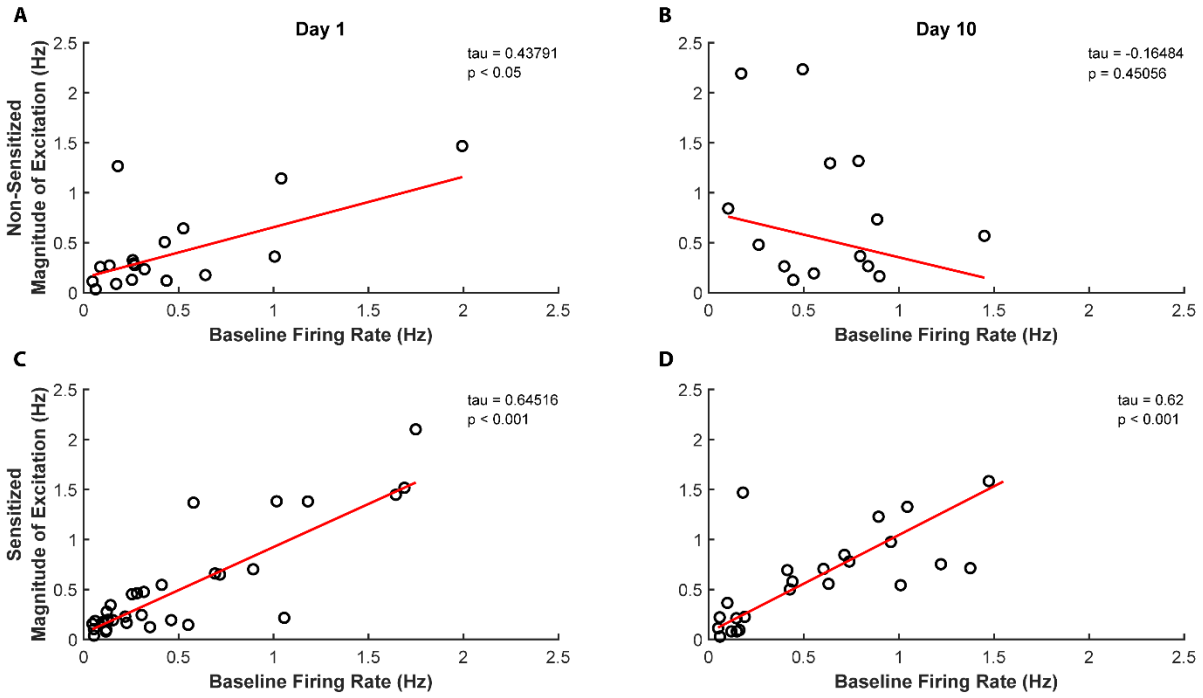

FIGURE 10 Individual differences in AMPH excitability modulation during repeated AMPH administration in freely moving rats.
The correlation between magnitude of change in firing rate of excited pMSNs (magnitude of excitation) and firing rate during baseline was analyzed using a Kendall correlation. A-B) Non-sensitized rats: a significant direct correlation between magnitude of excitation and baseline firing rate was observed in day 1 (A) but not in day 10 (B). C-D) Sensitized rats: a significant direct correlation between magnitude of excitation and baseline firing rate were observed in both day 1 (C) and day 10 (D). Each circle represents a pMSNs data. Red line corresponds to a Theil-Sen robust linear regression.

Interestingly, no significant correlation between AMPH magnitude of excitation with baseline firing rate was observed in non-sensitized rats on day 10 (tau = -0.16484, p=0.4506 Kendall Correlation).

Overall, these results show that DLS pMSNs AMPH excitability modulation in sensitized rats is preserved after repeated AMPH administration. In contrast, a loss of DLS pMSNs AMPH excitability modulation was observed in non-sensitized rats on day 10.

4.2.5. Individual differences in DLS firing pattern during repeated AMPH administration in freely moving rats

Firing pattern of DLS pMSNs was analyzed using Lv index (see methods 3.2.5). Similarly, to firing rate, baseline Lv index was measured for 30 minutes after saline ip injection and post-AMPH Lv index was measured for 60 minutes after AMPH 1.0 mg/kg ip injection on days 1 and 10 (Figure 11).

4.2.5.1 Baseline Lv index

pMSNs baseline Lv index was analyzed on days 1 and 10 between groups to assess individual differences in DLS firing pattern before a first AMPH injection and after withdrawal from repeated AMPH administration (Figure 11, white boxes). Interestingly, sensitized rats showed a higher baseline Lv index compared to non-sensitized rats on day 1 ($p<0.001$, Wilcoxon rank sum test , Wilcoxon rank sum test). In other words, a higher pMSNs baseline firing irregularity is observed in rats that will express AMPH locomotor sensitization. As in day 1, a higher baseline Lv index was found on day 10 in sensitized rats compared to non-sensitized rats ($p<0.05$ Wilcoxon rank sum test). Importantly, no significant differences were observed

between day 1 and day 10 within sensitized rats nor in non-sensitized rats (Non-sensitized: p=0.5221, Sensitized: p=0.0949, Wilcoxon sign rank test).

In summary, these results indicate that rats that will become sensitized show a higher DLS pMSNs firing irregularity before a first AMPH injection compared to those rats that not will not express AMPH locomotor sensitization. Also, repeated AMPH administration did not modify DLS baseline firing irregularity within groups, showing that the initial differences in firing irregularity between non-sensitized and sensitized rats were preserved after withdrawal.

4.2.5.2 Post-AMPH Lv index

Next, post-AMPH Lv index was analyzed between groups to determine individual differences in DLS firing pattern during the first (day 1) and last (day 10) AMPH injection (Figure 11, gray boxes). Post-AMPH Lv index in sensitized rats was significantly higher compared to non-sensitized rats on day 1 ($p<0.05$, Wilcoxon rank sum test). In contrast, no significant changes were observed on day 10 (p=0.2896, Wilcoxon rank sum test). Also, no significant differences were observed in post-AMPH Lv index between day 1 and day 10 within sensitized rats nor within non-sensitized rats (Non-sensitized: p=0.7013, Sensitized: p=0.1555, Wilcoxon sign rank test).

In relation to within groups analysis between baseline and post-AMPH, a decrease Lv index on both days 1 and 10 in sensitized rats was observed comparing baseline and post-AMPH (Day 1: $p<0.001$; Day 10: $p<0.01$, Wilcoxon sign rank test). Interestingly, a significant decrease in Lv index was observed only on day 1 of non-sensitized rats between post-AMPH and baseline (Day 1: $p<0.01$; Day 10: p=0.2254, Wilcoxon sign rank test).

In summary, these results indicate that individual differences in DLS pMSNs firing pattern were observed on both day 1 and day 10 of the repeated AMPH administration protocol. In other words, sensitized rats showed a higher DLS firing irregularity compared to non-sensitized rats after a first AMPH injection and AMPH injection only decreased DLS pMSNs firing irregularity in sensitized rats on the last AMPH injection.

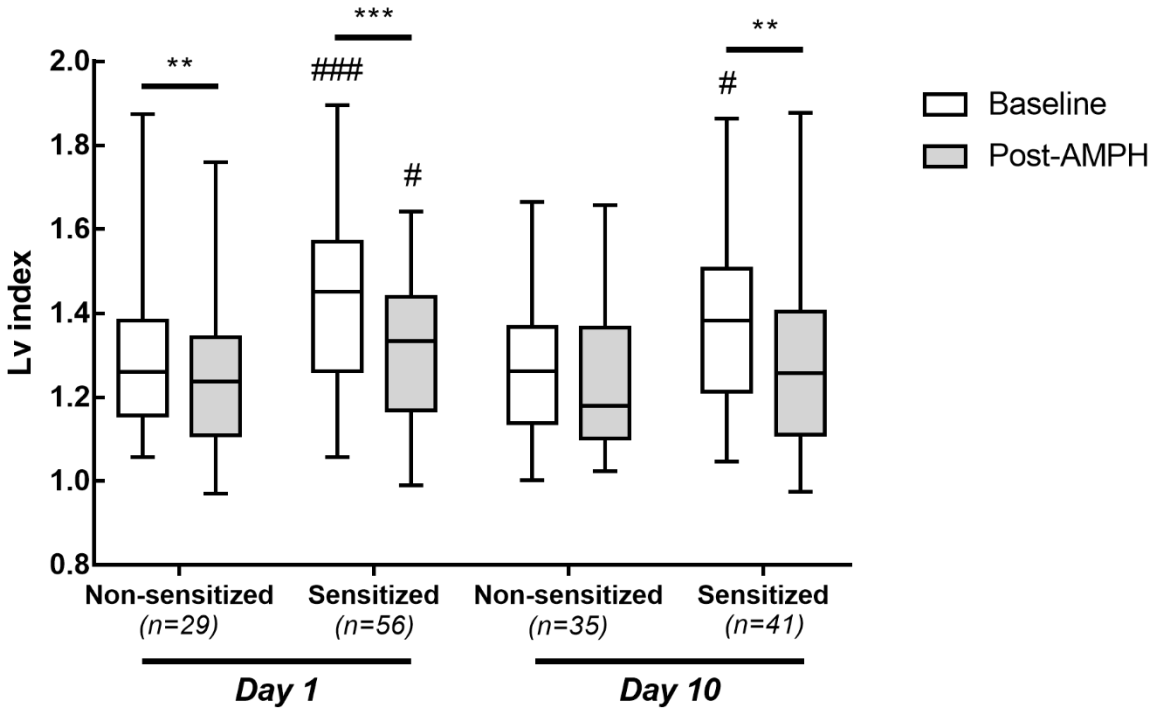

FIGURE 11 Individual differences in firing pattern of pMSNs during repeated AMPH administration in freely moving rats.

DLS pMSNs Lv index was analyzed for 30 minutes after saline ip injection (baseline) and for 60 minutes after AMPH 1.0 mg/kg ip injection (post-AMPH). Baseline Lv index was significantly higher in both days 1 and 10 of sensitized rats compared to non-sensitized rats. No significant differences in baseline Lv index were observed between day 1 and day 10 within groups ($p>0.05$, Wilcoxon rank sum test). Post-AMPH Lv index was significantly higher in day 1 of sensitized rats compared to non-sensitized rats. No significant differences in post-AMPH Lv index were observed between day 1 and day 10 within groups ($p>0.05$, Wilcoxon rank sum test). A significant decrease in Lv index was observed after AMPH 1.0 mg/kg ip injection in non-sensitized rats on day 1. Sensitized rats show a decrease in Lv index after AMPH 1.0 mg/kg ip injection on both days 1 and 10. ** $p<0.01$, *** $p<0.001$ using Wilcoxon sign rank test; # $p<0.05$, ### $p<0.001$ vs same day and condition in non-sensitized rats, Wilcoxon rank sum test. Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers).

4.2.6 Individual differences in the correlation between locomotor activity and DLS firing rate during repeated AMPH administration in freely moving rats.

4.2.6.1 Subpopulations of pMSNs showing a direct or inverse correlation between firing rate and locomotor activity.

It has been previously shown that a 36% of DLS pMSNs show a direct correlation between firing rate and locomotor activity, whereas a 20% show an inverse correlation (Wiltschko, Pettibone and Berke, 2010). Here, pMSNs firing rate and locomotor activity (FR/L) correlation during both baseline and post-AMPH epochs were analyzed (as showed in section 3.2.4.). Both firing rate and locomotor activity during baseline (after Saline ip injection) and after AMPH 1.0 mg/kg ip injection were grouped in 1 minute bins and normalized by their corresponding maximum value. Then, a Kendall correlation was performed.

Figure 12 shows three representative examples of pMSNs FR/L correlations during baseline and post-AMPH epochs. Figure 12A shows an example of a pMSN with no FR/L correlation during baseline (tau=0.0217, p=0.8851 Kendall Correlation). On the other hand, a direct FR/L correlation was observed in this same pMSN after an AMPH 1.0 mg/kg ip injection (Figure 12B, tau=0.4617, $p<0.001$ Kendall Correlation). In contrast, figure 12C-D show a pMSN with a direct FR/L correlation during both baseline (Figure 12C, tau = 0.4294, $p<0.01$ Kendall Correlation) and post-AMPH epochs (Figure 12D, tau = 0.4618, $p<0.001$ Kendall Correlation). Finally, figure 12E-F show a pMSN with an inverse FR/L correlation during both baseline (Figure 12E, tau=-0.5468, $p<0.001$ Kendall Correlation) and post-AMPH (Figure 12F, tau=-0.2346, $p<0.01$ Kendall Correlation) epochs.

In order to determine individual differences in the percentage of DLS pMSNs showing FR/L correlation, the percentage of FR/L correlated pMSNs ($p<0.05$, Kendall correlation) during both baseline and post-AMPH periods were obtained on days 1 and 10. Neurons were classified according to their Kendall tau value: $tau>0$ and $p<0.05$ were classified as directly FR/L correlated pMSNs (Figure 13) and $tau<0$ and $p<0.05$ were classified as indirectly FR/L correlated pMSNs (Annexes Figure S3). pMSNs with $p>0.05$ were classified as non-correlated neurons and not were further analyzed.

Overall, our results indicate that between a 16% to 58% of the pMSNs showed a direct FR/L correlation during repeated AMPH administration protocol (Figure 13). On the other hand, between a 3% to 13% of the pMSNs showed an inverse FR/L correlation (Annexes Figure S3). Given low percentage of pMSNs with inverse correlation, there were not considered for additional analysis. Therefore, pMSNs showing a direct FR/L correlation were further analyzed.

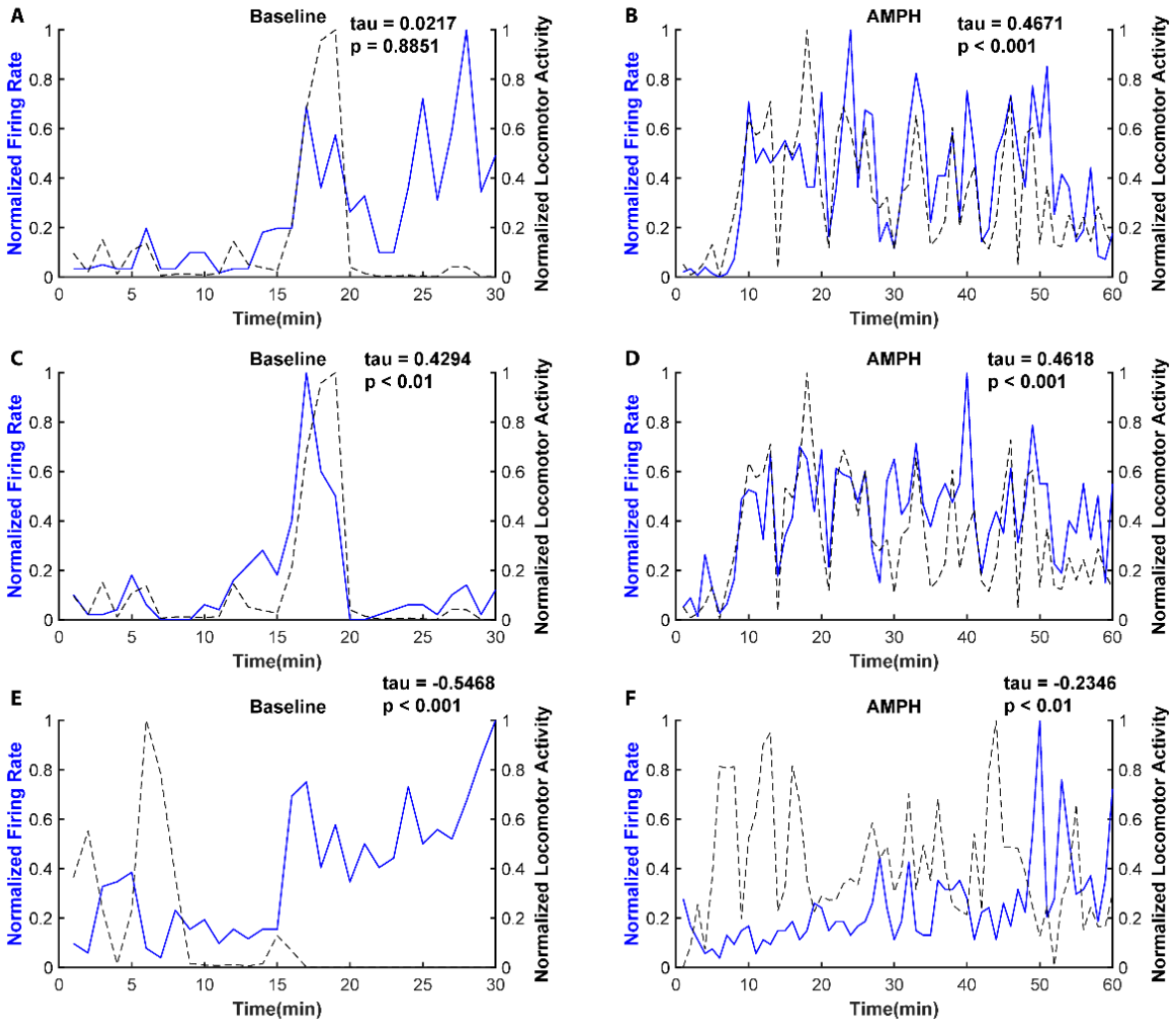

FIGURE 12 Examples of correlation between DLS pMSNs firing rate and locomotor activity during baseline and after AMPH 1.0 mg/kg ip injection in freely moving rats. Normalized firing rate (blue line) and normalized locomotor activity (dotted line) during baseline (after saline ip) and AMPH 1.0 mg/kg ip injections are plotted. A Kendall correlation between firing rate and locomotor activity was performed (bin= 1 min). A-B) Example of a pMSN with no correlation with locomotion during baseline (Figure 12A, tau = 0.0217, p=0.8851) and a significant direct correlation with locomotion after AMPH 1.0 mg/kg ip injection (Figure 12B, tau = 0.4671, p<0.001). C-D) Example of a pMSN with a significant direct correlation with locomotion during both baseline (Figure 12C, tau = 0.4294, p<0.01) and after AMPH 1.0 mg/kg ip injection (Figure 12D, tau = 0.4618, p<0.001). E-F) Example of a pMSN with a significant inverse correlation with locomotion during both baseline (Figure 12E, tau = -0.5468, p<0.001) and after AMPH 1.0 mg/kg ip injection (Figure 12F, tau = -0.2346, p<0.01).

4.2.6.2 Baseline FR/L correlation.

The percentage of pMSNs showing a direct FR/L correlation was analyzed to assess individual differences in the percentage of FR/L correlated pMSNs before AMPH exposure (day 1) and after withdrawal (day 10) from repeated AMPH administration (Figure 13, white bars). Interestingly, non-sensitized rats showed a higher percentage of correlated pMSNs compared to sensitized rats during baseline activity on day 1 ($p<0.01$ Fisher Exact test). In contrast, no significant differences were found between groups on day 10, although a trend of increase is observed in non-sensitized rats ($p=0.06309$, Fisher Exact test). Furthermore, no significant differences were observed between day 1 and day 10 within sensitized rats nor in non-sensitized rats (Non-sensitized: $p>0.99$; Sensitized: $p=0.08767$, Fisher Exact test).

Overall, these results indicate that individual differences were observed in the percentage of DLS pMSNs showing a direct FR/L correlation before AMPH exposure, i.e. rats vulnerable to express AMPH locomotor sensitization show a low percentage of pMSNs with a direct FR/L correlation during baseline activity compared to sensitization-resistant rats on day 1.

4.2.6.3 Post-AMPH FR/L correlation

Next, the percentage of pMSNs showing a direct FR/L correlation during AMPH epoch was compared between groups to determine individual differences in the percentage of FR/L correlated pMSNs during the first (day 1) and last (day 10) AMPH injection (Figure 13, gray bars). In this sense, the percentage of correlated pMSNs during AMPH epoch was not different between groups. No significant changes in the percentage of correlated pMSNs during post-AMPH were observed between groups on day 1 ($p=0.6453$ Fisher Exact test). Also, no significant differences were found between groups on day 10 ($p=0.8174$ Fisher Exact test).

Furthermore, no significant differences were observed between day 1 and day 10 within sensitized rats nor in non-sensitized rats ($p>0.99$, Fisher Exact test).

Regarding within groups analysis between baseline and post-AMPH, a significant increase in the percentage of correlated pMSNs on both days 1 and 10 was observed comparing post-AMPH and baseline in sensitized rats (Day 1: $p<0.001$; Day 10: $p<0.05$ Fisher Exact test). On the other hand, no significant differences were found between post-AMPH and baseline percentage of correlated pMSNs within non-sensitized rats on days 1 nor on day 10 (Day 1: $p>0.99$; Day 10: $p>0.99$ Fisher Exact test).

In summary, these results indicate that an increase in the percentage of DLS pMSNs showing a direct correlation is observed only in sensitized rats during the first and last AMPH injection of repeated AMPH administration protocol. Also, these results show that a similar percentage of DLS pMSNs correlate during AMPH epoch between groups.

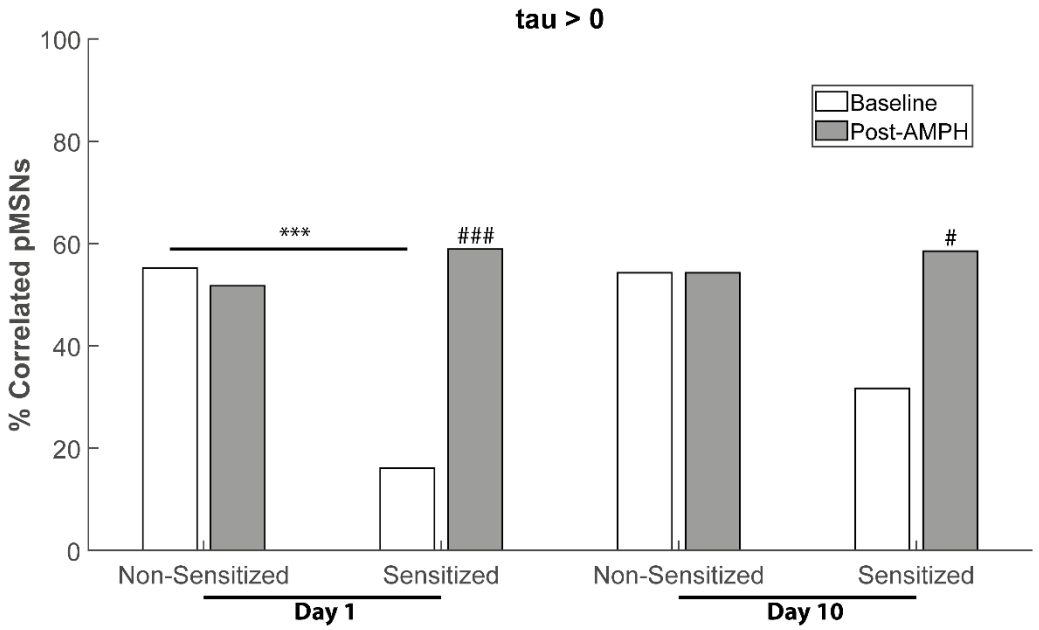

FIGURE 13 Individual differences in the percentage of FR/L correlated DLS pMSNs during repeated AMPH administration in freely moving rats.

A Kendall correlation between firing rate and locomotor activity were performed during baseline (after saline ip) and post-AMPH epochs. Neurons with a significant correlation ($p<0.05$) and tau>0 correspond to directly correlated pMSNs. During baseline activity on day 1, non-sensitized rats showed a significantly higher percentage of correlated pMSNs compared to sensitized rats. An increase in the percentage of correlated pMSNs was observed between baseline and AMPH epochs in sensitized rats on days 1 and 10. *** $p<0.001$ Fisher Exact test; # $p<0.05$, ### $p<0.001$ vs sensitized baseline in the same day, Fisher Exact test. Bar chart represent the total percentage of correlated pMSNs in each condition.

4.2.7 Individual differences in the correlation between locomotor activity and DLS firing pattern during repeated AMPH administration in freely moving rats.

No previous data showing the correlation between locomotor activity with firing pattern of pMSNs has been reported. Both Lv index and locomotor activity during baseline and after AMPH 1.0 mg/kg ip injection were grouped in 1 minute bins and normalized by their corresponding maximum value (as showed in section 3.2.4.). Then, a Kendall correlation was performed between Lv index and locomotor activity during baseline and post-AMPH epochs.

Figure 14 show two representative DLS pMSNs were Lv index vs locomotor activity (Lv/L) correlation was performed. Figure 14A shows an example of a pMSN with no Lv/L correlation during baseline (tau= -0.1838, p=0.2192 Kendall Correlation). Then, a direct Lv/L correlation was observed in the same pMSN after AMPH 1.0 mg/kg ip injection (Figure 14B, tau= 0.4934, $p<0.001$). On the other hand, Figure 14C-D show a pMSN with no Lv/L correlation during both baseline (Figure 14C, tau = -0.0692, p=0.6047 Kendall Correlation) and post-AMPH epochs (Figure 14D, tau = -0.0237, p=0.7937 Kendall Correlation).

The percentage of pMSNs showing a Lv/L correlation on days 1 and 10 was analyzed to assess individual differences in the percentage of correlated DLS pMSNs during repeated AMPH administration protocol (Figure 15). Overall, between 11% and 26% of the pMSNs showed a Lv/L correlation. No significant differences were observed between groups on each day during baseline (Day 1, p=0.7899 Fisher Exact test; Day 10, p=0.7930 Fisher Exact test) nor in AMPH epochs (Day 1, p=0.2719 Fisher Exact test; Day 10, p=0.5327, Fisher Exact test). Furthermore, no significant differences within conditions were observed in non-sensitized (Day 1: p=0.7297;

Day 10: p=0.3420, Fisher Exact test) nor in sensitized rats (Day 1: p>0.99; Day 10: p=0.4241, Fisher Exact test).

In summary, these data indicate that a low percentage of DLS pMSNs covariate their firing irregularity with locomotor activity and that individual differences were not observed in the percentage of DLS pMSNs showing a Lv/L correlation during repeated AMPH administration protocol.

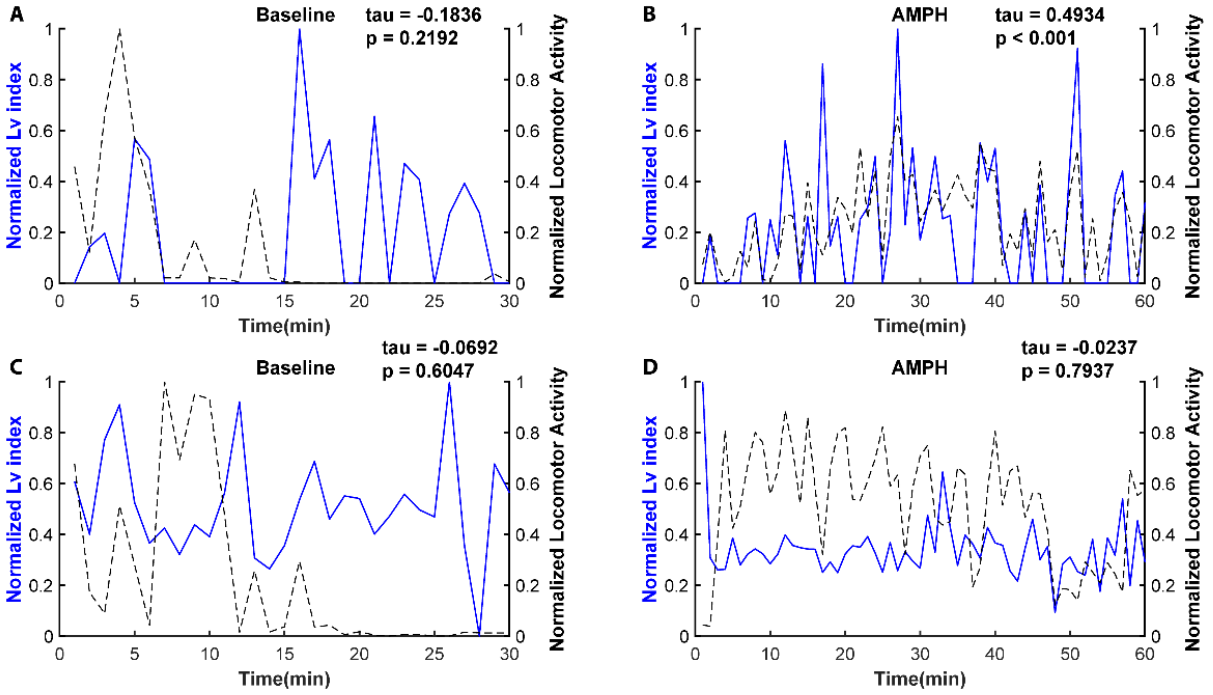

FIGURE 14 Examples of correlation between DLS pMSNs Lv index and locomotor activity during baseline and after an AMPH 1.0 mg/kg ip injection in freely moving rats. Normalized Lv index (blue line) and normalized locomotor activity (dotted line) during baseline (after saline ip) and after AMPH 1.0 mg/kg ip injection are plotted. A Kendall correlation between Lv index and locomotor activity was performed (bin= 1 min). A-B) Example of a pMSN with no correlation with locomotion during baseline (Figure 14A, tau = -0.1836, p=0.2192) and with a significant direct correlation locomotion after AMPH 1.0 mg/kg ip injection (Figure 14B, tau = 0.4934, p<0.001). C-D) Example of a pMSN with no correlation with locomotion during both baseline (Figure 14C, tau = -0.0692, p=0.6047) and after an AMPH 1.0 mg/kg ip injection (Figure 14D, tau = -0.0237, p=0.7937).

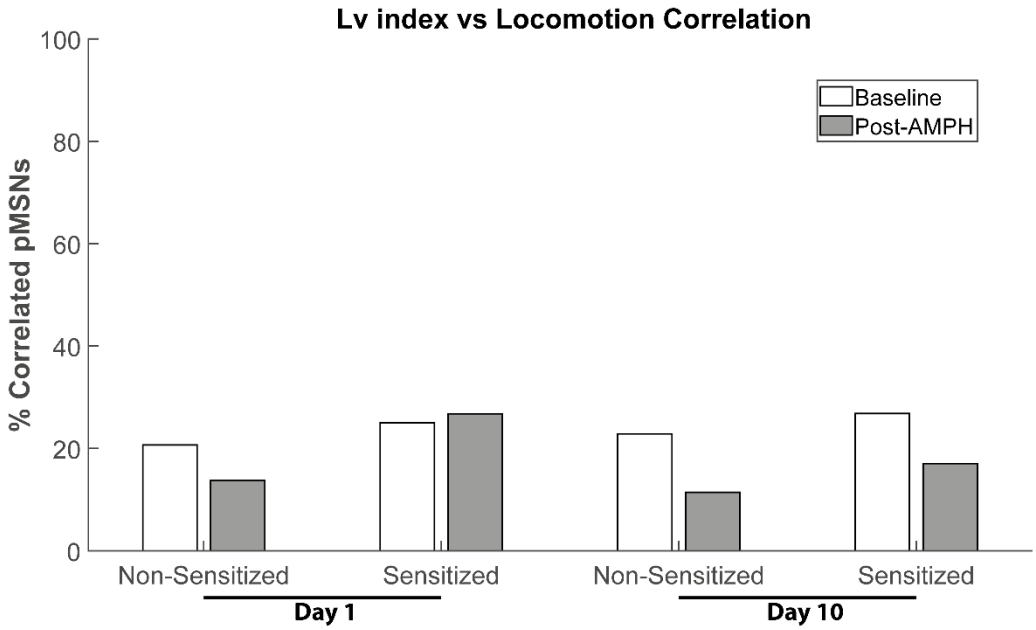

FIGURE 15 Individual differences were not observed in the percentage of Lv/L correlated DLS pMSNs during repeated AMPH administration in freely moving rats.
A Kendall correlation between Lv index and locomotor activity were performed during baseline (after saline ip) and post-AMPH epochs. Neurons with a significant correlation ($p<0.05$) correspond to correlated pMSNs. Bar chart represent the total percentage of correlated pMSNs in each condition. A low percentage of pMSNs showed a significant Lv/L correlation. Furthermore, no significant differences were observed between groups, conditions or within groups ($p>0.05$ Fisher Exact test).

4.3.- To quantify the gephyrin density in the entopeduncular nucleus, substantia nigra pars reticulata and globus pallidus after amphetamine repeated administration.

One disadvantage of our single unit experiments is that does not allow us to differentiate between dorsal striatum MSNs from direct and indirect pathway. Then, to quantify possible changes in the density of GABAergic synapses on basal ganglia direct and indirect pathway after repeated AMPH administration, profiles of the inhibitory postsynaptic marker Geph were counted in the EP, SNr and GP.

4.3.1 Gephyrin immunofluorescence testing and striatal outputs identification

In order to get reliable labeling, optimal conditions for gephyrin immunofluorescence were evaluated. Sections from rats brains were obtained and immunofluorescence protocol gephyrin was used (see methods 3.3.2.). To evaluate which secondary antibody use for gephyrin, two options were tested: anti-mouse AlexaFlour488 and anti-mouse Cy3 secondary antibody. High-magnification Geph immunofluorescence images are showed in Figure 16. A strong Geph staining was observed in both conditions, showing profiles arranged in fiber-like structures (Figure 16A-B). In general, the tested fluorescence channels for Geph staining were optimal. It is also important to remark that the antibody penetration was up to 4-5 µm in both conditions, making these immunofluorescence protocol correct to further perform stereological analysis of Geph profiles. We ended using the anti-mouse Cy3 secondary antibody, because it allowed more reliable immunofluorescence of Geph across sampled sections, compared to anti-mouse AlexaFlour488.

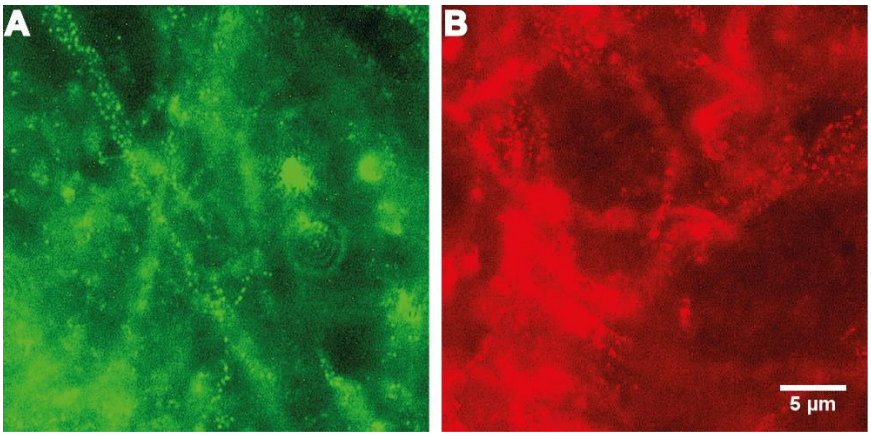

FIGURE 16 Gephyrin profiles using different immunofluorescent secondary antibodies in SNr sections.
High magnification fluorescence photomicrographs of sections that contains SNr. A) Geph in green, using anti-mouse AlexaFlour488 secondary antibody. B) Geph in red, using anti-mouse Cy3 secondary antibody. Both channels were optimal for stereological counting. Scale bar: 5 µm.

Both Geph and TH immunofluorescence (using an anti-rabbit AlexaFlour488 secondary antibody) were used to demarcate the EP, SNr and GP (Figure 17). The high GABAergic input on EP allow the identification of this area using Geph, that was observed with a high immunofluorescence within the internal capsule (ic, Figure 17A). SNr demarcation was performed using TH (green color channel, Figure 17B). SNr lies between the SNc and the cerebral peduncle (cp). Finally, GP demarcation cannot be performed only using Geph, as both dorsal striatum and GP have a large GABAergic innervation. Instead, we used both Geph used TH immunofluorescence to identify the GP (Figure 17C-E). TH immunofluorescence is high in dorsal striatum but low in GP (Figure 17C), compared to Geph that shows a high immunofluorescence in both dorsal striatum and GP (Figure 17D). Then, the merged imaged allow the identification of GP in the section (Figure 17E).

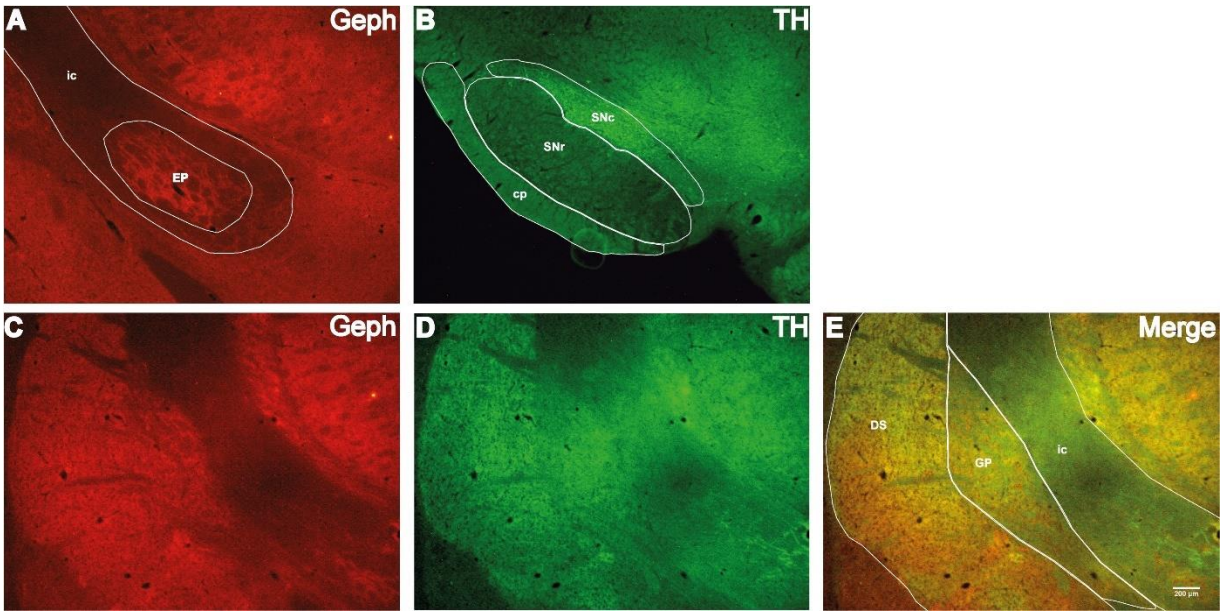

FIGURE 17 Immunofluorescence identification of GP, EP and SNr.
Representative low magnification fluorescence photomicrographs of coronal sections containing GP, EP and SNr. A) Identification of EP, using Geph in red. B) Identification of SNr, using TH in green. C-E) GP demarcation. Using Geph in red (C) and TH in green (D), the merged image (E) from TH and Geph allow the identification of GP. cp: cerebral peduncle, DS: dorsal striatum, ic: internal capsule, SNc: substantia nigra pars compacta. Scale bar: 200 µm.

4.3.2 Individual differences in density of gephyrin profiles on EP, SNr and GP after repeated AMPH administration

Serial sections containing EP, SNr and GP were obtained from rats brains from freely moving experiments and gephyrin immunofluorescence was carried out (see methods 3.3.2.). Then, stereological estimates of the density of Geph profiles in the EP, SNr and GP was carried out. The volume for each of the structures under study was also estimated using the planimetry function of the optical fractionator probe of Stereo Investigator (see methods 3.3.3.). The optical fractionator probe was used with the following settings: Section Evaluation Interval: 8; Counting Frame Size: 3 x 3 µm; Grid Size: 120 x 120 µm for EP, 320 x 320 µm for SNr, 420 x 420 µm for GP; Dissector Height: 3 µm; Top Guard Zone: 1 µm. Using these settings, a total of 1 to 22 counting areas per section were obtained and used for counting. The Gundersen's coefficient of error for each of the estimates (EP, SNr or GP in each animal) was lower than 0,1, which show the reliability of the measurement. One example of Geph profiles counting in the SNr is showed in Figure 18.

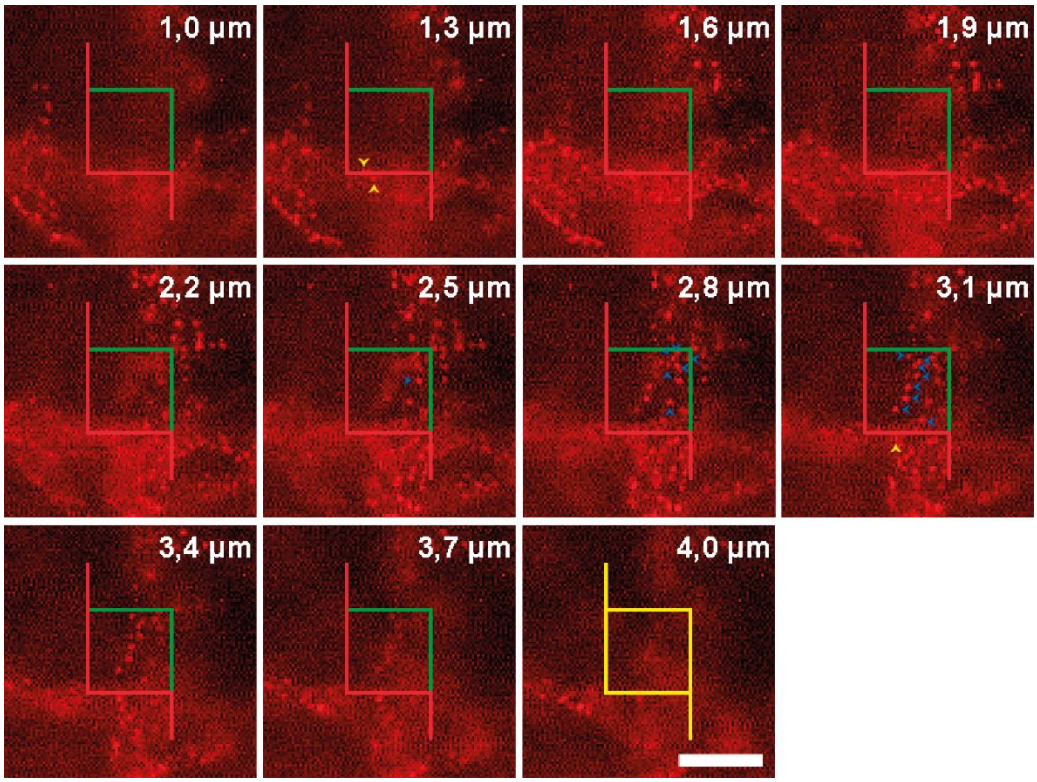

FIGURE 18 Example of a stereological counting of gephyrin profiles using the optical fractionator.

Images at different z depths are showed for a SNr region, stained for Geph (red). Counting frame: 3 x 3 µm; dissector height: 3.0 µm; guard zone: 1.0 µm. Gephyrin profiles on focus that were inside the counting frame or intersecting the green axis are counted, whereas the profiles that intersected the red axis were not counted. Blue arrow heads indicate profiles that were in focus at the given z depth and were counted. Yellow arrows indicate the profiles that were not counted. The change in color of the dissector indicates the bottom of the dissector, thus, no more counting is allowed. Scale bar: 5 µm.

To address if volume of EP, SNr and GP is modified by repeated AMPH administration, comparisons were carried out between treated rats and controls (i.e. rats without previous treatments or injections). The smaller region was EP, with a mean volume of 0.36 mm^3 in control rats (Figure 19A). The largest areas were the SNr and GP, with a volume of 2.11 mm^3 and 4.15 mm^3, respectively, in control rats (Figure 19B-C, also analyzed in Annexes Figure S5B). No significant differences in volume were observed between groups in each area after repeated AMPH administration (Figure 19; EP: $F_{(2,8)}$= 0.03447, p=0.7185 One-way ANOVA; SNr: $F_{(2,7)}$= 0.3588, p=0.7106 One way ANOVA; GP: $F_{(2,8)}$= 0.7112, p=0.5197 One-way ANOVA).

Next, to determine individual differences in the density of GABAergic synapses in the direct and indirect striatal pathways after repeated AMPH administration, gephyrin density was analyzed in EP, SNr and GP between groups (Figure 20). No significant differences were observed on gephyrin density in EP ($F_{(2,8)}$= 0.1584, p=0.8561 One-way ANOVA). Also, Geph density was not modified on SNr between groups ($F_{(2,7)}$= 1.569, p=0.2734 One-way ANOVA). On the other hand, a significant increase in gephyrin density on GP was observed in non-sensitized rats compared to control and sensitized rats ($F_{(2,8)}$= 5.705, $p<0.05$ One-way ANOVA; $p<0.05$ Non-sensitized vs Control and Sensitized rats, unpaired t test).

In summary, expression of AMPH locomotor sensitization is not accompanied by changes in nuclei volume, nor with changes in the density of GABAergic synapses in EP, SNr and GP. In contrast, an increased density of GABAergic synapses in GP, but not in EP or SNr, accompanies the lack of locomotor sensitization following repeated AMPH administration.

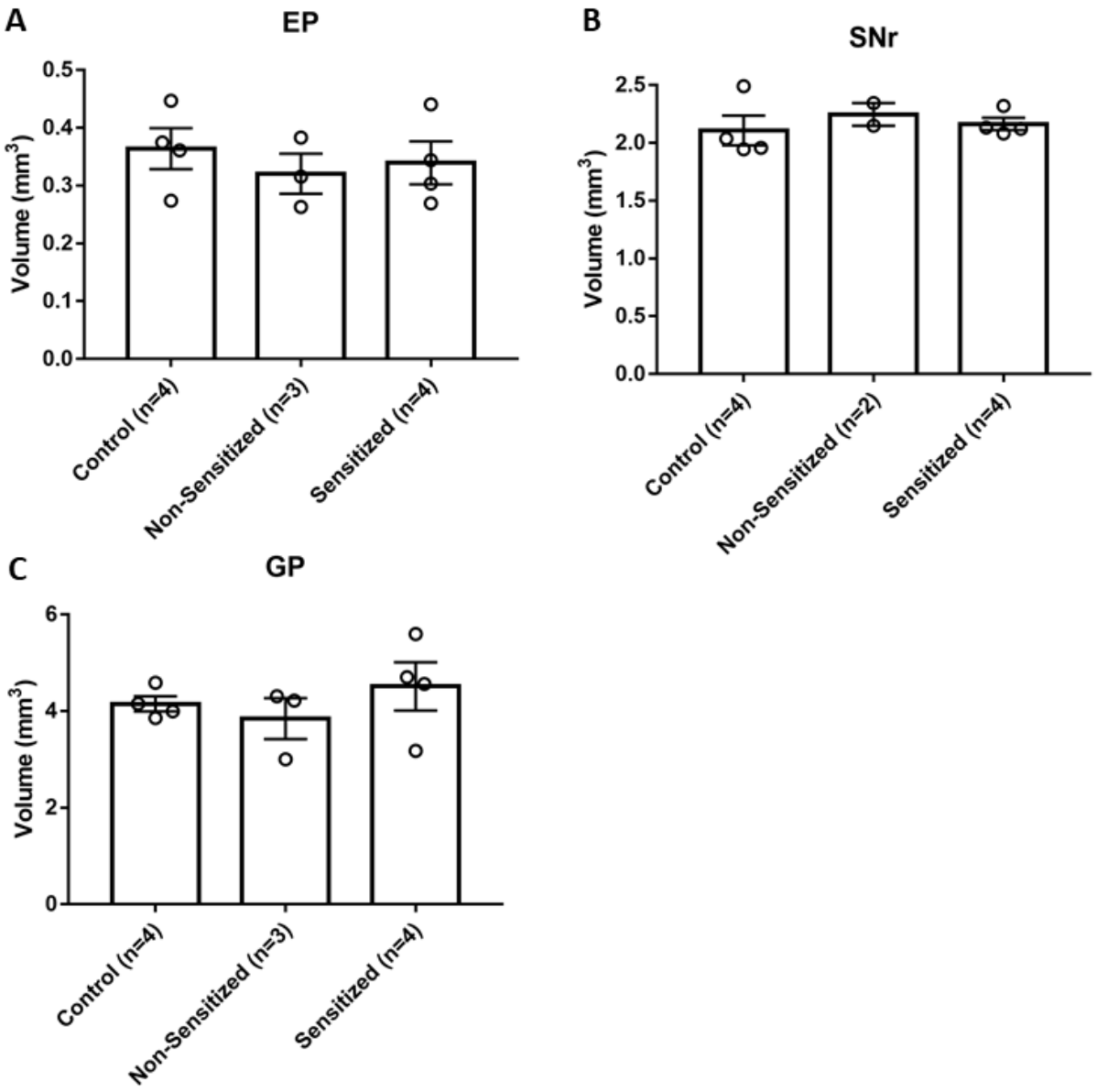

FIGURE 19 No differences in volume of EP, SNr and GP were observed after repeated AMPH administration.

A-C) Volume estimation was performed using the planimetry function of the optical fractionator in EP (A), SNr (B) and GP (C). No significant differences were observed between groups in each area ($p>0.05$, Bonferroni post test). Each bar represents mean $\pm$ SEM.

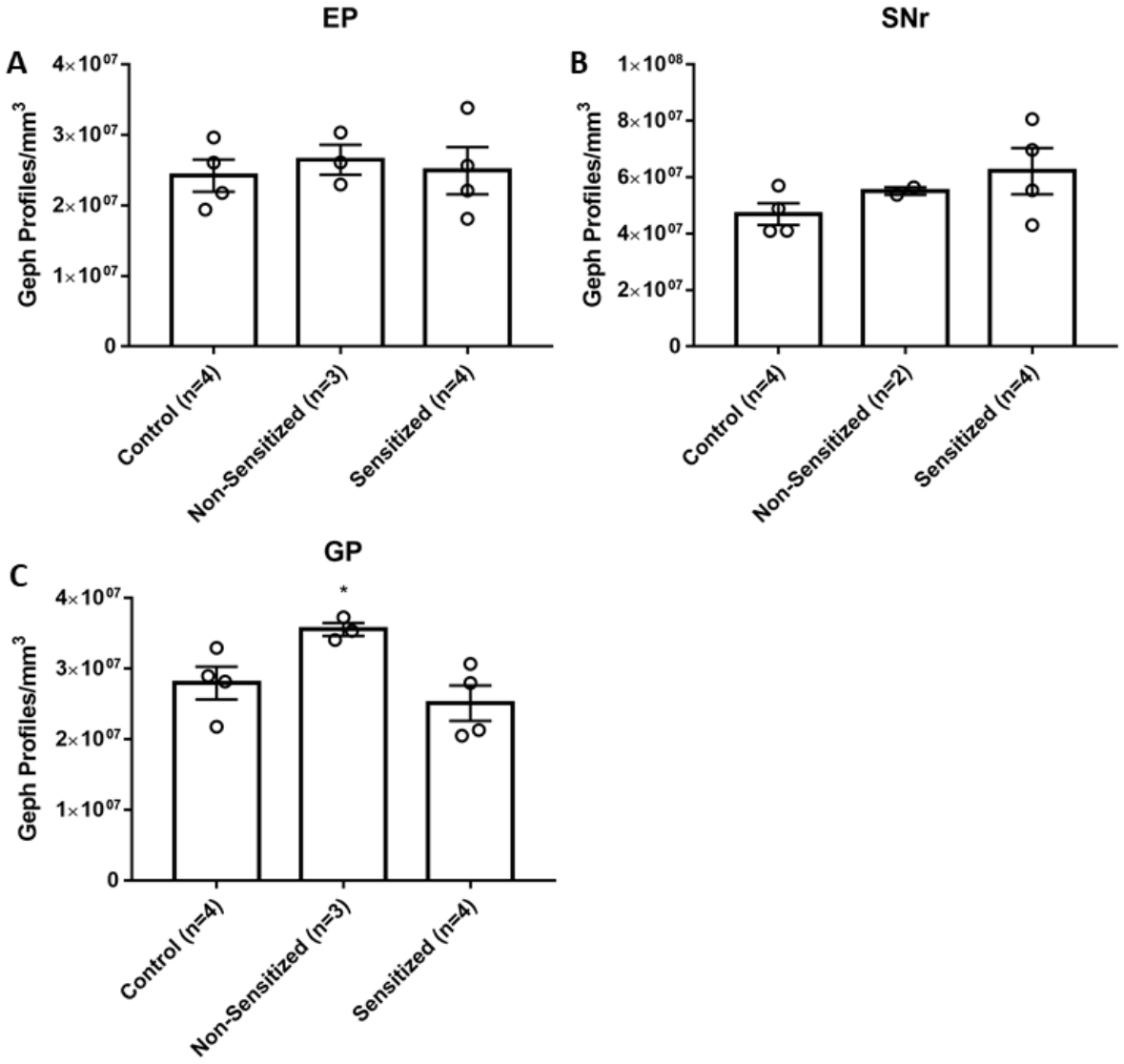

FIGURE 20 Individual differences in Geph density on GP, but not EP and SNr were found after repeated AMPH administration.

A-C) Stereological counting of gephyrin on EP (A), SNr (B) and GP (C) was performed using the optical fractionator. Number of counted profiles were normalized with the total volume of each area. * $p<0.05$ compared to control and sensitized rats, using unpaired t-test. Each bar represents mean $\pm$ SEM.

5. DISCUSSION

It has been stablished that differential changes in neural pathways are related to individual differences in the behavioral responses to drugs of abuse (Kasanetz et al., 2010). Then, not all the subjects repeatedly exposed to drugs of abuse develop addiction (Deroche-Gamonet et al., 2004).

In this doctoral thesis, DLS neuronal activity and the number of GABAergic synapses in dorsal striatum outputs were studied to address the role of dorsal striatum in individual differences observed after repeated AMPH administration. Our main findings are:

1.- In anesthetized rats, 24 hours after the expression of AMPH locomotor sensitization, DLS pMSNs activity was characterized by an increase in baseline firing rate and a decrease in Lv index after an acute AMPH injection;

2.- In freely moving rats, on day 1, a higher DLS pMSNs baseline Lv index and a low percentage of FR/L correlated pMSNs during baseline activity were found in rats vulnerable to express AMPH locomotor sensitization. Both non-sensitized and sensitized rats showed a high percentage of neurons showing change after AMPH, a direct correlation between AMPH excitation vs baseline firing rate and a decrease in Lv index after AMPH injection;

3.- In freely moving rats, on day 10, rats that expressed locomotor sensitization showed similar AMPH-effects on DLS neuronal activity compared to day 1. Instead, the lack of expression of AMPH locomotor sensitization was accompanied by a decrease in the percentage of neurons showing change after AMPH, no correlation between AMPH excitation vs baseline firing rate and no changes in Lv index after AMPH injection;

4.- No modifications in Geph density were observed on EP, SNr nor GP of sensitized rats. In contrast, an increase in Geph density on GP of non-sensitized rats was observed.

Overall, our results indicate that DLS and dorsal striatum outputs are not modified after the expression of AMPH locomotor sensitization. On the other hand, electrophysiological changes in DLS and an increase in the GABAergic synapses on GP accompanies the lack of expression of AMPH locomotor sensitization. Furthermore, individual differences in DLS firing pattern and in the correlation between firing rate and locomotor activity predict the expression of AMPH locomotor sensitization.

5.1. To determine the firing rate and firing pattern of dorsolateral striatum neurons after amphetamine repeated administration in anesthetized rats.

In the present objective, we observed that 55% of the rats repeatedly injected with AMPH 1.0 mg/kg ip expressed locomotor sensitization (Figure 3). Interestingly, an increase in DLS pMSNs baseline firing rate (Figure 4) and a decrease in post-AMPH Lv index (Figure 6) were only observed in sensitized rats. Furthermore, a decrease in Lv index after an acute AMPH injection was only observed in sensitized rats. Together, these data show that differential changes in neuronal activity in the DLS accompanies the individual differences in locomotor sensitization.

Individual differences in DLS pMSNs baseline firing rate were observed after repeated AMPH administration. Specifically, sensitized rats show an increased pMSNs baseline firing rate compared to non-sensitized and control rats (Figure 4). An increase in baseline firing rate of dorsal striatum neurons is also observed after both repeated administration of cocaine (Takahashi et al., 2007) and MDMA locomotor sensitization (Ball et al., 2010). Our data is consistent with these results (Figure 4). Altogether, this augmented pMSNs activity could facilitate the habitual intake of psychostimulants drugs (Nelson and Killcross, 2006). Recently, it has been shown that the expression of AMPH locomotor sensitization is also accompanied with an increase in basal DA levels in DLS (Azocar et al., 2019). We have extended this recent finding by showing no changes in the basal DA levels of non-sensitized rats (Gatica et al., 2019 under review). This hyperfunction in nigrostriatal dopaminergic activity after AMPH locomotor sensitization could be underlying the increase in DLS baseline firing rate. For instance, an increase in the strength of glutamate excitation modulated by DA could accompanies the increment in pMSNs baseline firing rate in sensitized rats (Kiyatkin and Rebec, 1996). In fact, the repeated administration of amphetamines (Szumlinski et al., 2017; Xue et al., 1996) and cocaine (Parikh et al., 2014; Reid and Berger, 1996) is accompanied by an increase in extracellular glutamate levels on striatum. Additional experiments could address if changes in extracellular glutamate levels are observed in the dorsal striatum of non-sensitized rats.

As previously shown (Wiltschko et al., 2010), a heterogenous response is observed in DLS single units firing rate after an acute AMPH injection. Our results are consistent with this, as they show that in control rats less than a half of the DLS pMSNs change their firing rate after an acute AMPH injection. On the other hand, a significant percentage of pMSNs change their firing rate after an acute AMPH injection in both sensitized and non-sensitized rats compared

to control rats (Figure 5). In fact, the percentage of neurons that increase and decrease their firing rate after AMPH is similar comparing non-sensitized and sensitized rats (Annexes Figure S1). These results suggest that repeated AMPH administration increase the recruitment of DLS pMSNs that respond to an acute exposure of AMPH, independent of individual differences observed in locomotor sensitization.

It have been previously shown that an acute AMPH injection modifies burst firing pattern in mPFC (Aguilar-Rivera et al., 2015). To analyze the effect of AMPH on firing irregularity in DLS pMSNs we used Lv index, an measure of firing pattern, that has the advantage of detect the instantaneous variability of ISIs (Shinomoto et al., 2009). The median value of pMSNs baseline Lv index in control rats is about 1.2, an Lv index value that is classified as a bursty firing pattern and that is comparable to other areas like hippocampus and mPFC (Mochizuki et al., 2016). Interestingly, after an acute AMPH injection, a significant decrease in pMSNs Lv index is observed in control rats compared to their respective baseline condition (Figure 6) suggesting a fast modification in the intrinsic firing irregularities induced by AMPH. Given that calcium uptake modulates burst firing in striatal neurons (Cooper and White, 2000; Kerr and Plenz, 2002), a transient decrease in AMPA-related calcium uptake could underlie the decrease in Lv index induced by AMPH (Yu et al., 2005). A decrease in burst activity after an acute AMPH injection was also observed in mPFC (Aguilar-Rivera et al., 2015), suggesting that AMPH produces similar responses on firing pattern in pyramidal and striatal neurons. Noteworthy, an increase in bursting activity is observed after AMPH 5.0 mg/kg (Stanford and Gerhardt, 2001), that compared to ours 1.0 mg/kg ip dose, show that AMPH effect on firing pattern is dose-dependent.

Alike to that observed in baseline firing rate, our results also show individual differences in DLS pMSNs Lv after repeated AMPH administration. The expression of AMPH locomotor sensitization is accompanied by a significant decrease in pMSNs baseline Lv index compared to control rats. These results indicate that a decreased DLS pMSNs baseline burst activity underlie locomotor sensitization to AMPH. Burst firing has been related to synaptic plasticity and efficiency of information transfer (Izhikevich et al., 2003; Lisman, 1997). In this sense, a decrease in AMPA/NMDA ratio has been observed in NAc after repeated cocaine administration, evidencing a synaptic depression (Kourrich et al., 2007). Together, these data suggest that the expression of AMPH locomotor sensitization is accompanied with a decrease in synaptic plasticity and information output of DLS pMSNs.

As with control rats, sensitized rats show a decrease in pMSNs Lv index after an acute AMPH injection compared to their respective baseline condition (Figure 6). Interestingly, non-sensitized rats pMSNs Lv index was not modified after an acute AMPH injection, indicating that the effect of AMPH on firing irregularity shows individual differences after repeated AMPH administration. Importantly, this change in pMSNs Lv index after an acute AMPH injection it was not accompanied by differences in population response to AMPH (figure 5) in both non-sensitized and sensitized rats. Together, we suggest that the persistence of AMPH-decreasing effect in firing irregularity on DLS pMSNs is the electrophysiological measure that differentially contributes to the expression of locomotor sensitization after repeated AMPH administration. Ours results are in line with previous evidence showing that the impairment on long-term depression (LTD) observed during the early exposure to cocaine persist in ‘addicted’ rats, but is reverted in ‘non-addicted’ rats (Kasanetz et al., 2010). Kasanetz and collaborator propose that the anaplasticity - i.e. the failure to counteract initial drug induced changes - is one

key feature that characterize the transition to addiction. Then, it is tempting to suggest that a loss of AMPH-decreasing effect on pMSNs Lv index is a neuroplastic adaptation that allow to counteract the increase in locomotor activity after repeated AMPH administration.

5.2. To determine neuronal activity and locomotion correlation of dorsolateral striatum neurons during amphetamine repeated administration in freely moving rats.

In the present objective, the two main findings were found before and after repeated AMPH administration. A high DLS pMSNs baseline firing irregularity prior to AMPH injection was associated with the expression of locomotor sensitization after repeated AMPH administration (Figure 11). After repeated AMPH administration, a persistence of AMPH-effect on DLS neuronal activity was observed in sensitized rats (Figure 9-11). On the other hand, the lack of expression of AMPH locomotor sensitization was accompanied by a decrease in AMPH population response to AMPH, a loss in AMPH excitability modulation and no changes in firing irregularity after AMPH injection (Figure 9-11). Taken together, these results suggest the persistence of AMPH effect on DLS is necessary for the expression of AMPH locomotor sensitization. Also, initial differences in DLS firing irregularity could induce differential neuroadaptations after repeated AMPH administration.

5.2.1 Dorsolateral striatum neuronal activity as a predictor for the expression of amphetamine locomotor sensitization.

As mentioned in the introduction (Section 1.3.), prior studies have noted that the vulnerability to express locomotor sensitization is associated to specific behavioral traits (Herrera et al., 2013; Piazza et al., 1989; Yamamoto et al., 2013) and differential activity of dopaminergic pathways (Marinelli and White, 2000). Here, we report that DLS pMSNs baseline firing rate was not

different between groups on day 1 (Figure 8). On the other hand, pMSNs baseline Lv index was higher in rats that will become sensitized compared to those who will not express locomotor sensitization (Figure 11). Together, these data indicate that rats showing a higher DLS baseline firing irregularity are more vulnerable to express locomotor sensitization after repeated AMPH administration. Furthermore, the differences observed in firing pattern are not related to changes in the total number of spikes during baseline activity, showing that without modifications in firing rate, a distinct baseline firing pattern differentiate the susceptibility to express locomotor sensitization. Our results are in line with other evidence showing individual differences in burst firing of dopaminergic neurons in SNc and VTA: rats with a high locomotion during novelty (HR rats) present a higher burst firing compared to rats with a low locomotion during novelty (LR rats, Marinelli and White, 2000). In fact, it was previously found that LR rats are more vulnerable to express locomotor sensitization after repeated AMPH administration (Piazza et al., 1989). A trend of decreased locomotor activity during novelty was observed in sensitization-vulnerable rats (Figure 7A), suggesting that LR rats show a higher DLS pMSNs firing irregularity. As burst firing is related to efficiency of information transfer and synaptic plasticity (Izhikevich et al., 2003; Lisman, 1997), our results suggest that initial differences in baseline firing irregularity define the progression to specific DLS neuroadaptations after repeated AMPH administration.

Various mechanism could explain this difference in baseline Lv index on day 1 between sensitization-vulnerable and sensitization-resistant rats. Irregular firing is one well stablished feature in pMSNs (Wilson, 1993). During awake intracellular recordings, MSNs firing pattern is mainly characterized for temporally disorganized depolarizing events, mainly related to uncorrelated firing of cortical neurons that innervate striatal neurons (Mahon et al., 2006). In

this sense, it has been stablished that a synchronization of cortical inputs increase firing irregularity (Stevens and Zador, 1998; Svirskis and Rinzel, 2000). Then, one plausible explanation of the differential pMSNs baseline Lv index observed on day 1 is a higher synchrony of excitatory cortical inputs on MSNs in rats with increased risk to express AMPH locomotor sensitization. Another mechanism could be related to intrinsic properties of striatal neurons, which are also relevant to determine firing pattern (Cooper, 2002). Dendritic calcium signals correlates with burst strength (Kerr and Plenz, 2002), with L-type calcium channels being more available during burst generation (Carter and Sabatini, 2004). Moreover, using the L-type calcium antagonist diltiazem, a significant decrease in burst duration was observed in NAc (Cooper and White, 2000). Given this, it is possible to think that a differential activity or expression of L-type calcium channels in pMSNs dendrites could underlie the individual differences in baseline Lv index.

In addition to individual differences in baseline Lv index, post-AMPH Lv index was significantly higher in rats that were become sensitized compared to those who were not express locomotor sensitization (Figure 11). Relevantly, this was accompanied by a lack of differences between groups on population response to AMPH (Figure 9) or in AMPH excitability modulation (Figure 10). Together these data indicate that a differential DLS pMSNs firing pattern during a first AMPH injection, without differences in AMPH effect on firing rate, define the susceptibility to express locomotor sensitization. The decrease in Lv index after AMPH injection could be explained due to a transient decrease in AMPA-related calcium uptake observed after AMPH (Yu et al., 2005), considering that calcium uptake modulates burst firing in striatal neurons (Cooper and White, 2000; Kerr and Plenz, 2002). This mechanism could be associated to both non-sensitized and sensitized rats, but a relevant difference arise. The

individual differences observed in baseline Lv index could be important for the differential post-AMPH Lv index between groups. It is tempting to suggest that baseline Lv index reflects the potential of pMSNs to undergo through specific AMPH-related neuroadaptations, i.e. pMSNs may show specific neuroplastic modifications after a first AMPH injection, which could be relevant for the expression of AMPH locomotor sensitization. This will be further discussed in the next section (5.2.2.).

The correlation between DLS neuronal activity parameters (firing rate and Lv index) and locomotor activity was also assessed. One main finding was that firing rate showed a high percentage of pMSNs with significant correlation with locomotor activity, i.e. 16-58% of pMSNs showed FR/L correlation (Figure 13). Our results are consistent with previous evidence showing that around a 60% of pMSNs correlate their firing rate with locomotor activity (Wiltschko et al., 2010). On the other hand, only a 11-26% of pMSNs presented a Lv/L correlation (Figure 15). To our knowledge, the correlation between Lv index and locomotor activity has been not been studied before. Overall, these data suggest that the firing pattern of a DLS pMSNs does not encode locomotion. A plausible explanation may be due to Lv index low variability across time (Shinomoto et al., 2009), which make it less probable to be related with a highly variable measure like locomotion. Taking together with firing irregularity results (Figure 11), we suggest that DLS pMSNs Lv index is relevant to assess intrinsic properties of neurons and neuroplasticity, which predict the vulnerability to express AMPH locomotor sensitization, but not to encode locomotion.

Focusing on FR/L correlation, individual differences were observed in the percentage of pMSNs correlated during baseline on day 1, with sensitization-vulnerable rats showing a lower

percentage of correlated pMSNs than sensitization-resistant rats (Figure 13). Also, AMPH injection increased the percentage of correlated pMSNs only in rats that will become sensitized to AMPH. Previously, it has been found that MSNs show a differential encoding of locomotor activity that is related to context (Yamin et al., 2013). In other words, some pMSNs correlate their firing rate with locomotor activity only during the exposure of a novel environment, while others only correlate during a familiar environment exposure, or others correlate during both situations. Given this, we suggest that MSNs encoding of locomotion define the individual differences during the course of locomotor sensitization. Sensitization-resistant rats may show MSNs that encode both locomotor activity during baseline activity and after AMPH injection, whereas MSNs of sensitization-vulnerable rats are mainly engaged with AMPH-induced locomotion. The low baseline dopaminergic activity showed by sensitization-vulnerable rats could underlie this finding (Marinelli and White, 2000), considering that DA activity in DLS modulates locomotion (Howe and Dombeck, 2016). Then, the increase DLS DA levels after AMPH injection (Azocar et al., 2019) could allow to set MSNs encoding of locomotion in a similar level than sensitized-resistant rats, animals that show a higher baseline levels of dopaminergic activity (Marinelli and White, 2000). Finally, although with single unit recordings we cannot confirm if pMSNs were from the direct or indirect pathways, recent evidence have shown that neuronal activity MSNs in both pathways is similar during locomotion (Barbera et al., 2016). This last evidence suggest that direct pathway neurons promotes behaviors, whereas indirect pathway neurons inhibit competing behaviors. Considering this, an optogenetic approach coupled with single unit recordings could allow to determine which specific pathway modulates behavior during baseline activity and after AMPH injection.

5.2.2 Role of dorsolateral striatum neuronal activity in the expression of amphetamine locomotor sensitization

Previous evidence have shown the relevance of DLS on drug addiction and locomotor sensitization. An increase in DA and glutamate levels on DLS was observed after psychostimulant sensitization and self-administration, with D1 and AMPA receptors antagonism blocking the expression of these processes (Azocar et al., 2019; Parikh et al., 2014; Wang et al., 2013; Willuhn et al., 2012). Also, DLS dendritic spines density showed an increase after MDMA and methamphetamine sensitization (Ball et al., 2010; Jedynak et al., 2007). Furthermore, an increased baseline firing rate was observed during the expression of MDMA locomotor sensitization (Ball et al., 2010). Yet, there is also evidence showing a lack of DLS neuroadaptations after repeated drug exposure. Using a puzzle cocaine self-administration procedure, that allows the development of drug seeking behaviors without habit formation, the role of DA neurotransmission on NAc core and DLS was evaluated (Singer et al., 2018). The infusion of DA antagonist α-flupenthixol on NAc core, but not in DLS, decreased drug seeking in this paradigm. Furthermore, Coffey and collaborators using a cocaine self-administration paradigm, showed no longitudinal changes in DLS baseline firing rate (Coffey et al., 2015). Also, no modifications in the surface expression of AMPA or NMDA receptors in DLS were observed after the expression of AMPH locomotor sensitization (Nelson et al., 2009). This last result is further supported by evidence showing no alterations in DLS AMPA/NMDA ratio in AMPH sensitized rats, indicating the lack of a synaptic potentiation (Wang et al., 2013). In summary, although evidence seems to be contradictory, some relevant differences can be taking into consideration.

One general finding that can be observed in the literature is that changes in DLS after repeated drug administration are mostly presynaptic, i.e changes in dopaminergic and glutamatergic neurotransmission (Azocar et al., 2019; Parikh et al., 2014). Albeit it has been shown post-synaptic adaptations after the expression of psychostimulant sensitization, such as an increase in DLS spiny density, long withdrawal periods (Ball et al., 2010) and differences in drug protocols (like escalating dosing of methamphetamine, Jedynak et al., 2007) could underlie this neuronal adaptations. On the other hand, other post-synaptic changes, like modifications in the expression of AMPA and NMDA receptors subunits and changes in AMPA/NMDA ratio were not observed in the DLS of AMPH sensitized rats, after short or long withdrawal (Nelson et al., 2009; Wang et al., 2013). Then, taking together these presynaptic modifications (Azocar et al., 2019; Parikh et al., 2014) without post-synaptic adaptations, an increased DLS baseline firing rate and firing irregularity was expected during the expression of AMPH locomotor sensitization. The increase in pMSNs firing was hypothesized considering that glutamate and DA are key modulators of MSNs activity (Kiyatkin and Rebec, 1996). In this sense, our results showing no changes in baseline pMSNs firing rate (Figure 8) and firing irregularity (Figure 11) could be explained due to changes in receptors function. A decrease in AMPA and NMDA receptor function could be a compensatory adaptation for the basal hyper-glutamatergic state during the observed in sensitized animals. Also, as calcium regulation is relevant for firing pattern (Cooper, 2002), modifications in calcium channels expression or function could allow an stabilization of baseline Lv index. Together, these mechanisms may act like a compensatory response, to maintain the baseline DLS pMSNs function of rats that express AMPH locomotor sensitization.

Our data is in line with other electrophysiological findings showing that a preserved DLS function is observed after repeated drug exposure. During the course of cocaine self-administration, while a change from inhibitory response to no response was observed in NAc, a maintenance of DLS response was found (Coffey et al., 2015). Also, using an odor-discrimination task it was shown that, whereas NAc encoding of odor outcomes was impaired, DLS encoding was unaltered in rats repeatedly exposed to cocaine (Takahashi et al., 2007). Our results showed that population response to AMPH (Figure 9D) and AMPH excitability modulation of AMPH excitation (Figure 10C-D) were preserved in AMPH sensitized rats. Together, this evidence suggest that the persistence of DLS drug-related information processing is necessary for AMPH locomotor sensitization. In this sense, Takahashi and collaborators proposed that DLS role in drug addiction could function on an Actor/Critic framework (Takahashi et al., 2008). This neural computation model assign the role of the 'Critic' to areas that are related to predict the value of an outcome. The limbic system have the role of 'Critic' in this model, with the NAc being the main integrative area. On the other hand, the 'Actor' role is characterized for action selection and execution, which are the main role of DLS. In this model, the 'Critic' help the 'Actor' to choose the actions with better outcomes. Taken together, Takahashi and collaborators suggest that a modification in the 'Critic' but not in the 'Actor' are necessary for the progression of addictive behavior. In other words, impaired outcome predictions could be relevant in the transition to addictive behavior, with action selection and execution unaltered. As a bulk of evidence have shown (Section 1.2), NAc and other limbic areas like mPFC are substantially modified after the expression of locomotor sensitization. Specifically, whereas modulation of AMPH firing rate is impaired in the mPFC of sensitized rats (Aguilar-Rivera et al., 2015), our results show a maintenance of the AMPH excitability

modulation in sensitized rats (Figure 10). Together, our data further support the hypothesis that a persistence of DLS processing of drug-related information is required during the course of locomotor sensitization. But, what about non-sensitized rats?

Surprisingly, without changes in baseline activity, DLS neuronal activity and firing pattern during AMPH was different between non-sensitized and sensitized rats. A decrease in population response to AMPH (Figure 9D), accompanied with a loss of AMPH excitability modulation (Figure 10A-B) and no changes in Lv index after AMPH injection (Figure 11) were found in non-sensitized rats on day 10. These results indicate a reversal of AMPH-effect on DLS neuronal activity in non-sensitized rats compared to sensitized rats. In general, studies have shown that non-sensitized rats does not undergo through neural adaptations after repeated drug administration. Dorsal striatum neuronal parameters were not altered in MDMA non-sensitized rats (Ball et al., 2010) and we have shown that basal DA levels in DLS were not modified in these animals (Gatica et al, 2019 under review). Similar findings have been found in other areas. In mPFC, stimulated DA levels were unaltered when comparing controls to AMPH non-sensitized rats (Casanova et al., 2013). Also, total DA levels in mPFC and NAc were not modified in rats that do not expressed AMPH locomotor sensitization (Scholl et al., 2009). Even though our results seems rather surprising, there are also evidence that support changes in non-addicted prone individuals. For instance, Kasanetz and collaborator proposed that anaplasticity - i.e. the failure to counteract initial drug induced changes - is one key feature that characterize the transition to addiction. Whereas cocaine addicted rats were characterized with a permanent loss of NAc LTD induction, cocaine non-addicted rats reverted this loss of LTD induction (Kasanetz et al., 2010). Our results complement this finding, suggesting that a reversal of DLS

anaplasticity is one mechanism that accompanies the lack of expression of AMPH locomotor sensitization.

AMPH-information processing was altered in rats resistant to express AMPH locomotor sensitization. A decrease in the percentage of pMSNs showing change after AMPH (Figure 9D) and loss of AMPH excitability modulation (Figure 10) were observed in non-sensitized rats. These modification of AMPH information processing in DLS of non-sensitized rats could be further understood using the Actor/Critic framework (Takahashi et al., 2008). As stated before, changes in the 'Critic' function without modifications in the 'Actor' are critical for the transition to addiction. Considering that evidence has shown that mPFC and NAc of remain unaltered in non-sensitized rats (Casanova et al., 2013; Scholl et al., 2009), our results indicate that DLS neuroadaptations are relevant to block the expression of AMPH locomotor sensitization. Then, a modified 'Actor' (DLS) changes how it interpret the orders of an operational 'Critic' (NAc-mPFC). Relevantly, the percentage of FR/L correlated pMSNs was not modified after repeated drug administration (Figure 13). Given this, these data suggest that the modification in DLS function in non-sensitized rats is not related to normal DLS function. In turn, what is modified is the processing of AMPH-related information after repeated drug administration(Figure 9,10), that underlies the lack of expression of AMPH locomotor sensitization.

As discussed before, sensitized rats neuroadaptations could be related to changes in NMDA and AMPA receptors, and calcium channels function, that can be associated to a compensatory mechanism that accompanies the increase glutamate and DA after the expression of locomotor sensitization (Azocar et al., 2019; Parikh et al., 2014). Conversely, whereas presynaptic adaptations have been not observed in non-sensitized rats (Casanova et al., 2013; Scholl et al.,

2009), post-synaptic adaptations such as an increase in LTD induction (Kasanetz et al., 2010) could underlie the reversal of AMPH-effect in DLS neuronal activity. Relevantly, Bock and collaborators showed that, while rats with compulsive cocaine use showed no modifications in AMPA/NMDA ratio in NAc indirect pathway, rats resistant to develop compulsive cocaine use showed an increase in AMPA/NMDA ratio, indicating a synaptic potentiation of this neuronal pathway (Bock et al., 2013). Then, it is plausible to suggest that the differential susceptibility to express locomotor sensitization is related to a neuroplastic changes on indirect pathway MSNs. Considering that basal ganglia indirect pathway activation is related to decrease in movement (Kravitz et al., 2010), the increase in AMPA/NMDA ratio in striato-pallidal MSNs could underlie as a protective mechanism against locomotor sensitization. Our results further complement Bock and collaborators findings, also suggesting that this differential modification in AMPA/NMDA ratio may be related to the initial differences observed in baseline Lv index (Figure 11). The implications of indirect pathway in AMPH locomotor sensitization will be further discussed on section 5.3.

5.2.3. Differences between anesthetized and freely moving single unit recordings

When our single unit recordings in freely moving rats were compared with those in anesthetized rats, some differences were observed. Whereas an increase in baseline firing rate was observed in sensitized rats on anesthetized experiments (Figure 4), no changes in baseline firing rate were observed between groups in freely moving recordings (Figure 8). Also, a decrease in baseline Lv index was observed between sensitized and control rats in anesthetized experiments (Figure 6), while no modifications within groups were observed in freely moving recordings (Figure 11).

This contrast in the results may arise from differences related the animal vigilance state. pMSNs intracellular membrane potential is characterized by a transition between up-down states, with the up states being associated with action potentials and burst generation (Wilson and Kawaguchi, 1996). Relevantly, Mahon and collaborators showed that the transition between up and down states is not present during wakefulness, with these transitions being found only during slow wave sleep (Mahon et al., 2006). In this sense, the authors indicate that these two-state MSNs neuronal activity is not an artifact of anesthesia. Instead, is related to a a synchronized pattern of cortical activity during slow wave sleep.

To further understand these differences between behavioral states in DLS activity, baseline firing rate and Lv index were compared between anesthetized and freely moving rats (Annexes Figure S4). Freely moving rats showed a higher baseline firing rate compared to anesthetized rats (Annexes Figure S4). In contrast, no differences were observed in baseline Lv index between groups (Annexes Figure S4). Our results related to pMSNs baseline firing rate are in line with evidence showing a decrease in firing rate after the induction of anesthesia with urethane (West, 1998). The differences in baseline firing rate between anesthetized and freely moving rats may be related to the differential synaptic drive observed in each behavioral state (Mahon et al., 2006). In relation to firing pattern, no previous studies have focused in analyzing the differences in this parameter between anesthesia and freely moving animals on pMSNs. In this sense, our results indicate that pMSNs Lv index is a stable measure across wakefulness and sleep cycles. Furthermore, is relevant to point out that in both anesthetized and freely moving experiments a loss of AMPH effect on Lv index in non-sensitized is observed, i.e. after an acute AMPH injection on anesthetized recordings (Figure 6) and during day 10 of freely moving recordings (Figure 11). Together, these results show that, using Lv index, AMPH-related

neuronal adaptations on DLS pMSNs can be observed during both slow wave sleep and wakefulness.

5.3. To quantify the gephyrin density in the entopeduncular nucleus, substantia nigra pars reticulata and globus pallidus after amphetamine repeated administration.

One disadvantage of our single unit experiments is that does not allow us to differentiate between dorsal striatum MSNs from direct and indirect pathway. In order to address this problem, as well as getting a detailed view of morphological changes related to sensitization, we decided to study the structural output of basal ganglia direct and indirect pathway, i.e. the GABAergic synapses on EP, SNr (direct pathway) and GP (indirect pathway). GABAergic synapses were studied using Geph, an anchoring protein of $GABA_A$ receptors, largely used to quantify GABAergic synapses (Groeneweg et al., 2018). Our main results were that, whereas no modifications in Geph density were observed in EP, SNr nor GP of sensitized rats, an increase in Geph density on GP was observed in non-sensitized rats (Figure 20). Together, these results indicate that an increase in GABAergic synapses on dorsal striatum indirect pathway underlies the lack of expression of AMPH locomotor sensitization.

GABAergic terminals are the main inputs of EP, SNr and GP (Dudman and Gerfen, 2015) and, among these inputs, those from dorsal striatum are the most numerous (DiFiglia et al., 1982). For example, is estimated that around an 80% of the synapses in GP are from dorsal striatum (Dudman and Gerfen, 2015). To our knowledge, our results are the first quantification of Geph in these areas using stereological methods (West et al., 1991). In control rats, the total number of Geph profiles was significantly higher in SNr and GP compared to EP (Annexes, Figure S5A). Considering that the total volume between areas were significantly different (Annexes,

Figure S5B), Geph density was analyzed instead. Geph density on SNr was significantly higher compared to EP and GP (Annexes, Figure S5C). These results show a differential degree of GABAergic synaptic density between the direct (EP, SNr) and indirect (GP) pathway.

Previous data using DREADD have shown a relevant role of the basal ganglia direct and indirect pathway in locomotor sensitization. Whereas a decrease in dorsal striatum direct pathway activity via a DREADD M4 $G_{i/o}$ activation disrupt the expression of AMPH locomotor sensitization, a decrease in indirect pathway neurons activity potentiate the expression of locomotor sensitization (Ferguson et al., 2011). Conversely, an increase in striatopallidal activity via a DREADD M3 $G\alpha_{olf}$ activation blocks the expression of AMPH locomotor sensitization (Farrell et al., 2013). Given this, our hypothesis was that an increase direct pathway (EP, SNr) GABAergic synapses, together with a decrease in GABAergic synapses in indirect pathway (GP) accompanies the expression of AMPH locomotor sensitization. On the other hand, it was hypothesized that an increase in indirect pathway GABAergic synapses accompanies the lack of expression of locomotor sensitization. Our data did not confirm the first hypothesis, given that no modifications in Geph density were observed in EP, SNr nor GP of sensitized rats (Figure 20). On the other hand, an increase in GABAergic synapses on GP was observed in rats that did not expressed AMPH locomotor sensitization, supporting our second hypothesis. Basal ganglia direct and indirect pathway are known to have a bidirectional role on behavior (Dudman and Gerfen, 2015). Optogenetic activation of direct pathway increase locomotion, whereas indirect pathway activation decrease locomotion (Kravitz et al., 2010). In this sense, our data suggest that the potentiation of only of the indirect pathway is necessary to block the increase in AMPH hyperlocomotion related to the expression of AMPH locomotor sensitization.

The increased GABAergic synapses in GP of non-sensitized rats could be explained with neuroplastic modifications in this basal ganglia region. The increase of striatopallidal activity via a DREADD M3 $G\alpha_{olf}$ blocked the expression of AMPH locomotor sensitization and also increased the AMPA/NMDA ratio of NAc shell striatopallidal neurons, showing an potentiation in the synaptic strength of these neurons (Farrell et al., 2013). Similar findings were also observed using cocaine self-administration, were only rats resistant to increase drug taking showed an increase in AMPA/NMDA ratio in NAc indirect pathway neurons (Bock et al., 2013). Relevantly, our single unit recordings of DLS pMSNs showed a reversal of AMPH-effect on population response, AMPH excitability modulation and firing irregularity (Figures 9-11) only in non-sensitized rats after repeated AMPH administration, suggesting neuroplastic modifications. Then, is plausible to suggest that an increase in synaptic strength of MSNs projecting to GP is observed in non-sensitized rats, resulting in an increase in the GABAergic synapses in GP. This differential modifications between direct and indirect pathway MSNs has been proposed to be related with an increased vulnerability of D2 MSNs to neuroplastic changes (Cepeda et al., 2008). Overall, this further support the important role of dorsal striatum indirect pathway neuroadaptations as a protector mechanism for drug related disorders.

In relation to sensitized rats, our findings related to Geph density are in line with the lack of changes observed in pMSNs single unit recordings (Figures 8-11). As previously stated, the expression of AMPH locomotor sensitization is not accompanied with changes in AMPA/NMDA ratio in dorsal striatum neurons (Wang et al., 2013). These results are supported by no changes in AMPAR and NDMAR subunits levels in DLS and NAc of AMPH sensitized rats (Nelson et al., 2009). Overall, these results suggest that no neuroplastic modifications are observed in sensitized rats direct and indirect pathway. As stated previously (Section 5.2.2),

using the Actor/Critic framework, a functional 'Actor' is required for the transition in addiction behavior. Then, the persistence of AMPH-effect on DLS neuronal activity and the lack of changes in the density of GABAergic synapses in dorsal striatum output areas could be necessary for the expression of AMPH locomotor sensitization. Considering the relevance of the mPFC and NAc for the expression of locomotor sensitization (as showed in section 1.2.), it could be relevant to study if changes in glutamatergic and GABAergic synapses on these areas are observed in sensitized rats after repeated AMPH administration.

A large withdrawal period could be necessary for neuroplastic modifications in sensitized rats. An increase in DLS dendritic spines was observed in previous studies using withdrawal periods of 2 weeks (Ball et al., 2010) or 3 months (Jedynak et al., 2007). In other areas, like mPFC, a decrease in Geph expression levels was observed after 2 weeks withdrawal from cocaine place preference (Yang et al., 2017). Also, a larger period of drug treatment (2-3 weeks) and/or higher doses of psychostimulants, like escalating doses of methamphetamine (Jedynak et al., 2007), have been used. In this sense, we suggest that an increase in the withdrawal and AMPH treatment periods could be accompanied with changes in the number GABAergic synapses in the direct pathway.

Finally, one topic that could be further studied is that if basal differences in Geph density on EP, SNr and GP are observed in rats that are vulnerable to express AMPH locomotor sensitization compared to resistant rats. As explained earlier, the increased risk to express locomotor sensitization it has been associated with a low locomotor activity during the exposure to a novel environment (Piazza et al., 1989), i.e. LR express locomotor sensitization, while HR rats not. Our results showed a trend of a lower locomotor activity during baseline activity in rats that will

become sensitized compared sensitization-resistant rats (Figure 7A). Albeit our results only suggest that a differential locomotor activity during novelty is associated with the vulnerability to express locomotor sensitization, our electrophysiological data on day 1 show that a higher DLS pMSNs baseline Lv index (Figure 11), accompanied with a low percentage of pMSNs with a FR/L correlation during baseline (Figure 13) were observed in rats that will express AMPH locomotor sensitization. Then, considering the changes observed in GP of non-sensitized rats, is plausible to propose that HR rats shown a higher Geph density in GP compared to LR rats? Although we did not measure novelty-related locomotor activity in control rats, the variance of Geph density on GP in this group was not high (Figure 20), suggesting that initial differences in GABAergic synapses are not observed. Measuring locomotor activity during novelty exposure in control rats could allow to confirm this suggestion. Nevertheless, our current results suggest that a differential plasticity induced by DLS pMSNs, related to the higher baseline firing irregularity (Figure 11), underlies the expression of AMPH locomotor sensitization (as discussed in 5.2.). Then, initial differences in GABAergic synapses in dorsal striatum outputs could be not necessary for the individual differences observed after repeated AMPH administration. Instead, the modifications observed in Geph expression in GP may be related to dorsal striatum-induced neuroplasticity during the course of repeated AMPH administration in sensitization-resistant rats.

6. CONCLUSIONS

In summary, the main conclusion of this doctoral thesis is that individual differences are observed in DLS neuronal activity and the density of GABAergic synapses on dorsal striatum outputs after repeated AMPH administration. The expression of AMPH locomotor sensitization is accompanied by an increase in DLS neuronal activity during anesthetized recordings, although not in freely moving experiments. In freely moving rats, AMPH-effects on DLS neuronal activity were similar between the first and last AMPH injection. Interestingly, rats resistant to express AMPH locomotor sensitization reverted AMPH-effects on DLS neuronal activity. This differential DLS activity after repeated AMPH administration was preceded by individual differences in baseline firing irregularity and in the correlation between firing rate and locomotor activity during baseline firing rate. Finally, whereas no changes in GABAergic synapses density were found in dorsal striatum outputs of sensitized rats, an increase in GABAergic synapses density on GP of non-sensitized rats was observed. Together, these data indicate that a persistence of AMPH-effects on DLS neuronal activity accompanies the expression of AMPH locomotor sensitization. On the other hand, a reversion of AMPH-effect on DLS neuronal activity, accompanied with a potentiation of dorsal striatum indirect pathway, are observed in rats resistant to express locomotor sensitization after repeated AMPH administration.

ANNEXES

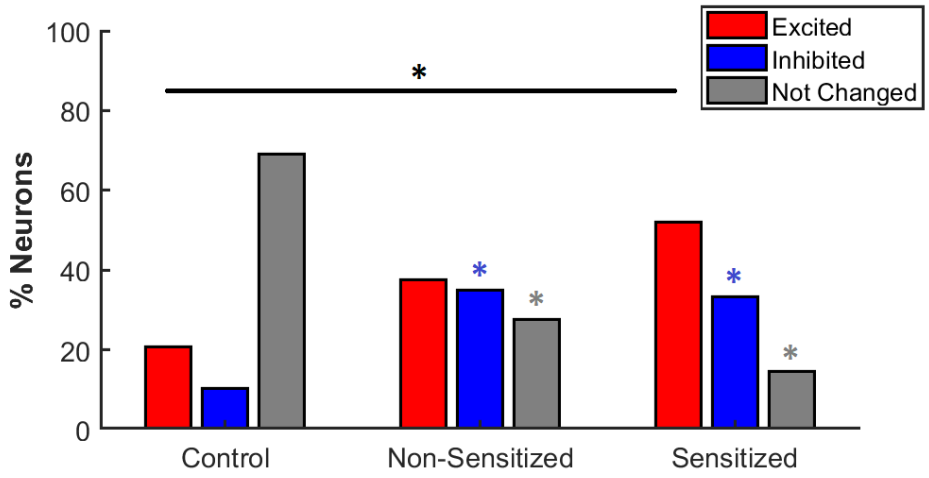

FIGURE S1 DLS pMSNs population response to an acute AMPH injection after repeated AMPH administration, in anesthetized rats.
Bar chart shows the percentage of excited, inhibited or not changed pMSNs after an acute AMPH 1.0 mg/kg ip injection. A significant increase in the percentage of excited neurons after AMPH was observed in Sensitized group compared to control (red * $p<0.05$, Fisher Exact test). Also, the percentage of inhibited neurons was higher in both non-sensitized and sensitized rats compared to control rats (blue *$p<0.05$, Fisher Exact test). Conversely, the percentage of not changed neurons was lower in both non-sensitized and sensitized rats compared to controls (gray * $p<0.05$, Fisher Exact Test).

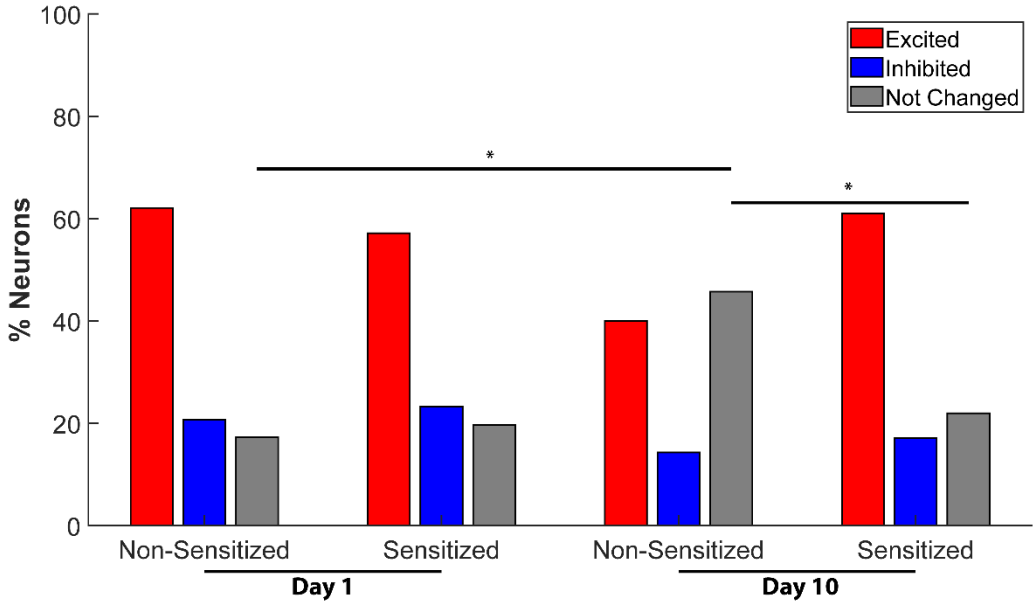

FIGURE S2 DLS pMSNs population response to an AMPH injection during repeated AMPH administration, in freely moving rats.

Bar chart shows the percentage of excited, inhibited or not changed pMSNs after an AMPH 1.0 mg/kg ip injection on days 1 and 10 of repeated AMPH administration. No significant changes were observed between percentage of excited pMSNs between groups or between day 1 and day 10 within each group ($p>0.05$ Fisher Exact test). Similarly, no significant changes were observed between percentage of inhibited pMSNs between groups or between day 1 and day 10 within each group ($p>0.05$ Fisher Exact test). On the other hand, an increase in the percentage of not changed pMSNs between day 10 and day 1 of non-sensitized rats was observed (* $p<0.05$, Fisher Exact test). Moreover, non-sensitized rats percentage of not changed pMSNs was significantly higher compared to sensitized rats on day 10 ($p<0.05$, Fisher Exact test).

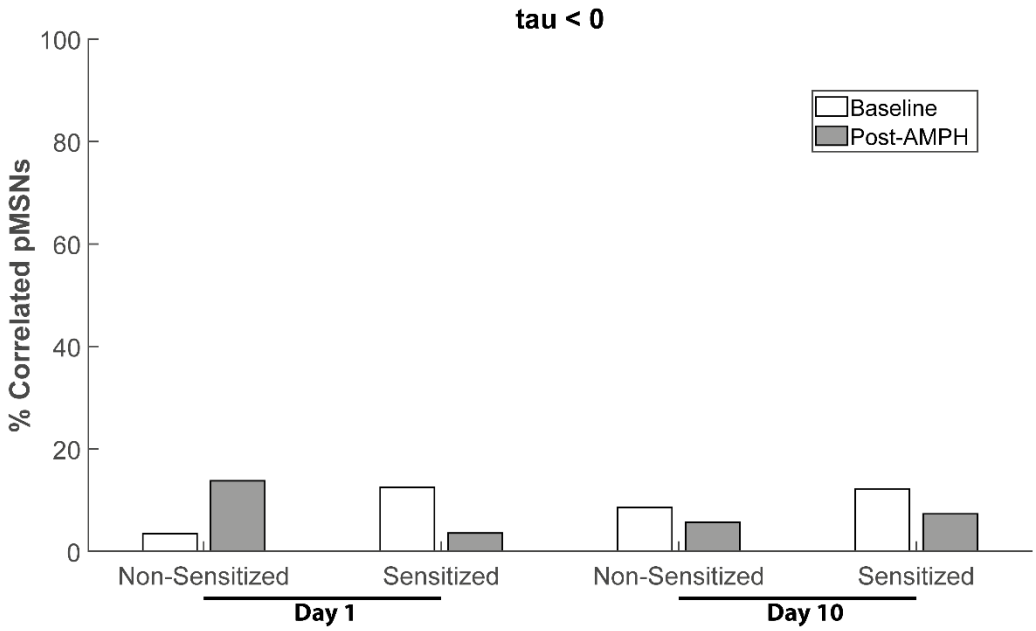

FIGURE S3 A low percentage of pMSNs show an inverse correlation between firing rate vs locomotor activity.

Kendall correlation between firing rate and locomotion were performed during basaline (after saline ip) and post-AMPH epochs. Neurons with a significant correlation ($p<0.05$) and $tau<0$ correspond to inversely correlated pMSNs. No significant differences were observed between or within groups, or between conditions ($p>0.05$ Fisher Exact test). Bar chart represent the total percentage of correlated pMSNs in each condition.

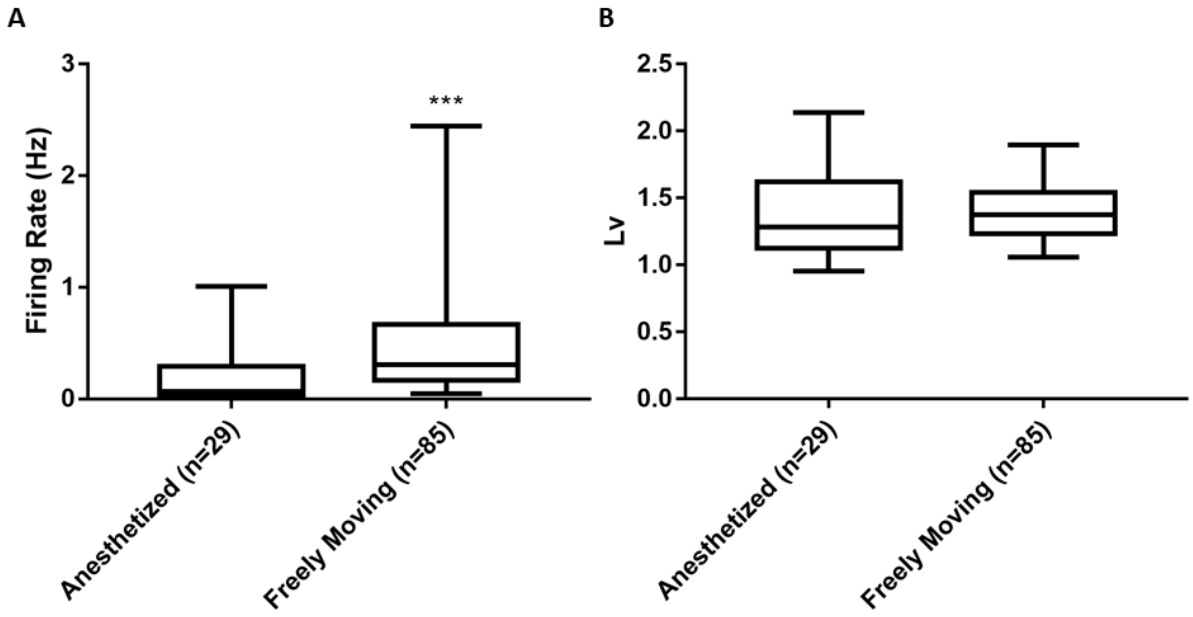

FIGURE S4 Comparison of DLS pMSNs baseline firing rate and Lv index between anesthetized and freely moving single unit recordings.

pMSNs of control rats anesthetized experiments (n=29) and both non-sensitized and sensitized rats day 1 of freely moving experiments (n=85) were used to compare baseline firing rate and baseline Lv index between conditions. A) pMSNs baseline firing rate between anesthetized and freely moving rats. A higher baseline firing rate was observed in freely moving rats compared to anesthetized rats ($p<0.001$, Mann-Whitney test). B) pMSNs baseline Lv index between anesthetized and freely moving rats. No significant differences were observed between conditions ($p>0.05$, Mann-Whitney test). Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers).

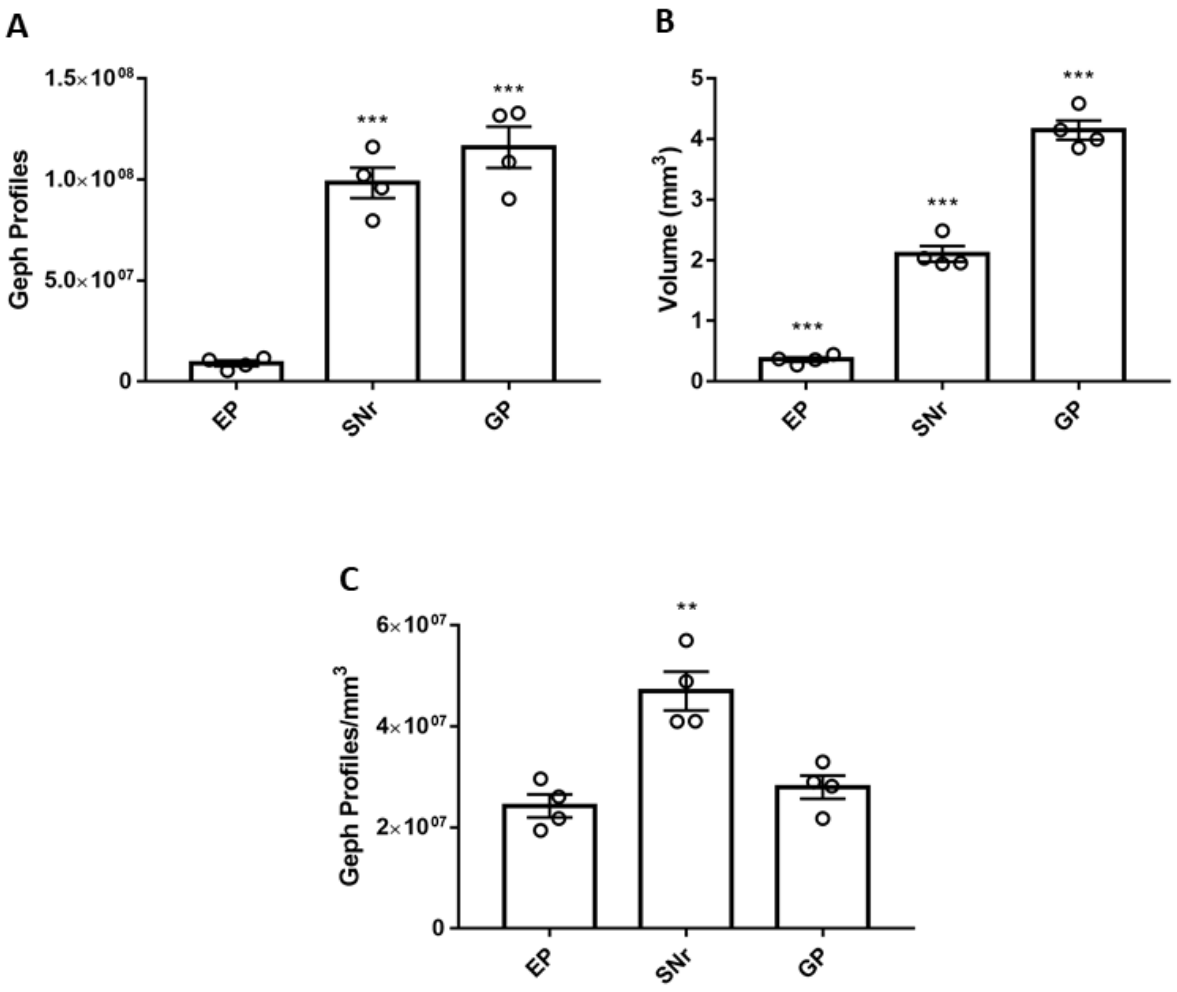

FIGURE S5 Characterization of Geph profiles on EP, SNr and GP.
Estimation of volume and total number of Geph profiles were performed in EP, SNr and GP of control rats (n=4), using the optical fractionator. A) Total number of Geph profiles in EP, SNr an GP. Geph profiles on SNr and GP were significantly higher compared to EP (*** $p<0.001$ vs EP, Bonferroni post-test).B) Volume of EP, SNr and GP of control rats. All the areas volume were significantly differently between each other (*** $p<0.001$ vs the other areas, Bonferroni post-test). C) Geph density in EP SNr and GP. SNr Geph density was significantly higher compared to EP and GP (** $p<0.01$ vs EP and GP, Bonferroni post-test). Each bar represents mean $\pm$ SEM.

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